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

Tese de Doutorado

A AMPLITUDE DO CONCEITO DE FENÓTIPO ESTENDIDO EM GALHAS DE INSETOS

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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.

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"From the day we arrive on the planet, (...)

there's more to see than can ever be seen, more to do than can ever be done;

there's far too much to take in here, more to find than can ever be found (...).

It's the circle of life, and it moves us all..."

(Circle of Life – Tim Rice)

Desde o dia em que chegamos ao planeta, (...)

há mais para ver do que jamais será visto, mais a fazer do que jamais será feito;

há coisas demais para se internalizar, mais a ser descoberto do que jamais se descobrirá (...).

São os ciclos da vida que nos movem a todos...

(Circle of life – Tim Rice)

AGRADECIMENTO

Nasceu, há 29 anos, em uma época quando tudo era muito diferente de hoje. Ainda que não planejado, jamais foi indesejado. Foi criado no seio de uma família cuja maior riqueza é imaterial e, por isso, tornou-se consciente de que valores não significam cifrões. Foi enganado por ter aprendido a ser bom. Foi discriminado por não ser medíocre. Diminuiu-se por querer agradar a todos. Nunca nutriu rancor de seus algozes. Ao contrário, procurou impulsionar-se no negativismo alheio para seguir em direção oposta ao sofrimento.

Da realidade destes idos tempos, muitas coisas mudaram e somente o essencial foi conservado... Percebeu que sua maior força não era física e que poderia se impor pela força das suas ideias. Reaprendeu a confiar nas pessoas. Permitiu-se. Fascinou-se pelo mundo e todas suas formas de vida. Descobriu que amigos verdadeiros nem sempre são somente aqueles de infância, e que eles também são família. Viveu, não sobreviveu! Hoje, olha para trás com orgulho e saudosismo. Pelo seu passado e presente, é somente gratidão. Pelo seu futuro, é todo esperança. Pelos caminhos que percorreu, de percalços e vitórias, é futuro Dr. Renê Gonçalves da Silva Carneiro. Um mestre, biólogo, botânico, cidadão, filho, irmão, amigo; feliz e grato por tudo! Aqui, nomes e palavras adicionais não se fazem necessários; o registro da influência de cada um nesta história são sentimentos indeléveis. E estes, de tão puros e genuínos, não são passíveis de tradução...

ÍNDICE

PREFÁCIO

Desde os meus primeiros passos após ingressar no laboratório de Anatomia Vegetal da UFMG, venho trabalhando com *Psidium myrtoides* e as galhas associadas. Desde a época em que me foi apresentada pela Dra. Anete Formiga, em 2006, muitos trabalhos foram conduzidos com esta espécie. Com a evolução dos meus trabalhos e parcerias, principalmente durante o período trabalhado com o Dr. Denis Oliveira durante seu doutoramento, amadureci a ideia de utilizar esta espécie como modelo para estudos estruturais, ultraestruturais, histoquímicos e imunocitoquímicos. Este era um sistema interessante do ponto de vista do desenvolvimento, pois, como pude observar durante o meu mestrado, embora o ciclo de vida das galhas fosse de um ano, as fases eram bem demarcadas. Isso facilita o processo de coleta do material, o que, muitas vezes, constitui-se um empecilho para os estudiosos do desenvolvimento de galhas.

Dos estudos com esta planta durante meu mestrado, veio a minha primeira publicação como primeiro autor, que descreve a biologia de *Nothotrioza myrtoidis*, espécie e gênero novos, em parceira com o especialista em taxonomia de Psylloidea, Dr. Daniel Burckhardt, pesquisador do Museu de História Natural em Basel, Suíça. Inicialmente, quando o Dr. Daniel Burckhardt analisou os insetos, imaginou ser *Neotrioza tavaresi*, coletado e descrito por Crawford em 1925, com uma identificação duvidosa da planta hospedeira – uma Malpighiaceae. Entretanto, *Neotrioza tavaresi* já havia sido descrito como o galhador das folhas de *Psidium cattleianum*, ocorrente no sul do Brasil. Disso surgiu um impasse, uma vez que era pouco provável que o mesmo inseto galhador induzisse galhas em dois hospedeiros diferentes, dada a especificidade das relações que resultam na formação de galhas. Com o devido tratamento taxonômico dado pelo Dr. Daniel Burckhardt, observou-se então, que existiam três espécies de galhadores: *Nothotrioza tavaresi* Burck. comb. n., que substitui *Neotrioza tavaresi* Crawf., e as duas novas espécies de *Nothotrioza – N. cattleiani* associado a *P. cattleianum* e *N. myrtoidis* associado a *P. myrtoides*. A espécie hospedeira de *Nothotrioza tavaresi* permanece incógnita.

Com estas novas informações, surgiu a ideia de trabalhar com os sistemas duplamente co-genéricos, ou seja, *Nothotrioza* spp. – *Psidium* spp. O projeto de doutorado, até então formulado para tratar do desenvolvimento de galhas em Myrtaceae induzidas por galhadores com diferentes hábitos alimentares, *N. myrtoidis – P.*

myrtoides (sugador) e Cecidomyiidae – *Myrcia itambensis* (raspador), mudara. *Myrcia itambensis*, assim como suas galhas, nunca foram localizadas na área previamente descrita, o campus Pampulha – UFMG, o que constituiu um motivo adicional para a mudança. Como a espécie hospedeira de *N. tavaresi* era desconhecida, elegeu-se o sistema *N. cattleiani* – *P. cattleianum* para o desenvolvimento da tese. Assim, ao invés de analisarmos puramente as diferenças induzidas por galhadores com hábitos alimentares diferentes, nos propusemos – eu, a orientadora Dra. Rosy Isaias e todos os nossos colaboradores – a estudar as minúcias do desenvolvimento de galhas morfologicamente muito semelhantes. E assim, nasceu a tese que vos é apresentada: "A amplitude do conceito de fenótipo estendido em galhas de insetos". Esta tese é composta de seis capítulos que serão apresentados em seguida.

O primeiro e segundo capítulos tratam de gradientes histoquímicos e do desenvolvimento anatômico e imunocitoquímica de parede celular nas galhas de *N. myrtoidis* em *P. myrtoides*, respectivamente, publicados nos periódicos South African Journal of Botany (2014, v. 92, pp. 97-104 – Cap. 1) e Plant Cell Reports (2014, v. 33, pp. 2093-2106 – Cap. 2). Em ambos os artigos, compilei dados obtidos durante o mestrado – reanalisados e reformulados para a publicação – adicionados de novas análises conduzidas durante o doutorado. O terceiro capítulo trata do desenvolvimento citológico das galhas de *N. myrtoidis* em *P. myrtoides*, publicado no periódico Protoplasma (2014, DOI 10.1007/s00709-014-0709-x). O quarto e quinto capítulos tratam, respectivamente, do desenvolvimento anatômico e imunocitoquímica, e do desenvolvimento citológico e histoquímica nas galhas de *N. cattleiani* em *P. cattleianum.* Os respectivos manuscritos estão submetidos aos periódicos PLOS ONE e AOB Plants. O sexto e último capitulo é fruto de uma parceria com o Dr. Claudio Donnici, meu co-orientador do departamento de química da UFMG. Este capítulo trata da identificação dos perfis espectroscópicos na faixa do infravermelho das superfícies interna e externa das galhas, relacionados à composição péctica das paredes celulares, conduzidas com as galhas de *N. myrtoidis* em *P. myrtoides.* Assim, resumem-se estes quatro anos de trabalho; intensamente vividos e apreciados, sempre sobre a égide do maravilhoso e intrigante mundo das galhas.

RESUMO

Galhas se desenvolvem através da reprogramação das células vegetais, para que linhagens celulares assumam formas e funções diferentes daquela do padrão ontogenético do órgão hospedeiro. A maneira como cada inseto galhador modula o comportamento celular nas diferentes espécies hospedeiras é única e determina padrões de fenótipo celular em galhas. Estes padrões levam ao surgimento de diferentes morfotipos de galhas, isto é, os fenótipos estendidos dos galhadores. Entretanto, é comum observar-se a convergência de formas em galhas de diferentes sistemas inseto galhador – planta hospedeira. Em sistemas em que há convergência morfológica das galhas e os insetos e plantas hospedeiras são táxons filogeneticamente relacionados entre si, surgem algumas perguntas: Os mecanismos celulares envolvidos na geração de formas similares são conservativos? Existem padrões de alterações estruturais e metabólicas que conferem funcionalidades únicas para cada galha? As galhas podem ser consideradas como o fenótipo estendido de seus galhadores nos níveis celulares e subcelulares? A presente tese discute mecanismos celulares de determinação da forma e função dos tecidos vegetais, sob a perspectiva do desenvolvimento de galhas, a neo-ontogênese vegetal.

Palavras-chave: Anatomia vegetal, Biologia celular vegetal, *Nothotrioza cattleiani*, *N. myrtoidis*, *Psidium cattleianum*, *P. myrtoides.*

ABSTRACT

Galls develop by the reprogramming of plant cells, so that cell lineages assume forms and functions different from that of the standard ontogenesis of the host organ. The way galling insects modulate cell behaviour in the different host plants is unique, and determines patterns of cell phenotypes in their galls. Such cellular phenotypic patterns lead to the organogenesis of different gall morphotypes, i. e., the extended phenotypes of the gall inducers. Nevertheless, convergent gall shapes are observed for different galling insect – host plant systems. Systems in which galls are morphologically convergent, and both the insects and host plants are phylogenetically related, some questions arise: Are the cellular mechanisms involved in the generation of similar conservative forms? Are there patterns of structural and metabolic alterations that confer unique functionalities to each gall? Can the galls be considered the extended phenotypes of their inducers at the cellular and subcellular levels? This thesis discusses different cellular mechanisms for the determination of forms and functions in plant tissues, under the perspective of gall development, a plant neo-ontogenesis.

Keywords: *Nothotrioza cattleiani, N. myrtoidis,* Plant anatomy, Plant cell biology, *Psidium cattleianum, P. myrtoides*.

INTRODUÇÃO GERAL

Insetos galhadores são parasitas especialistas que induzem a formação de estruturas de diferentes formatos nos órgãos vegetais, a partir das quais obtêm abrigo e nutrição (Price et al. 1987). A manipulação do programa de desenvolvimento padrão dos vegetais leva à morfogênese das galhas através da fina regulação da plasticidade fenotípica celular pelos estímulos químicos e mecânicos dos insetos (Redfern e Askew 1992, Rohfritsch 1992). Desta forma, as galhas induzidas por insetos tendem a apresentar crescimento limitado e bem definido, sendo altamente conservativas em termos morfogenéticos, levando à determinação de formatos repetitivos na natureza (Raman 2007).

Na região Temperada, onde a biodiversidade de insetos galhadores é menor do que a dos Neotrópicos (Price et al. 1998, Espírito-Santo & Fernandes 2007), muitos aspectos dos sistemas de plantas hospedeiras – insetos galhadores, tais como taxonomia, ecologia, estrutura e química já são bem delimitados (Price et al. 1987, Abrahamson e Weis 1997, Inbar et al. 1998, Kamata et al. 2002, Irwin e Lee 2003, Gish e Inbar 2006, Kamata et al. 2006, Craig et al. 2007). Por outro lado, a ausência de conhecimento taxonômico dos insetos galhadores Neotropicais dificulta a descrição destes sistemas, de forma que as galhas são referidas como morfotipos, de acordo com seu formato tridimensional (Isaias et al. 2013). Este tipo de classificação tem sido utilizada eficientemente para expressar, em termos genéricos, a diversidade de insetos galhadores Neotropicais, já que os morfotipos de galhas são tidos como o fenótipo estendido dos seus indutores (*sensu* Dawkins 1982). Entretanto, a classificação de galhas segundo aspectos estritamente macro morfológicos deve ser utilizado com cautela e associado a outras análises, sempre que possível.

Os estudos com galhas Neotropicais mostram não somente a riqueza e diversidade dos morfotipos de galhas, mas também aspectos fenológicos, anatômicos, histoquímicos e citológicos nos sistemas planta hospedeira– inseto galhador (Gonçalves et al. 2009, Moura et al. 2009, Oliveira e Isaias 2009, Sá et al. 2009, Campos et al. 2010, Oliveira e Isaias 2010, Oliveira et al. 2010, Detoni et al. 2011, Formiga et al. 2011, Isaias et al. 2011, Oliveira et al. 2011). Tais estudos mostram padrões de alterações estruturais e fisiológicas nos sítios das galhas, além de peculiaridades tidas como específicas de cada sistema. Por exemplo, ferramentas anatômicas e histoquímicas permitem elucidar a dinâmica de linhagens celulares que determinam as relações de

forma e função das galhas em diferentes sistemas. Estas ferramentas são importantes para o reconhecimento dos morfotipos de galhas como entidades únicas pelas suas peculiaridades.

Galhas induzidas por *Nothotrioza myrtoidis* em *Psidium myrtoides* e por *N. cattleiani* em *P. cattelainum* são morfologicamente similares e representam um impasse à validade do conceito de fenótipo estendido, isto é, a expressão fenotípica de genes que se estende para fora das células nas quais exercem influência bioquímica imediata (Dawkins 1982). De fato, as galhas induzidas por *N. cattleiani* em *P. cattleianum*, e *N. myrtoidis* em *P. myrtoides* são ambas arredondadas, glabras, e são protrudidas para a superfície abaxial da lâmina foliar. Sua coloração pode variar do verde escuro ao verde amarelado, chegando até o vermelho intenso (Fig. 1A, B). Assim, ambas as galhas são classificadas como sendo globoides (*sensu* Isaias et al. 2013), e sua morfologia externa não é suficiente para individualizá-las. A coloração de galhas, por exemplo, pode mudar devido aos diferentes estágios de desenvolvimento das galhas (Sáiz e Nuñes 1997), à efetividade da alimentação do galhador (Dias et al. 2013), ou à presença de parasitoides (Carneiro et al. 2013). Como em ambos os sistemas, *Nothotrioza* spp. são atacados por parasitoides (Butignol and Pedrosa-Macedo 2003, Carneiro et al. 2013), as galhas podem apresentar respostas similares que levam a uma convergência fenotípica entre elas.

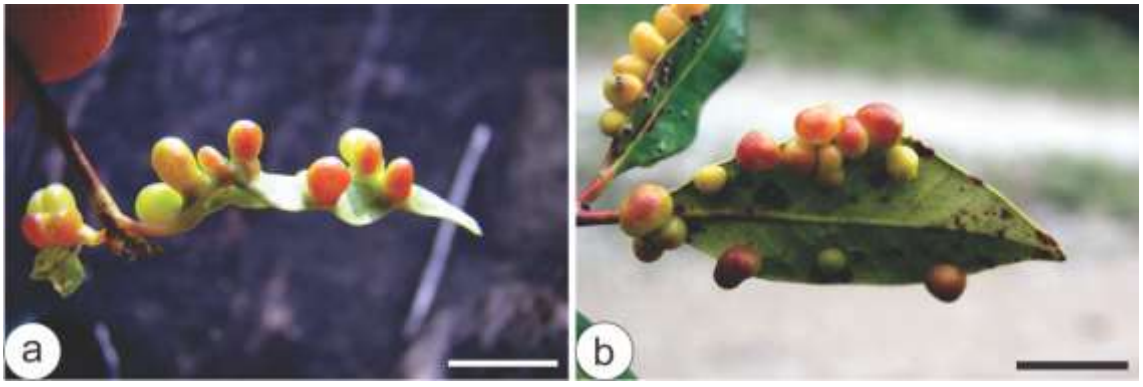


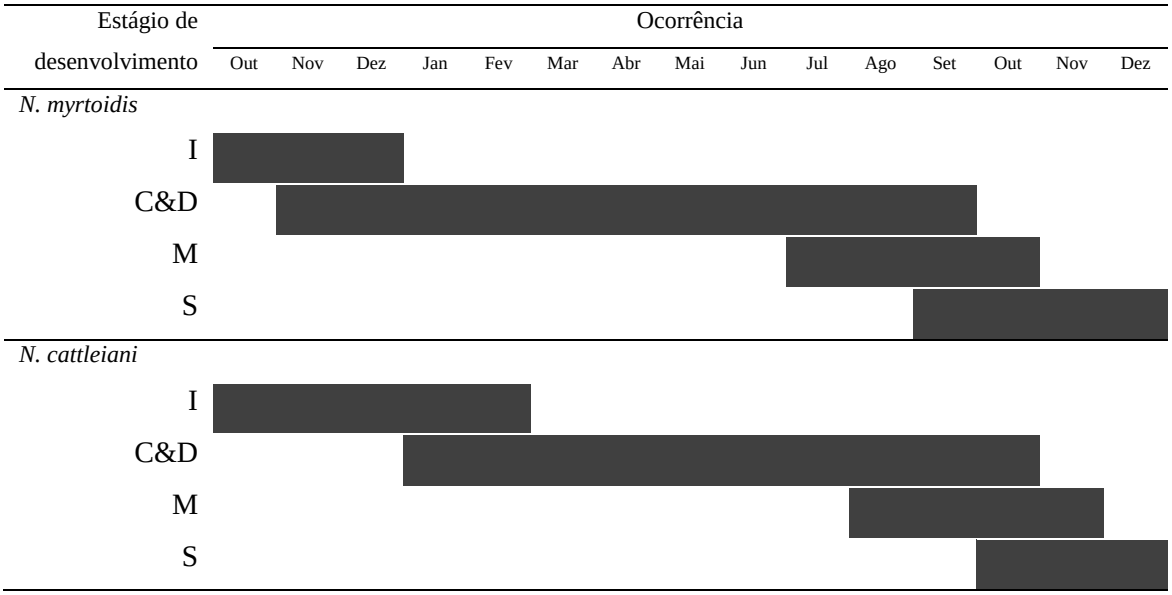
Figura 1. Macro morfologia das galhas de *Nothotrioza* spp. induzidas em *Psidium* spp.. **a** Galhas globoides de *N. myrtoidis* em *P. myrtoides*; **b** Galhas globoides de *N. cattleiani* em *P. cattleianum*. Ambas as galhas são glabras e apresentam as mesmas variações de coloração. Barras: 1 cm.

O ciclo de vida das espécies de *Nothotrioza* dura um ano (Tabela 1), e as galhas possuem estágios de desenvolvimento parecidos. A fase de indução, caracterizada por

pequenos pontos na superfície adaxial das lâminas foliares das espécies de *Psidium* (*sensu* Carneiro et al. 2013), começa em Outubro e dura de um a três meses, podendo ser observada até o início de Fevereiro. A fase de crescimento e desenvolvimento dura de nove a dez meses, sendo observada desde um mês após a indução, até Outubro do ano seguinte. Esta fase é caracterizada pelo maior aumento em biomassa das galhas. A fase de maturação é curta, geralmente durando dois meses, e é observada desde Julho até Novembro. A partir de Setembro até Dezembro, galhas em fase de senescência podem ser observadas em *P. myrtoides* e *P. cattleianum* (Tabela 1). A senescência das galhas é caracterizada por um processo de deiscência no qual as galhas se abrem espontaneamente.

Embora a fenologia das galhas de *N. cattleiani* e *N. myrtoidis* seja ligeiramente diferente, acredita-se que as fenofases mais prolongadas observadas para *N. cattleiani*, ou seja, as menores taxas de desenvolvimento, sejam devidas às temperaturas mais baixas do seu local de ocorrência, como descrito por Butignol e Pedrosa-Macedo (2003). Como pode ser visto, apesar das análises morfológicas e fenológicas serem bastante informativas, estas não foram capazes de revelar peculiaridades dos sistemas *Nothotrioza* spp. – *Psidium* spp. suficientes para a individualização das galhas.

Tabela 1. Distribuição temporal dos estágios de desenvolvimento das galhas induzidas por *Nothotrioza* ssp. (Hemiptera: Psylloidea) em *Psidium* ssp. (Myrtaceae)



Barras horizontais indicam a ocorrência de cada estágio de desenvolvimento das galhas durante o ano: indução (I), crescimento e desenvolvimento (C&D), maturação (M) e senescência (S).

Dentro deste cenário de similaridades, hipotetizou-se a existência não somente de caracteres conservativos, consequência da proximidade filogenética das espécies envolvidas, mas também de caracteres distintivos que revelariam as peculiaridades únicas de cada sistema. Para isso, conduzimos análises anatômicas, citológicas e imunocitoquímicas do desenvolvimento destas galhas e seus órgãos hospedeiros, visando acessar os perfis de alterações estruturais nas galhas. Ainda, análises histoquímicas de galhas e folhas maduras foram realizadas para a observação de perfis de alterações bioquímico-metabólicas. Juntos, este conjunto de dados seria crucial para o entendimento das novas relações de forma e função estabelecidas nos tecidos vegetais para o desenvolvimento das galhas enquanto verdadeiros fenótipos estendidos de seus indutores.

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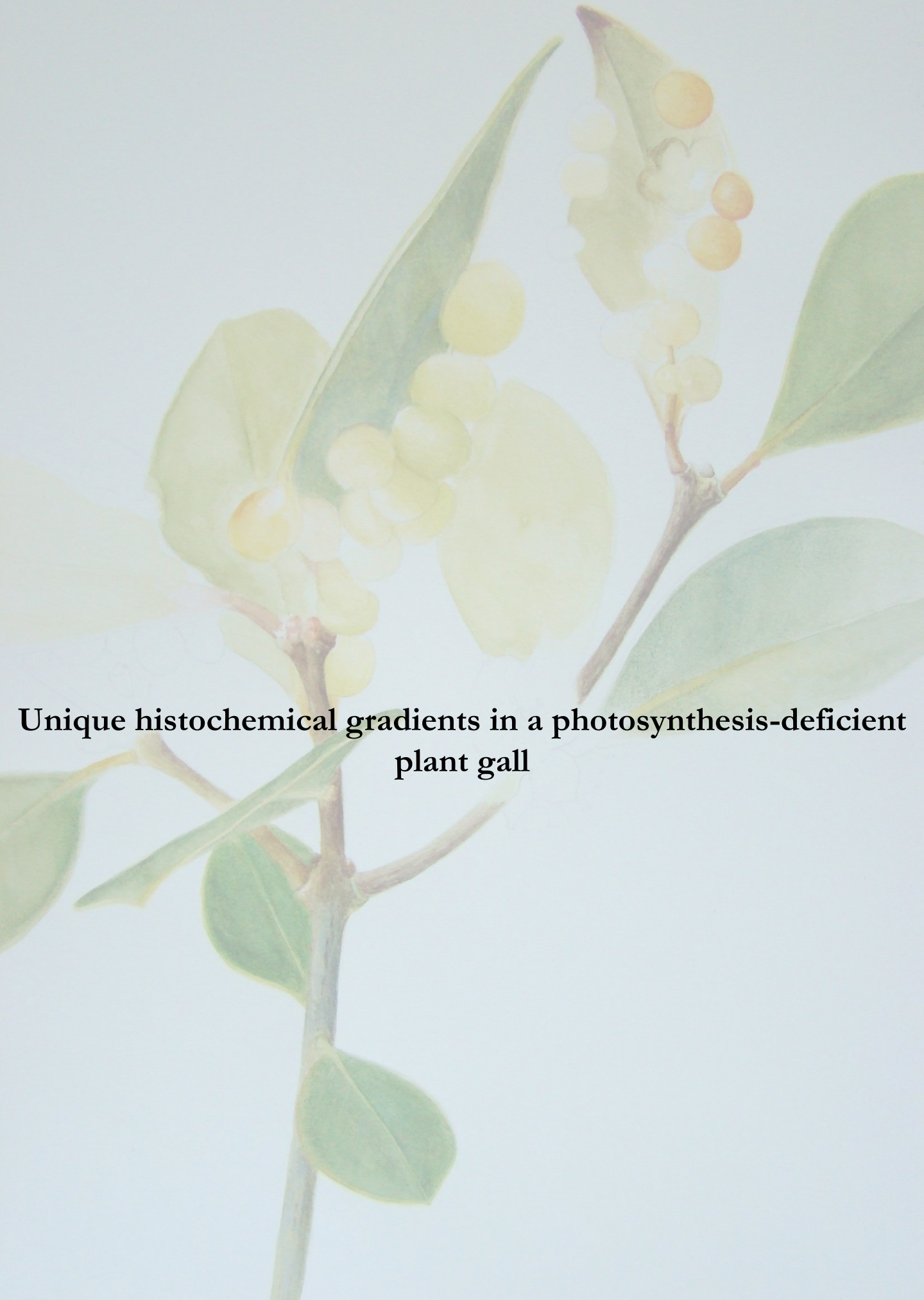

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Unique histochemical gradients in a photosynthesis-deficient plant gall

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ABSTRACT

Galls usually present low chlorophyll content, and their metabolism may vary depending on the *taxa* of the inducer and on the complexity of the gall structure. Primary and secondary plant metabolites allocated in gall tissues are evidenced with histochemical tests and may indicate the physiological status of such tissues. The histochemical and biochemical profiles of the galls induced by *Nothotrioza myrtoidis* Burck. (Hemiptera: Psylloidea) on *Psidium myrtoides* (Myrtaceae) were compared to those reported for some other Neotropical galls. The extralaminar galls of *N. myrtoidis* have low chlorophyll and nitrogen contents, but they accumulate more polysaccharides than the non-galled leaves. The histochemical gradient of reducing sugars is guaranteed by the activity of acid phosphatase, and ensures the nourishment of the gall inducer, while the gradients of phenolics and proanthocyanidins are both related to protection of the gall inducer and modulation of plant cell growth. The accumulation of reactive oxygen species seems to play a major role on the determination of the extent of tissue alterations during gall morphogenesis. The lack of morphological continuum and physiological continuum between the extralaminar galls of *N. myrtoidis* and the leaves of *P. myrtoides*, together with the low impacting feeding activity of the sucking insect, determine the establishment of a photosynthesis-deficient structure with unique features among Neotropical galls.

1. Introduction

The physiological traits of gall tissues have been evaluated through the quantification of photosynthesizing pigments and nutrients (Yang et al., 2003; Castro et al., 2012, 2013), and by histochemical tests (Hartley, 1998), providing an overview of the neo-established functionalities of plant tissues during gall morphogenesis. Different techniques have been used to evidence the metabolic dependence and the photosynthetic deficiency of galls (Yang et al., 2003; Khattab and Khattab, 2005). Even though the reduction on the concentration of chlorophylls is a widespread characteristic of galls, it has been recently demonstrated that photosynthesis is not strictly related to chlorophyll content, since normal rates of electron transport may be maintained, as occurred in the intralaminar galls on *Aspidosperma australe* (Oliveira et al., 2011a). On the other hand, Castro et al. (2012) demonstrated that the extralaminar horn-shaped gall on *Copaifera langsdorffii* has low chlorophyll content, but also acts as a sink of nutrients, as it does not photosynthesize at the same level of its host leaves. These findings indicate that morphology may interfere directly on gall metabolism.

The metabolic complexity of the insect-induced plant galls (Bronner, 1992) and the accumulation of plant metabolites related to the defense or nutrition (Hartley, 1998) were both assessed using histochemical tests. The study of Cecidomyiidae galls in the Neotropics, for instance, revealed conservative patterns on the accumulation of carbohydrate and related enzymatic activity (Oliveira et al., 2010, 2011b) both in intralaminar and extralaminar gall morphotypes. Galls induced by sucking-insects, on the other hand, present variable metabolic features. The cytological and histochemical gradients on the intralaminar galls of *Pseudophacopteron* sp. (Hemiptera) (Oliveira and Isaias, 2010) are similar to those of Cecidomyiidae galls, while the extralaminar bivalve-shaped galls induced by *Euphalerus ostreoides* (Hemiptera) on *Lonchocarpus muehlbergianus* (Fabaceae) present low carbohydrate metabolism (Isaias et al., 2011). As far as the accumulation of defensive compounds is concerned, the conspicuous structure of such extralaminar galls may be aposematic due to their high content of secondary plant metabolites, as previously proposed for galls on *Pistacia* (Inbar et al., 2010).

As seen, not only the insect *taxa* are important on the determination of gall metabolism, but also the gall structure, since the metabolism of the extralaminar galls tends to be lower than that of the intralaminar ones. At this scenario, the extralaminar galls induced by *Nothotrioza myrtoidis* Burck. (Hemiptera: Psylloidea), a recently described species from the Neotropics (Carneiro et al., 2013), on *Psidium myrtoides* (Myrtaceae) were studied to check the occurrence of such physiological

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Table 1
Reagents used for histochemical detection of plant primary and secondary metabolites, reactive oxygen species and enzyme activity in hand-made cross sections of fresh non-galled leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*.

Test/Reagent–Substance	Reaction mediums	Reference
Primary and secondary metabolites		
Fehling's reagent–reducing sugars	Equal parts of "A" (II copper sulfate 6.93% w:v) and "B" (sodium potassium tartrate 34.6% and 12% sodium hydroxide m:m:v) solutions heated to pre-boiling temperature	Sass (1951)
Lugol's reagent–starch	1% potassium iodine–iodide solution for 5 min	Johansen (1940)
Sudan red B–total lipids	Saturated solution of Sudan red B in 70°GL ethanol for 5 min	Brundett et al. (1991)
Coomassie blue–total proteins	0.25% Coomassie blue solution for 5 min	Dunn (1993)
Ferric chloride–phenolics	1% ferric chloride solution for 5 min	Johansen (1940)
DMACA–proanthocyanidins	Fixation: 0.5% caffeine sodium benzoate in 90% butanol for 1–2 h. Reaction: 1% *p*-dimethylaminocinnamaldehyde for up to 30 min.	Feucht et al. (1986)
NADI–terpenoids	1% α-naphthol, 1% dimethyl-*p*-phenylenediamine in 0.01 M phosphate buffer (pH 7.2) for up to 30 min	David and Carde (1964)
Jeffrey's mixture–alkaloids	Equal parts of 10% nitric acid and 10% chromic acid for 5 min	Johansen (1940)
Wiesner reagent–lignins	2% phloroglucinol in acidified solution for 5 min	Johansen (1940)
Reactive oxygen species		
Diaminobenzidine (DAB)–ROS	0.5% DAB solution for 15 to 60 min	Rossetti and Bonnatti (2001)
Enzyme activity		
Acidic phosphatase	Incubation: 0.012% lead nitrate and 0.1 M potassium sodium glycerophosphate in 0.5 M acetate buffer (pH 4.5) for 24 h, at room temperature. Reaction: Wash in distilled water and immerse in 1% ammonium sulfate for 5 min	Gomori (1956)
Glucose-6-phosphatase	Incubation: 20 mg of potassium glucose-6-phosphate in 125 ml of 0.2 M Tris–maleate buffer (pH 6.7), 3 ml of 2% lead nitrate in 7 ml of distilled water for 15 min to 2 h, at 37 °C. Reaction: wash in distilled water and immerse in 1% ammonium sulfate for 5 min	Jensen (1962)
Phosphorylase	Incubation: 1% glucose-1-phosphate in 0.1 M acetate buffer (pH 6.0) for 2 h at room temperature. Reaction: Lugol's reagent for 5 min	Jensen (1962)
Sucrose synthase	Fixation: 2% paraformaldehyde with 2% polyvinylpyrrolidone and 0.005 M dithiothreitol for 1 h. Incubation: 5 ml of 150 mM NADH, 5 ml (1 U) of phosphoglucomutase, 5 ml of 3 mM glucose-1,6-biphosphate, 5 ml (1 U) of glucose-6-phosphate dehydrogenase, 5 ml (1 U) of UDPG–pyrophosphorylase, 280 ml of 0.07% aqueous nitro-blue tetrazolium (NBT), 350 ml of buffer, and 50 ml of substrate for 30 min. Buffer: 100 mM HEPES, 10 mM $MgCl^2$, 2 mM EDTA, 0.2% BSA, and 2 mM EGTA at pH 7.4. Substrate: 0.75 M sucrose, 15 mM UDP, and 15 mM pyrophosphate.	Wittich and Vreugdenhil (1998)
Invertases	Incubation: 0.38 mM sodium phosphate (pH 7.5), 0.024% NBT, 0.014% phenazin metasulfate, 30 U of glucose oxidase, 30 mM of sucrose at room temperature for 3 h.	Zrenner et al. (1995) and Doehlert and Felker (1987)

and histochemical patterns already described for other galls. The following questions are addressed: (1) Do the biochemical and histochemical profiles of *N. myrtoidis–P. myrtoides* system fit the patterns described for other extralaminar galls? (2) Are there metabolic gradients in the extralaminar galls of the sucking-insect *N. myrtoidis*? (3) Do the histochemical profiles indicate the establishment of new functional designs in the tissues of the galls?

2. Materials and methods

2.1. Study area

The population of *P. myrtoides* O. Berg (Myrtaceae) infested by *N. myrtoidis* is located in a trail 2 km away from the park headquarters in the Reserva Particular do Patrimônio Natural Serra do Caraça,

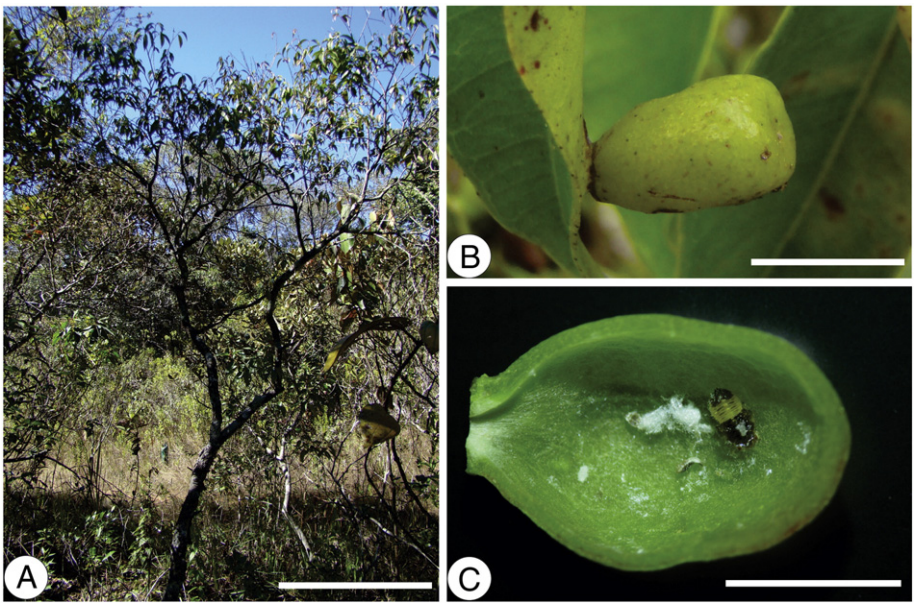

Fig. 1. Macroscopic characteristics of the *Psidium myrtoides–Nothotrioza myrtoidis* system. A—Habitus of *P. myrtoides*; B—Globoid leaf galls of *N. myrtoidis*; C—Gall in cross section evidencing the 4th instar nymph inside the broad nymphal chamber. Bars: A—70 cm; B and C—1 cm.

municipality of Catas Altas, Minas Gerais state, Brazil (20°06′22″ S–43°29′42″W). Individuals (n = 12) were marked, and samples of non-galled leaves (NGL) and galls (GA) were collected.

2.2. Dosage of photosynthetic and accessory pigments

Mature leaves and galls (n = 30) were collected from 10 individuals. Each sample of NGL consisted of four leaf disks of 0.44 cm^2, while GA were sampled in four halves, all previously weighted. Chlorophylls *a* and *b* and carotenoids were extracted in 80% acetone and quantified according to the equations proposed by Lichtenthaler and Walburn (1983). Total anthocyanins were extracted in 5 ml of 95% ethanol and 1.5 N HCl (99:1, v/v), and kept under refrigeration. After five days, the samples were macerated and centrifuged. Quantification followed the equation [total anthocyanins ($mg \cdot 100\ g^{-1}$) = absorbance · dilution factor · $(E^{1\%}1\ cm)^{-1}$], in which the value of $E^{1\%}1$ cm was 98.2, as stated by Lees and Francis (1972). All extracts were analyzed in a spectrophotometer at specific wavelengths, and the data were expressed in $\mu g \cdot g^{-1}$ FM.

2.3. Dosage of nitrogen and carbohydrates

The samples of NGL and GA were dried in an oven, macerated and stored free of humidity. Nitrogen quantification followed Kjeldahl's method (Tedesco et al., 1995). The material was digested in concentrated sulfuric acid and the residue was distilled (distiller Tecnal TE-0363). Ammonia released in the form of NH_4OH was collected in a solution of 2% boric acid, and titrated against 0.02 N hydrochloric acid previously standardized.

The dosage of carbohydrates – total soluble sugars (TSS), water-soluble polysaccharides (WSP) and starch – was based on phenol–sulfuric acid method (Dubois et al., 1956; modified by Chow and Landhäusser, 2004), and analyzed in a spectrophotometer. The material was dried in a microwave oven for 3 min (Marur and Sodek, 1995), kept in an oven with forced air circulation, and then macerated. The TSS were extracted with a solution of methanol:chloroform:water (12:5:3) (Bielski and Turner, 1966), the WSP with 10% ethanol (Shannon, 1968), and the starch with 30% perchloric acid (McCready et al., 1950).

2.4. Histochemical tests

Histochemical tests for primary and secondary plant metabolites, reactive oxygen species (ROS), and the activity of enzymes related to carbohydrate metabolism were performed as described in Table 1. Control tests were done according to the references and compared with blank sections. Sections were mounted on glass slides with Kaiser's glycerol gelatin (Kraus and Arduin, 1997), and photographed on a light microscope (Olympus BH-2).

2.5. Statistical analyses and graphical representation

Statistical analyses were performed using the software JMP® (SAS Institute, U.S., 1989–2002). Normal data (Shapiro–Wilk test) were compared by parametric tests of ANOVA followed by t-test or multiple tests of Tukey. Non-normal data were compared using the test of Kruskal–Wallis followed by Dunn's multiple tests. All tests used alpha = 0.05. The graphs were generated by GraphPad Prism® for Windows, version 5.0 (Motulsky, 1992–2009).

3. Results

3.1. General features

P. myrtoides is a shrubby or subarboreous plant (Fig. 1A) whose leaves are parasitized by *N. myrtoidis*. First-instar nymphs of *N. myrtoidis* induce galls on young leaves, which develop into a broad chambered, extralaminar structure containing a single insect (Fig. 1B, C).

Table 2
Content of photosynthetic and accessory pigments in non-galled leaves (NGL) and galls (G) of *Nothotrioza myrtoidis* (Hemiptera) in *Psidium myrtoides* (Myrtaceae) (n = 30, mean ± standard error; $mg \cdot g^{-1}$ FM).

Pigments	Samples	
	NGL	G
Chlorophyll *a*	734.9 ± 30.32a	44.43 ± 2.99b
Chlorophyll *b*	315.9 ± 13.92a	28.87 ± 1.781b
Chlorophyll *a*/*b*	2.34 ± 0.03a	1.56 ± 0.06a
Total chlorophylls	1062 ± 43.96a	79.24 ± 4.74b
Carotenoids	10.98 ± 0.33a	5.94 ± 0.37b
Anthocyanins	24.14 ± 0.61a	3.43 ± 0.25b

Means followed by the same letter in the lines are not significantly different (Kruskal–Wallis followed by Dunn's multiple test) (α = 0.05).

3.2. Biochemical profiles

The concentration of total chlorophylls was 13-fold higher in the NGL when compared to the GA (Table 2). Chlorophylls *a* and *b*, anthocyanins and carotenoids were higher in the NGL than in the GA, but the chlorophyll *a*/*b* ratio was similar (Table 2).

Carbohydrate contents were significantly different between the NGL and the GA. The total soluble sugars (TSS) were higher in the NGL (182.25 ± 29.88 $mg \cdot g^{-1}$ DM) than in the GA (101. 99 ± 41.70 $mg \cdot g^{-1}$ DM), differently from the water-soluble polysaccharides (WSP), which were higher in the GA (16.57 ± 2.63 $mg \cdot g^{-1}$ DM in NGL; 22.19 ± 2.61 $mg \cdot g^{-1}$ DM in GA). Starch storage was not significantly different between the NGL and the GA (35.94 ± 10.03 $mg \cdot g^{-1}$ DM in NGL; 30.67 ±

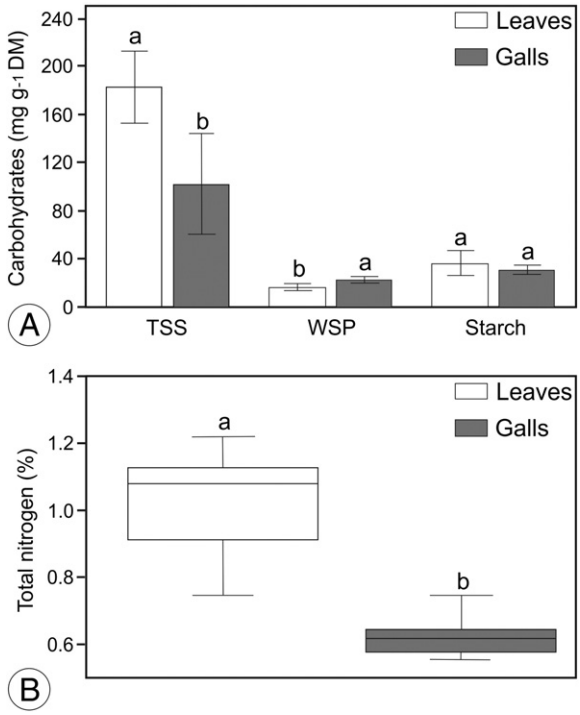

Fig. 2. Quantification of nutrients in the *Psidium myrtoides–Nothotrioza myrtoidis* system. A—Total soluble sugars (TSS), water soluble polysaccharides (WSP) and starch allocated in the leaves of *P. myrtoides* and leaf galls of *N. myrtoidis*; B—nitrogen content in the leaves of *P. myrtoides* and leaf galls of *N. myrtoidis*. Statistically different parameters are signed with different letters.

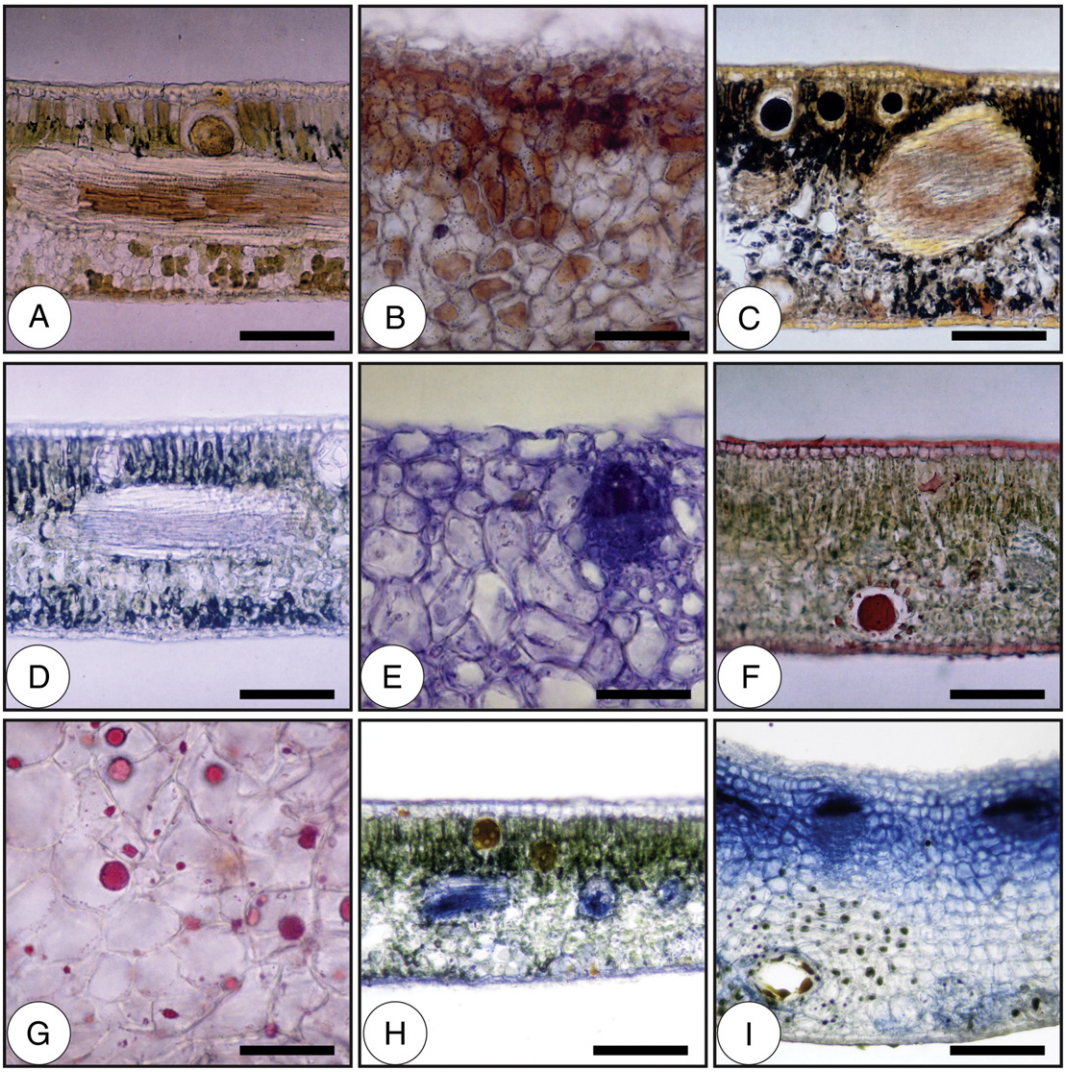

Fig. 3. Histolocalization of plant primary and secondary metabolites in the leaves of *P. myrtoides* and leaf galls of *N. myrtoidis*. A—Reducing sugar throughout leaf mesophyll; B—reducing sugars concentrated in the inner cortex of the gall; C—starch throughout leaf mesophyll; D—proteins throughout leaf mesophyll; E—proteins precipitated in the inner cortex of the gall; F—lipids throughout leaf mesophyll; G—lipids throughout gall cortex; H—essential oils concentrated on the phloem and oil glands in the leaf mesophyll; I—essential oils concentrated in the median and inner gall cortices. Bars: A, C, D, F, H, I—50 μm; B, E, G—25 μm.

2.98 $mg \cdot g^{-1}$ DM in GA) (Fig. 2A). The percentage of nitrogen in the NGL (1.04% ± 0.14) was higher than in the GA (0.62% ± 0.05) (Fig. 2B).

3.3. Histochemical profiles

Reducing sugars were detected as a brownish precipitate in the phloem, epidermal cells, chlorophyllous tissues, and epithelium of oil glands of the NGL (Fig. 3A). In the GA, reducing sugars formed a centripetal gradient from the outer cortex towards the nymphal chamber (Figs. 3B, 5). Primary starch grains stained in dark blue were only observed in the NGL mesophyll, mostly in the palisade parenchyma cells (Fig. 3C). Proteins were detected in the phloem and in the chlorophyllous tissues of the NGL (Fig. 3D), while in the GA they were mostly precipitated in the cortical parenchyma and in the vascular bundles (Fig. 3E). Lipids were revealed in the cuticle and in the lumen of glands of the NGL (Fig. 3F) and the GA, which also presented droplets scattered in the mesophyll (Figs. 3G, 5). Essential oils were observed in the secretion of glands, and in the phloem of the NGL (Fig. 3H). In the GA, essential oils were revealed in the median and inner cortices close to the nymphal chamber, especially in the phloem (Fig. 3I).

Total phenolics were observed throughout the mesophyll of the NGL, concentrated in the palisade parenchyma and more sparsely in the spongy parenchyma (Fig. 4A). In the GA, such phenolic compounds formed a centripetal gradient from the outer cortex until the median cortex, near the nymphal chamber (Figs. 4B, 5). Proanthocyanidins were detected in the epidermal cells, the adaxial layer of palisade parenchyma, and in isolated cells of the spongy parenchyma in NGL (Fig. 4C). In the GA, proanthocyanidins were revealed in the epithelium of the oil glands, in the parenchyma cells of the outer cortex, and in idioblasts scattered in the median cortex (Figs. 4D, 5). Alkaloids were weakly stained in the epidermis, mesophyll and collenchyma of the midrib in the NGL (Fig. 4E). In the GA, alkaloids were precipitated only in the epithelium of the oil glands (Figs. 4F, 5). Lignins were located in the mature xylem and associated fibers of the vascular bundles in the NGL, and in the lignified parenchyma in the outer cortex of GA (Figs. 4G, 5). ROS accumulated throughout the mesophyll of the NGL, and in the GA, the reaction was weak, but somewhat more evident in the outer cortex and vascular bundles (Figs. 4H, I and 5).

Phosphorylase, invertases and sucrose synthase activities were not detected either on the NGL or on the GA. Acid phosphatase activity was negative for the NGL and positive for the GA, while glucose-6-

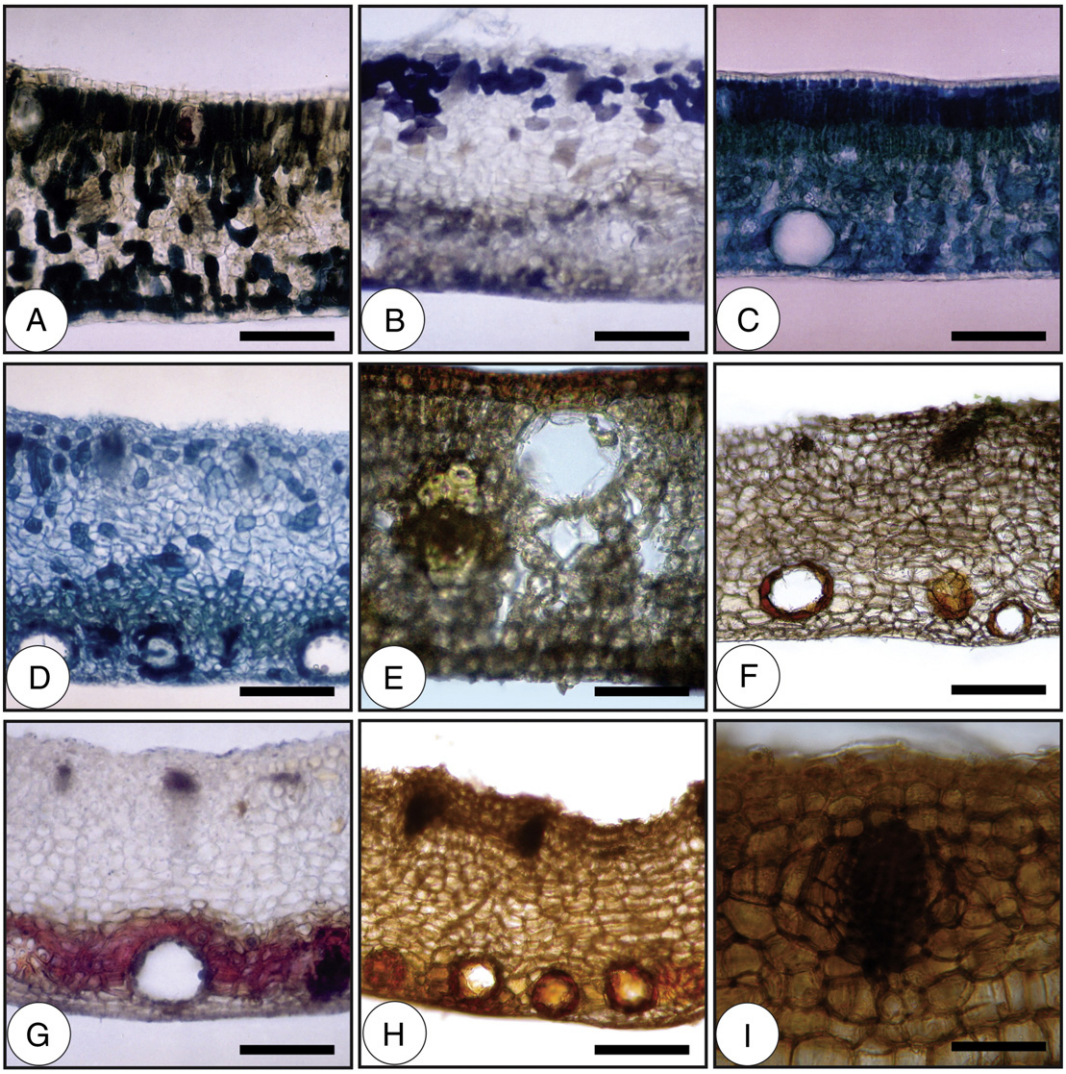

Fig. 4. Histolocalization of plant secondary metabolites and reactive oxygen species (ROS) in the leaves of *P. myrtoides* and leaf galls of *N. myrtoidis*. A—Total phenolics throughout leaf mesophyll; B—total phenolics concentrated in the outer and median cortices of the gall; C—proanthocyanidins throughout leaf mesophyll; D—proanthocyanidins concentrated in the outer and median cortices of the gall; E—alkaloids concentrated in the epidermis and leaf mesophyll; F—alkaloids concentrated in the epithelium of oil glands in the outer cortex of the gall. G—lignins on the sclerenchyma cells in the outer cortex of the gall; H—accumulation of ROS mainly in the outer and inner cortices of the gall; I—detail of intense ROS accumulation in the vascular bundles near the nymphal chamber. Bars: A, B, C, D, F, G, H—50 μm; E, I—25 μm.

phosphatase had positive results for the NGL and negative for the GA (Table 3, Fig. 5).

4. Discussion

4.1. Metabolic status on extralaminar galls

The galls induced by *N. myrtoidis*, i.e., extralaminar structures with few chlorophyllous parenchyma, and low content of photosynthetic pigments, are believed to be photosynthesis-deficient. Low content of photosynthetic pigments is a usual response of plant tissues to gall development (Yang et al., 2003; Khattab and Khattab, 2005; Oliveira et al., 2011a; Castro et al., 2012; Dias et al., 2013) for such structures have been classically described as sinks of photoassimilates (Burstein et al., 1994; Raman et al., 2006; Álvarez et al., 2009). Nevertheless, recent studies on the Neotropics have shown that galls may photosynthesize properly despite the decrease on the concentration of photosynthetic pigments. The intralaminar galls induced by *Pseudophacopteron* sp., a sucking-insect on *A. australe* (Oliveira et al., 2011a) present low anatomical alterations, and form a continuum with the leaf lamina, which does not imply in photosynthetic deficiency. On the other hand, the extralaminar structure of the horn-shaped galls studied by Castro et al. (2012) protrudes from the leaf lamina, and has few scattered chlorophyllous parenchyma. The absence of a morphological continuum similar to that of the intralaminar galls of *A. australe* is believed to impose major constraints on the physiological status of these extralaminar galls, similarly to the ones herein studied.

The galls induced by *N. myrtoidis* differentiate from their host non-galled tissues in a pre-established pattern for galls induced by Hemiptera (Meyer, 1987), whose structural changes are less drastic than those induced by Hymenoptera and Diptera. In fact, the lipids and essential oils present both in non-galled leaves of *P. myrtoidis* and in its galls represent a conservative pattern, as these compounds widely occur among the Myrtaceae (Ramos et al., 2010). Similarly, the presence of lipids in the galls of *Aceria lantanae*, a sap-sucking mite, on *Lantana camara* (Verbenaceae) was related to the characteristics of the host plant (Moura et al., 2008). Even though lipids are highly energetic molecules, this resource is not overstored in the galls of *N. myrtoidis*, indicating that their presence in the galls constitutes a constraint of plant metabolism that could neither be suppressed nor enhanced by the galling insect.

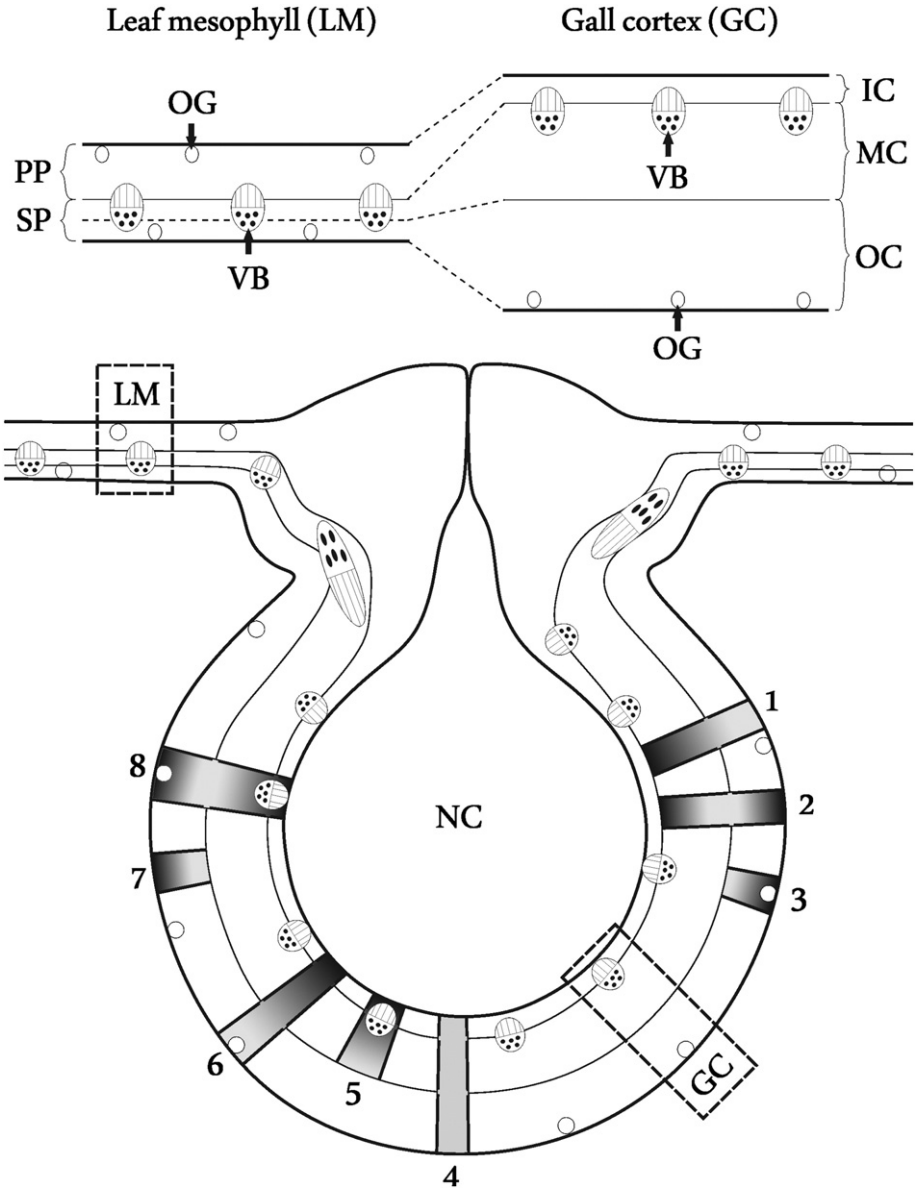

Fig. 5. Morpho-physiological continuum between the leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. The gall cortex (GC) is redifferentiated from the leaf mesophyll (LM), as the palisade parenchyma (PP) originates the inner cortex (IC), and the spongy parenchyma (SP) originates the median cortex (MC) and the outer cortex (OC). Vascular bundles (VB) are located near the nymphal chamber (NC) and oil glands (OG) occur only in the outer cortex of the galls. The intensity of gray color indicates the histochemical gradients. 1—phenolics; 2—proanthocyanidins; 3—alkaloids; 4—lipids; 5—acid phosphatase; 6—reducing sugars; 7—lignins; 8—ROS.

Nitrogen and carbohydrates, on the other hand, are commonly manipulated by the galling herbivores and may confer distinct metabolic status between the galls and the non-galled tissues (Hartley, 1998; Castro et al., 2012, 2013). The existence of a higher content of nitrogen implies a more intense protein metabolism (Heldt and Piechulla, 2010), which should be linked to the amount of RUBISCO in the photosynthesizing tissues of leaves. The contents of nitrogen and chlorophylls are lower in the galls induced by *N. myrtoidis* than in the non-galled leaves, fitting the pattern of a photosynthesis-deficient structure. As well, the total soluble sugars concentrated mainly in the non-galled leaves of *P. myrtoides*, as the galls of *N. myrtoidis* seem not to photosynthesize as its host leaves. Nevertheless, the galls accumulate as much starch as the leaves, and have more water soluble polysaccharides for they are sinks of photoassimilates. The activity of acid phosphatase in the galls of *N. myrtoidis* corroborates this metabolic feature, as this enzyme is important on the establishment of sinks in plant tissues (Koch, 2004), and form a gradient towards the nymphal chamber in the galls of *A. australe* (Oliveira and Isaias, 2010), where it probably breaks down starch into soluble sugars.

The nutritional analyses of galls induced by *N. myrtoidis*, together with the low content of photosynthesizing pigments, patterns of carbohydrates accumulation, and related enzyme activity indicate that these

Table 3
Histochemical detection of carbohydrate-related enzyme activity in non-galled leaves (NGL) and galls (G) of *Nothotrioza myrtoidis* (Hemiptera) in *Psidium myrtoides* (Myrtaceae).

Enzymes	Samples	
	NGL	G
Acid phosphatase	−	+
Glucose-6-phosphatase	+	−
Phosphorylase	−	−
Sucrose synthase	−	−
Invertases	−	−

Negative signs (−) represent non-detectable enzyme activity and positive signs (+) represent detectable enzyme activity.

galls function as sinks of photoassimilates. Moreover, even though the impact of *N. myrtoidis* does not determine the redifferentiation of highly specialized tissues, histochemical gradients for reducing sugars, and the activity of acid phosphatase were proven to be established towards the nymphal chamber. Current results partially corroborate the metabolic gradients previously described for other gall systems (Bronner, 1992; Oliveira et al., 2010, 2011b; Oliveira and Isaias, 2010), as these galls present such metabolic gradients, although limited to a lower extent when compared to galls induced by Cecidomyiidae and to other intralaminar galls induced by sucking-insects.

4.2. Histolocalization of defensive compounds and new functional designs

Histochemical tests allow the visualization of plant metabolites allocated in different tissues and cells. In the study of the morphogenesis of plant galls, cell redifferentiation (sensu Lev-Yadun, 2003) should lead to the establishment of new functional designs, which are related to the fitness of the galling insects (Weis and Abrahamson, 1986). Thus, the localization of secondary metabolites in gall tissues is often related to the protection of the gall inducer (Bronner, 1992). In the galls of *N. myrtoidis*, alkaloids, phenolics and proanthocyanidins confer repellency to the tissues, thus playing defensive roles. As a sucking insect that feeds mostly on the vascular bundles, *N. myrtoidis* benefits from the suppression of alkaloids accumulation on perivascular tissues observed on the host organs, but absent on galls, as these compounds are toxic for insects (Henriques et al., 2000). The reallocation of such compounds to the outermost tissues of gall cortex indicates an adaptive strategy that acts on the benefit of the galling insect (sensu Bronner, 1992) by changing the functionality of plant tissues. Also, phenolics and proanthocyanidins, which form a gradient from the outer towards the median cortex, may play secondary roles related to gall development.

Even though the phenolics are widespread in the leaves of *P. myrtoides*, this centripetal gradient protects the galling insect against its natural enemies (Abrahamson and Weis, 1997), and their allocation in specific tissues of the galls is known to improve the quality of their diet (Nyman and Julkunen-Tiitto, 2000). Despite being regularly associated with plant defenses against herbivory (Álvarez et al., 2008), the concentration of phenolics does not seem to affect gall infestation in all systems (Formiga et al., 2009) as gall-inducing insects are specialist herbivores and their saliva may readily degrade phenols (Hori, 1992). So, in the *N. myrtoidis–P. myrtoides* system, phenolic compounds are believed to modulate cell expansion and division, by acting as IAA oxidase inhibitors (Hori, 1992), thus allowing a higher rate of tissue growth. Furthermore, phenolics are known to be essential for the differentiation of vascular tissues (Aloni, 2001), which in the case of these galls should enable the neo-formation of vascular bundles that nourish the insect and help establishing these galls as physiological sinks.

Structural, histochemical and physiological modifications in plant tissues due to gall morphogenesis have been greatly explored, but the biochemistry that underlies this process is still poorly known. Nevertheless, the accumulation of ROS in gall tissues seems to determine the functionality of plant tissues due to gall induction. According to Rosseti and Bonnatti (2001), ROS production is one of the first steps in the hypersensitive response of plants to pathogens and, in the case of galls, may trigger gall morphogenesis (Isaias and Oliveira, 2012). The ROS usually accumulate in tissues with high metabolic activity, and galls may present sophisticated mechanisms of ROS scavenging (Oliveira et al., 2011a) to prevent damage to their photosynthetic apparatus. However, the parenchymatic galls of *N. myrtoidis* present a discrete gradient of ROS due to their low metabolism. This gradient differs from those described for galls induced by Cecidomyiidae which have nutritive tissues (Oliveira et al., 2010), and from other intralaminar galls induced by sucking insects which have storage tissues (Oliveira and Isaias, 2010; Isaias et al., 2011). The accumulation of proanthocyanidins in the same sites of ROS, where they probably act as antioxidants (Simmonds, 2003; Bouaziz et al., 2005), constitutes a possible strategy that prevent gall tissues to undergo major alterations in response to oxidative stress.

The determination of tissues with different functionalities in the galls seems to be related not only to the insect's feeding activity, but also to the structure and metabolism of the gall. In the *N. myrtoidis–P. myrtoides* system, the broad chambered structure of the gall, the dispersed feeding sites of the galling insect, and the allocation of proanthocyanidins hindered the accumulation of ROS, and determined minor alterations on gall structure. The functional design of these galls partially corroborate the literature concerning the allocation of secondary metabolites, as the roles of phenolics and proanthocyanidins do not seem to be strictly related to defense.

5. Conclusions

The features of the *N. myrtoidis–P. myrtoides* system are unique in the Neotropics. Differently from the extralaminar galls induced by Cecidomyiidae and the intralaminar galls induced by other sucking-insects, carbohydrate-related enzyme activity is poorly detected in the galls of *N. myrtoidis*. The activity of acid phosphatase and the histolocalization of reducing sugars indicate the establishment of a metabolic gradient that ensures the nutrition of the gall inducer and maintenance of the gall structure. The accumulation of water soluble polysaccharides in galls, even with reduced chlorophyll contents, indicates that these galls are sinks of photoassimilates. The lack of morphological continuum and physiological continuum between extralaminar galls of *N. myrtoidis* and their host plant organs, together with the limited feeding impact of the sucking insect, determines the establishment of a photosynthesis-deficient structure. The histochemical profiles of ontogenetically correspondent tissues in the leaves and the galls are distinct, and neo-established histochemical gradients were detected. The histochemical profile of secondary metabolites indicated the generation of neo-formed functional designs in the cortices of the galls, where phenolics may regulate gall growth, and proanthocyanidins may take part on the control of oxidative stress, thus modulating the physiological status of the gall.

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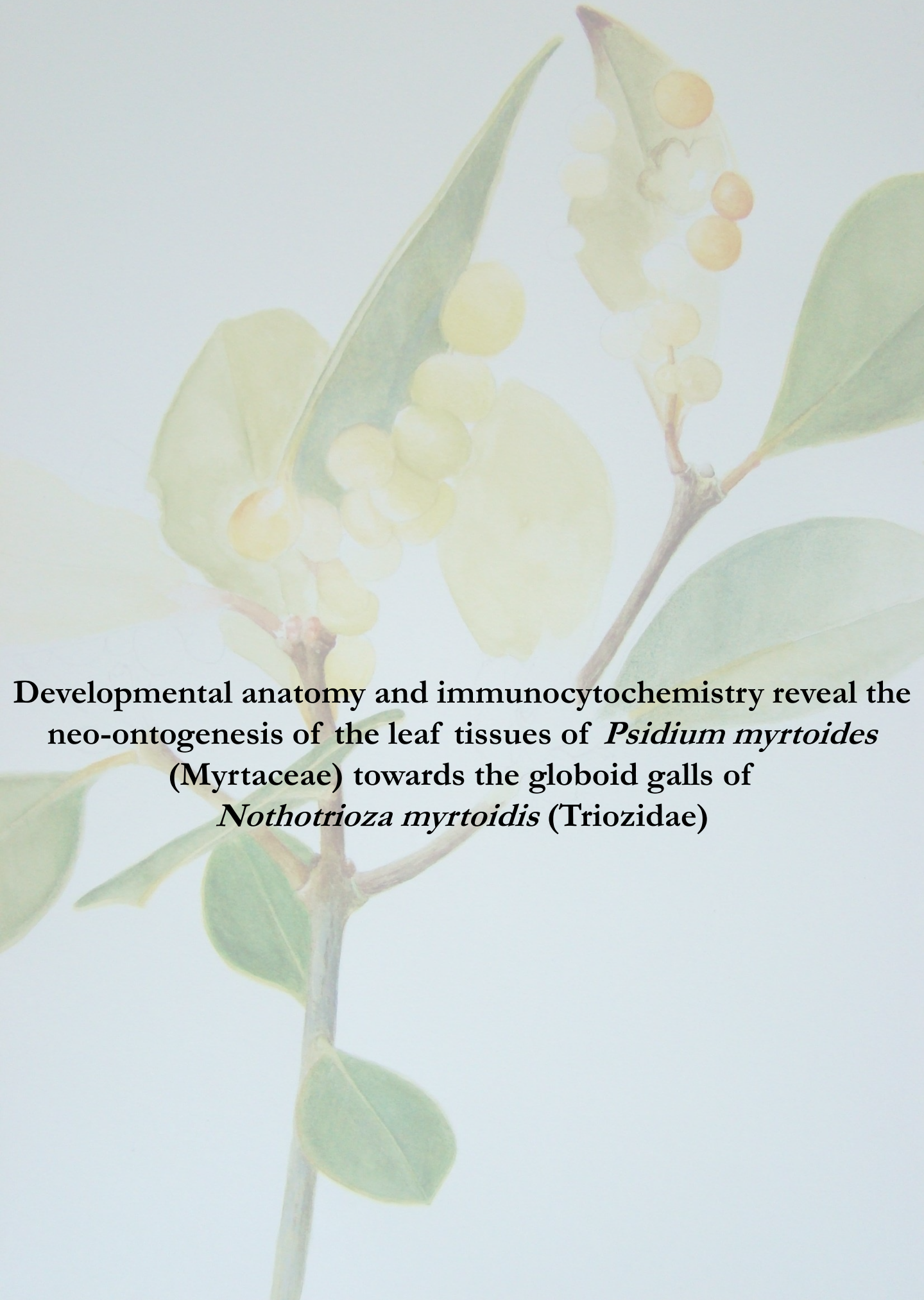

Developmental anatomy and immunocytochemistry reveal the neo-ontogenesis of the leaf tissues of *Psidium myrtoides* (Myrtaceae) towards the globoid galls of *Nothotrioza myrtoidis* (Triozidae)

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ORIGINAL PAPER

Developmental anatomy and immunocytochemistry reveal the neo-ontogenesis of the leaf tissues of *Psidium myrtoides* (Myrtaceae) towards the globoid galls of *Nothotrioza myrtoidis* (Triozidae)

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Abstract

Key message **The temporal balance between hyperplasia and hypertrophy, and the new functions of different cell lineages led to cell transformations in a centrifugal gradient that determines the gall globoid shape.**

Abstract Plant galls develop by the redifferentiation of new cell types originated from those of the host plants, with new functional and structural designs related to the composition of cell walls and cell contents. Variations in cell wall composition have just started to be explored with the perspective of gall development, and are herein related to the histochemical gradients previously detected on *Psidium myrtoides* galls. Young and mature leaves of *P. myrtoides* and galls of *Nothotrioza myrtoidis* at different developmental stages were analysed using anatomical, cytometrical and immunocytochemical approaches. The gall parenchyma presents transformations in the size and shape of the cells in distinct tissue layers, and variations of pectin and protein domains in cell walls. The temporal balance between tissue hyperplasia and cell hypertrophy, and the new functions of different cell lineages led to cell transformations in a centrifugal gradient, which determines the globoid shape of the gall. The distribution of cell wall epitopes affected cell wall flexibility and rigidity, towards gall maturation. By senescence, it provided functional stability for the outer cortical parenchyma. The detection of the demethylesterified homogalacturonans (HGAs) denoted the activity of the pectin methylesterases (PMEs) during the senescent phase, and was a novel time-based detection linked to the increased rigidity of the cell walls, and to the gall opening. Current investigation firstly reports the influence of immunocytochemistry of plant cell walls over the development of leaf tissues, determining their neo-ontogenesis towards a new phenotype, i.e., the globoid gall morphotype.

Keywords Anisotropy · Cell fate · Cytometry · Immunocytochemistry · Isotropy

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Introduction

Galls are new plant organs with conservative shapes (Raman 2007) and complex tissue organisation (Rohfritsch 1992). Plant organs are known to have peculiar dynamics of cell division and elongation, i.e., isotropic and anisotropic expansion (Baskin 2005), which lead to their typical shapes. In the Neotropics, developmental studies of plant galls have described detailed ontogenetical steps (Isaias et al. 2011; Oliveira and Isaias 2010; Moura et al. 2009; Magalhães et al. 2014) with special attention to cell hypertrophy and tissue hyperplasia that determine their size, new forms and functions. The functionality of plant tissues depends on cell structure, which varies during plant development (Buvat 1989). The composition of plant cell walls and the properties conferred by their different chemicals have already been described (Knox et al. 1990; Willats et al. 2000; Albersheim et al. 2011). However,

variations in the structure and functionality of the cell walls during gall development have only recently been focused.

The disposition of cellulose microfibrills in the cell walls of *Baccharis dracunculifolia*, and their rearrangement during gall development were demonstrated by Magalhães et al. (2014), as the cells acquired new sizes and shapes. Formiga et al. (2013) and Oliveira et al. (2014) provided interesting insights over the functionality of plant tissues by assessing the distribution of pectins in the cell walls of different gall morphotypes using specific monoclonal antibodies. Pectins are major components in the primary cell walls of higher plants that influence the porosity, adhesion, flexibility and defensiveness of cells (Jarvis 1984; Albersheim et al. 2011). They are synthesised and secreted in a high methylesterified form (Albersheim et al. 2011), and may be modified by pectin methylesterases (PMEs) during the development of plant cells. In such cases, the previously methoxylated carboxyl groups present other substituents (Jolie et al. 2010), which cross link with Ca^{2+} and increase the rigidity of the cell wall and of the whole tissue (Catoire et al. 1998; Albersheim et al. 2011). Thus, the use of monoclonal antibodies for investigating the degree of pectin methylesterification can indirectly demonstrate the activity of PMEs (Liu et al. 2013; Oliveira et al. 2014). Herein, the gall induced by *Nothotrioza myrtoidis* Burck. on *Psidium myrtoides* O. Berg. is used as a model of study of plant cell wall properties under the developmental approach. The dynamics of the cell size and shape, and the cell wall polysaccharides are analysed to elucidate the determination of gall globoid shape, and to confirm the functional roles defined by the composition of the cell walls as proposed in literature.

Nothotrioza myrtoidis is a recently described species of Triozidae (Hemiptera: Psylloidea) (Carneiro et al. 2013) that induces conspicuous globoid galls with a large nymphal chamber and unique metabolic features among the Neotropical galls (Carneiro et al. 2014). We hypothesise that there is a gradient of cell transformations in the tissues of the gall, which are responsible for the development of the globloid shape, and that these transformations depend on variations in the composition of the cell walls. The following questions are addressed: (1) how is the gradient of cell transformations established during the development of the galls?, (2) Are there particular dynamics of cell expansion and division at each developmental stage of the galls? And (3) how does the distribution of pectin and protein epitopes affect the structure and functionality of the tissue layers along the development of the galls?

Materials and methods

Study area

The studied population of *P. myrtoides* is located at the park *Reserva do Patrimônio Privado Natural da Serra do Caraça*, municipality of Catas Altas, state of Minas Gerais, Brazil (20°06′22″ S–43°29′42″ W). Individuals presenting galled leaves were accompanied during two cycles of the galls in 2012 and 2013.

Structural analyses

Samples of young and mature leaves, and galls at different developmental stages, i.e., induction, growth and development, maturation and senescence (sensu Carneiro et al. 2013) ($n = 5$ per developmental stage) were collected from different individuals and fixed in Karnovsky's solution in 0.1 M phosphate buffer (pH 7.2) (O'Brien and McCully 1981). The material was dehydrated in *n*-butyl series (Johansen 1940), embedded in Paraplast® (Kraus and Arduin 1997), sectioned (12–14 μm) with a rotary microtome (Jung biocut) and stained with 9:1 (v/v) solution of Astra blue and safranin (Bukatsch 1972, modified to 0.5 %). Fixed young leaves with the eggs of *N. myrtoidis* and early developmental stages of the galls were washed in phosphate buffer, post fixed in 1 % osmium tetroxide for 2 h, and dehydrated in ethanol series (Johansen 1940). The material was critical point dried with carbon dioxide (BAL-TEC® CPD030), mounted on aluminum stubs and covered with 30 ηm of gold (BAL-TEC® SCD050) for observation under the scanning electron microscope (LEO EVO® 40).

Cytometry and histometry

Digital images were obtained with an optical photomicroscope Zeiss® Primo Star, and analysed with the AxioVision® software, Zeiss Imaging Systems, version 4.7.2 (Zeiss® 2008). Cells from the adaxial and abaxial protoderm, the adaxial, median and abaxial layers of the ground meristem of non-galled leaves, as well as their redifferentiated gall tissues had their areas individually measured ($n = 150$; 5 cells of each tissue per image, 30 images per developmental stage). The thickness of the mesophyll was measured at different developmental stages, and the number of oil glands per area (0.37 mm^2; area of the image), and the area of the lumen + secretory epithelium were evaluated in mature non-galled leaves and galls.

Immunocytochemistry of plant cell wall epitopes

Galls at different developmental stages ($n = 3$; from different individuals) were fixed in Karnovsky's solution in 0.1 M phosphate buffer (pH 7.2) (O'Brien and McCully 1981), dehydrated in ethanol series (Johansen 1940), and embedded in glycolmethacrylate (Leica®). Sections (6–10 μm) were incubated with the monoclonal

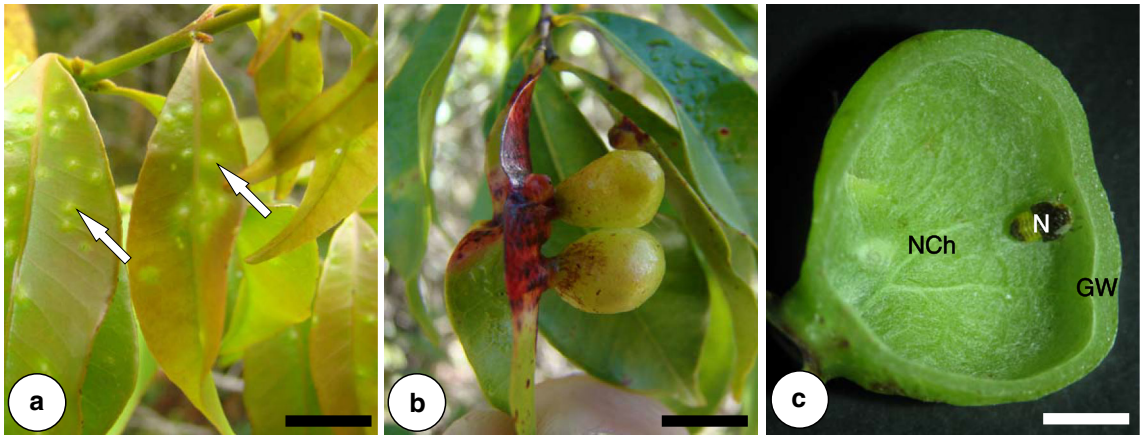


Fig. 1 Morphology of the galls induced by *Nothotrioza myrtoidis* on the leaves of *Psidium myrtoides*. **a** Induction of galls by the first instar nymphs (*arrows*) on the adaxial leaf surface. **b** Mature globoid gall morphotype protruded towards the abaxial leaf surface. **c** Sectioned gall evidencing thin gall wall (*GW*) and ample nymphal chamber (NCh) sheltering a single nymph (N). *Bars* **a**, **b** = 1 cm; **c** = 1 mm

antibodies JIM5, JIM7, LM1, LM2, LM5, and LM6 (Centre for Plant Sciences, University of Leeds, UK). These antibodies specifically bind epitopes of low methylesterified homogalacturonans (HGAs) (JIM5) (Vanderbosch et al. 1989; Knox et al. 1990; Willats et al. 2000; Clausen et al. 2004), high methylesterified HGAs (JIM7) (Knox et al. 1990; Willats et al. 2000; Clausen et al. 2004), extensins (LM1) (Smallwood et al. 1995; Cassab 1998; Sabba and Lulai 2005; Leroux et al. 2011), arabinogalactan proteins (APGs) (LM2) (Smallwood et al. 1996), galactans (LM5) (Jones et al. 1997), and arabinans (LM6) (Willats et al. 1998).

The sections were immersed in blocking solution with 3 % (w/v) powdered milk in PBS for 30 min to avoid cross labelling, and incubated with primary antibodies in PBS for 2 h at room temperature. For the control tests, the primary antibodies were suppressed. Sections were washed in PBS, and incubated in the secondary antibody anti-rat IgG—FITC (sigma) in PBS for 2 h in the dark. After washing in PBS, the sections were mounted in 50 % glycerin and analysed under a Confocal Zeiss 510 META microscope, with excitation wavelength of 488 ηm and 505–530 ηm emission filter.

Statistical analyses

Statistical analyses of the cytometric variables were performed using the JMP® software (SAS Institute, US, 1989–2002). Normal data (Shapiro–Wilk test) were compared by parametric tests of ANOVA followed by *t* test or multiple tests of Tukey. Non-normal data were compared using the non-parametric tests of Kruskal–Wallis followed by Dunn's multiple tests. All tests used $\alpha = 0.05$.

Results

Macroscopic features

First instar nymphs of *N. myrtoidis* induce galls on the adaxial surface of the young leaves of *P. myrtoides* (Fig. 1a). The leaf lamina invaginates and projects towards the abaxial surface, as the galls develop into a globoid structure (Fig. 1b). Mature galls are broad chambered and have thin walls (Fig. 1c).

Developmental anatomy of leaves and galls

The qualitative aspects of cell and tissue transformations during the developmental pathways of leaves and galls are quite distinct. Leaves develop and expand towards the common laminar pattern, while the globoid galls develop with the formation of a pocket in the leaf lamina, and the establishment of a gradient of cell size increment towards the outer surface.

Transverse sections of the leaves from the first node show a single-layered protoderm, and a multilayered ground meristem with interspersed procambial strands (Fig. 2a). Three regions can be distinguished in the ground meristem of developing leaves, namely, the single-layered adaxial meristem, the three-layered median meristem and the two-layered abaxial meristem (Fig. 2b). At the second node, the leaves have a single-layered adaxial meristem, three-layered median meristem and two-layered abaxial meristem (Fig. 2c). Subsequently, periclinal divisions add one cell layer to the abaxial meristem, and two cell layers to the adaxial meristem (Fig. 2d). By the fourth and fifth nodes, leaves have a nine-layered mesophyll (Fig. 2e) and, at the sixth node, the cells of the adaxial meristem elongate

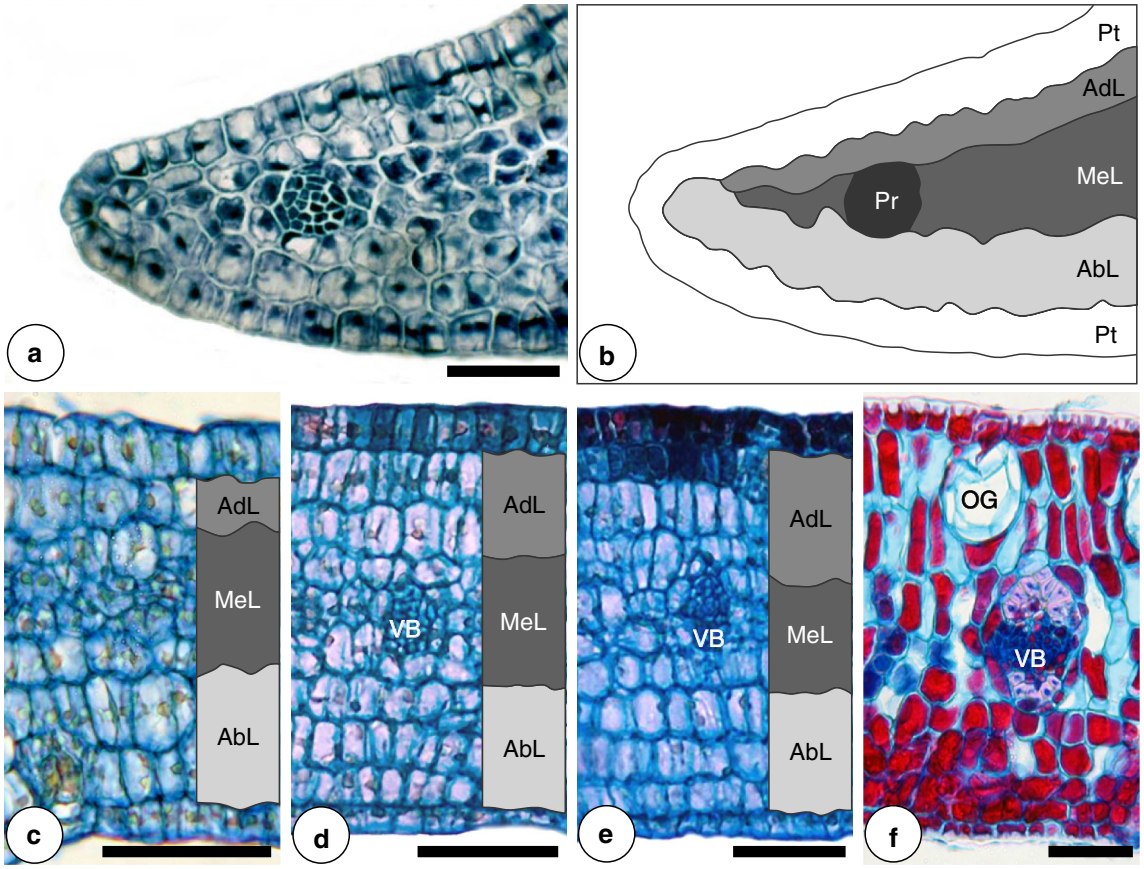


Fig. 2 Developmental anatomy of the leaves of *Psidium myrtoides* in transverse sections. **a** Leaf anatomy. **b** Schematic diagram. **a**, **b** First node leaf evidencing protoderm (*Pt*), procambium (*Pr*) and ground meristem divided into adaxial (*AdL*), median (*MeL*) and abaxial (*AbL*) layers. **c** Second node leaf with 1-layered AdL, 3-layered MeL and 2-layered AbL. **d** Third node leaf with 2-layered AdL, 3-layered MeL and 3-layered AbL. **e** Fourth/fifth node leaf with 3-layered AdL, 3-layered MeL and 3-layered AbL. **f** Sixth node mature leaf with 3-layered palisade parenchyma and 6-layered spongy parenchyma. *VB* vascular bundle, *OG* oil gland. *Bars* **a** = 10 µm; **c**–**f** = 25 µm

and differentiate into the palisade parenchyma, while the median meristem and the abaxial meristem give rise to the spongy parenchyma (Fig. 2f). Oil glands differentiate from both the adaxial and the abaxial surfaces of the epidermis in a schizogenous process and grow towards the adaxial and abaxial meristems of the mesophyll.

The margins of the leaves from the second to the fifth node are the preferable oviposition sites of *N. myrtoidis*, where the females insert the peduncle of their eggs (Fig. 3a, b). As the eggs hatch, the first instar nymphs move to the leaf lamina, where they feed and trigger gall morphogenesis (Fig. 3c). During the induction phase, cells underneath the body of the gall inducer hypertrophy, and cells from the adaxial meristem surrounding the nymph elongate and divide, covering the insect (Fig. 3d, e). At the end of the phase of induction and beginning of growth and development, the first instar nymphs are already surrounded by the plant tissues; the ostiole is on the adaxial portion of the gall and the cortex protrudes on the opposite side (Fig. 3f). As the galls continue to develop, the cells of the ostiole lignify. The cortex of the gall is divided into the outer, median and inner homogenous cortices, respectively, derived from the adaxial, median and abaxial meristems of the young and developing leaves, which have undergone cell hypertrophy and tissue hyperplasia (Fig. 3g). At the phase of maturation, the outermost layers of the outer cortex lignify, and the other cortical cells hypertrophy and vacuolize (Fig. 4a). At senescence, insect's stimuli trigger gall dehiscence, and the cortical cells accumulate phenolic substances at the sites of cell collapse and death (Fig. 4b, c). These events occur from the top towards the base of the galls; the wall ruptures, and the adult insects emerge (Fig. 4d).

The inner and outer surfaces of the epidermis of the galls are respectively derived from the adaxial and abaxial surfaces of the epidermis of the leaves. In the galls, the oil glands differentiate exclusively from the outer epidermis and they are more numerous ($6 \pm 1.54a$ in galls and

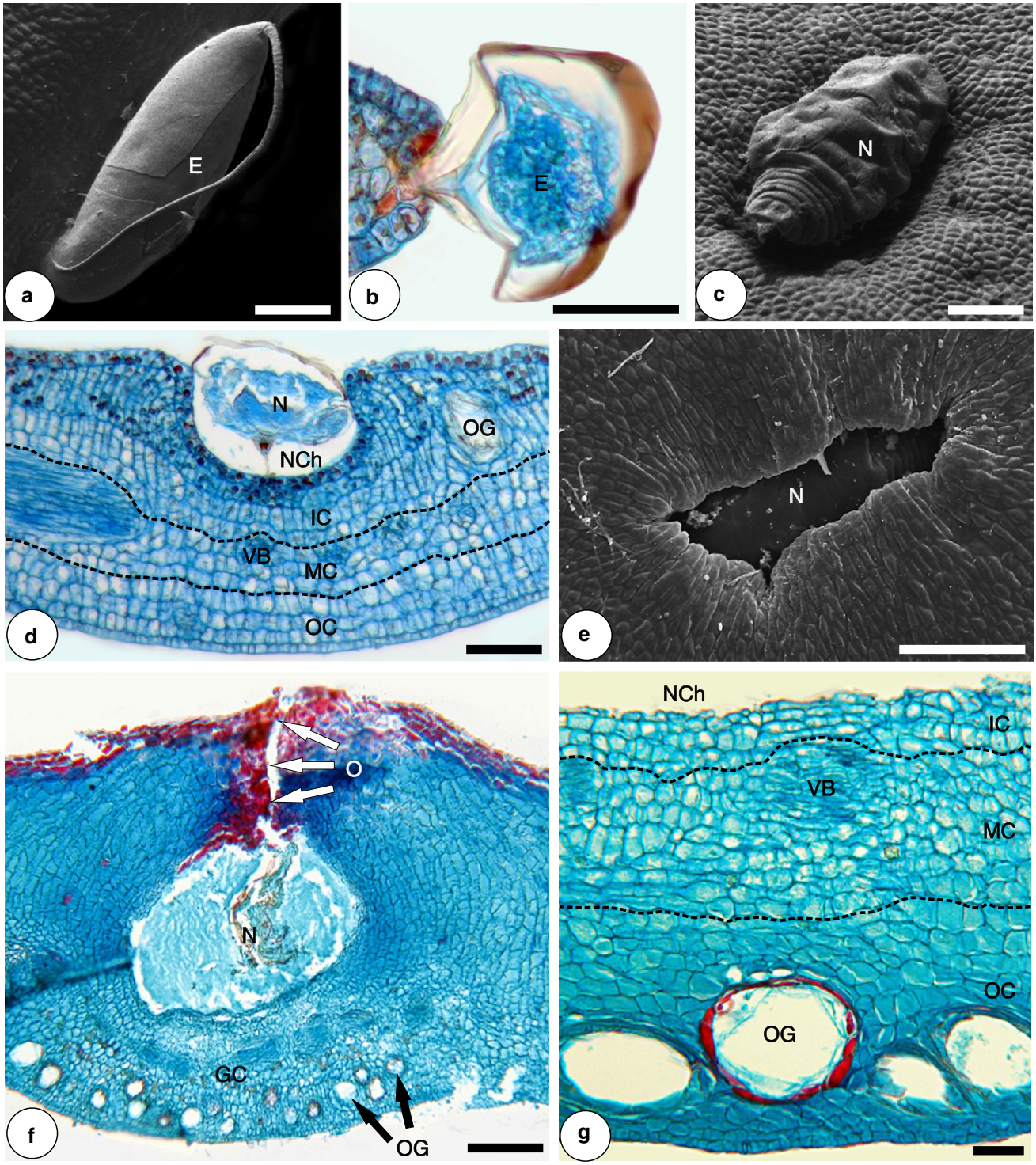


Fig. 3 Developmental anatomy of the galls induced by *Nothotrioza myrtoidis* on the leaves of *Psidium myrtoides*. **a**, **c**, **e** Scanning electron microscopy; **b**, **d**, **f**, **g** light microscopy; transverse sections. **a** An egg on the margin of the leaf. **b** Peduncle of the egg inserted in the tissues of the leaf margin. **c** First instar nymph on the adaxial surface of the leaf. **d** Induction phase. Gall cortex divided into inner (*IC*), median (*MC*) and outer (*OC*) cortices. **e** Detail of the leaf tissues covering the first instar nymph. **f** Endophytic stage of gall induction phase evidencing the formation of the ostiole (*O*) on the adaxial surface of the gall and the gall cortex (*GC*) on the abaxial surface. **g** Homogenous gall cortex during the phase of growth and development. It is divided into *IC*, *MC* with vascular bundles (*VB*), and *OC* with oil glands (*OG*). *Bars* 50 µm

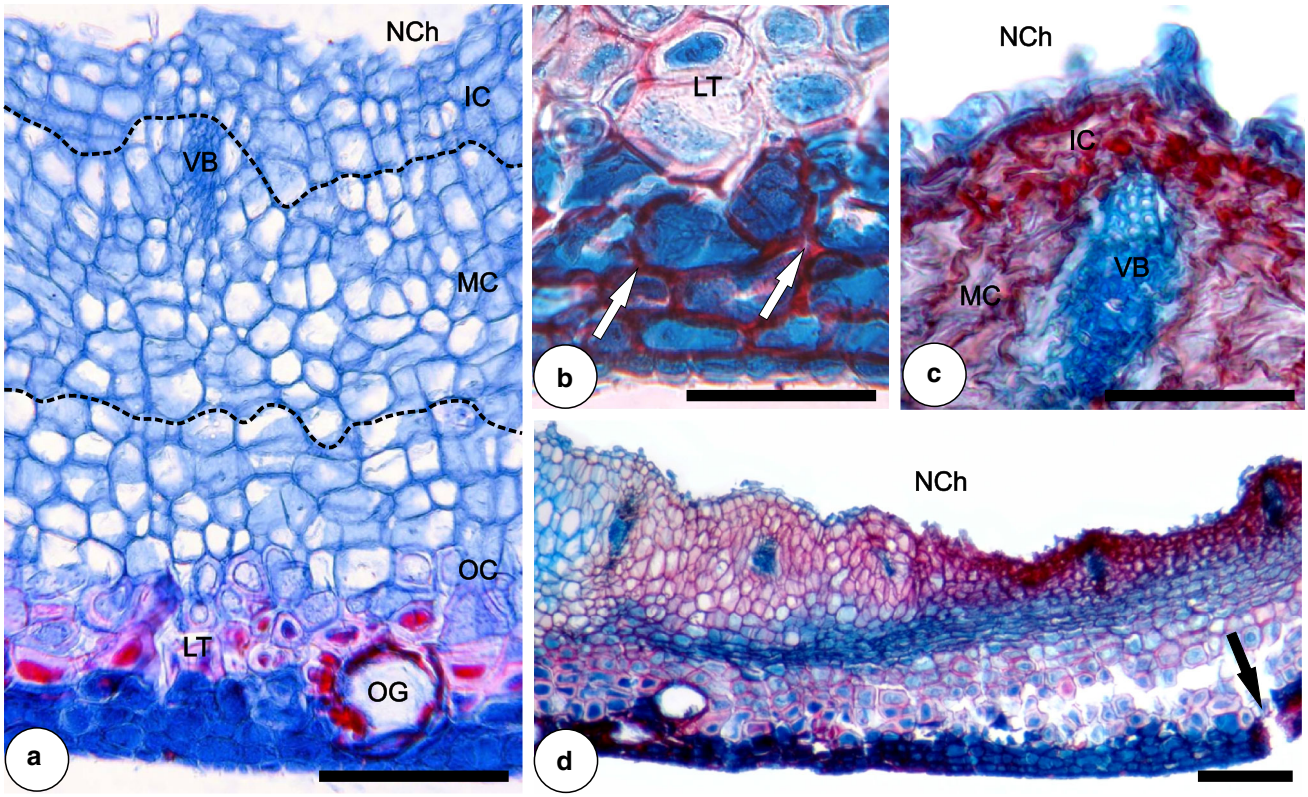


Fig. 4 Developmental anatomy of the galls of *Nothotrioza myrtoidis* on the leaves of *Psidium myrtoides*. Transverse sections. **a** Mature gall with hypertrophic inner (*IC*), median (*MC*) and outer (*OC*) cortices. The outermost layers of the OC have cells with lignified thickened walls. **b**, **c**, **d** Senescent gall. **b** Detail of cell wall degeneration in the *OC*. **c** Detail of cell collapse in the *IC* and *MC*. **d** Cell wall degeneration and cell collapse determining the rupture of the gall cortex (*arrow*) for the emergence of the adult galling insect. *Bars* **a**, **d** = 100 μm; **b**, **c** = 50 μm

4 ± 0.85*b* in leaves) (per area of the image −0.37 mm^2) and larger (4,972.37 ± 2,188.68*a* μm^2 in galls and 2,893.57 ± 718.23*b* μm^2 in the leaves) (mean ± standard deviation) at gall sites when compared to the leaves.

Dynamics of cell size and shape during gall development

The cells and tissues of *P. myrtoides* alter qualitatively and quantitatively during the morphogenesis of the globoid galls of *N. myrtoidis.* The increment in cell area together with new directions of cell elongation defines a centripetal gradient of cell transformations.

The cells from the adaxial surface of the epidermis of the leaves are anticlinally elongated due to anisotropic growth (Fig. 5a). The cells of inner surface of the epidermis in galls are significantly larger (Table 1) and also grow anisotropically, but elongate periclinally (Fig. 5b–d). The epidermis is the less altered tissue, and increases its size in about 170–190 % from the non-galled leaves until mature galls (Table 1). The inner, median and outer cortices of the galls are, respectively, derived from the adaxial, median and abaxial meristems of the non-galled leaves, and the type of cell growth changes from anisotropic to isotropic due to massive hypertrophy (Fig. 5a–l). The cells of the inner cortex increase in size mostly from the stage of growth and development to the stage of maturation, when they are about 530 % larger than the cells of the adaxial meristem of the leaves (Table 1). The median and the outer cortices present the most altered tissues and have similar dynamics, as their cells are 620 and 1,200 % larger than the cells of the median and abaxial meristems of the leaves, respectively (Table 1). The cells of the abaxial surface of the leaf epidermis present anisotropic expansion (Fig. 5m). They originate the cells of the outer epidermis of the galls which are about 300 % larger at maturation phase (Table 1), and are periclinally elongated due to anisotropic expansion (Fig. 5n–p). The wall of the gall thickens gradually during its development from the non-galled leaf (136.5 ± 0.52*d* μm) through the phases of induction (163.8 ± 1.63*c* μm), growth and development (372.0 ± 4.09*b* μm), until maturation (558.9 ± 8.30*a* μm),

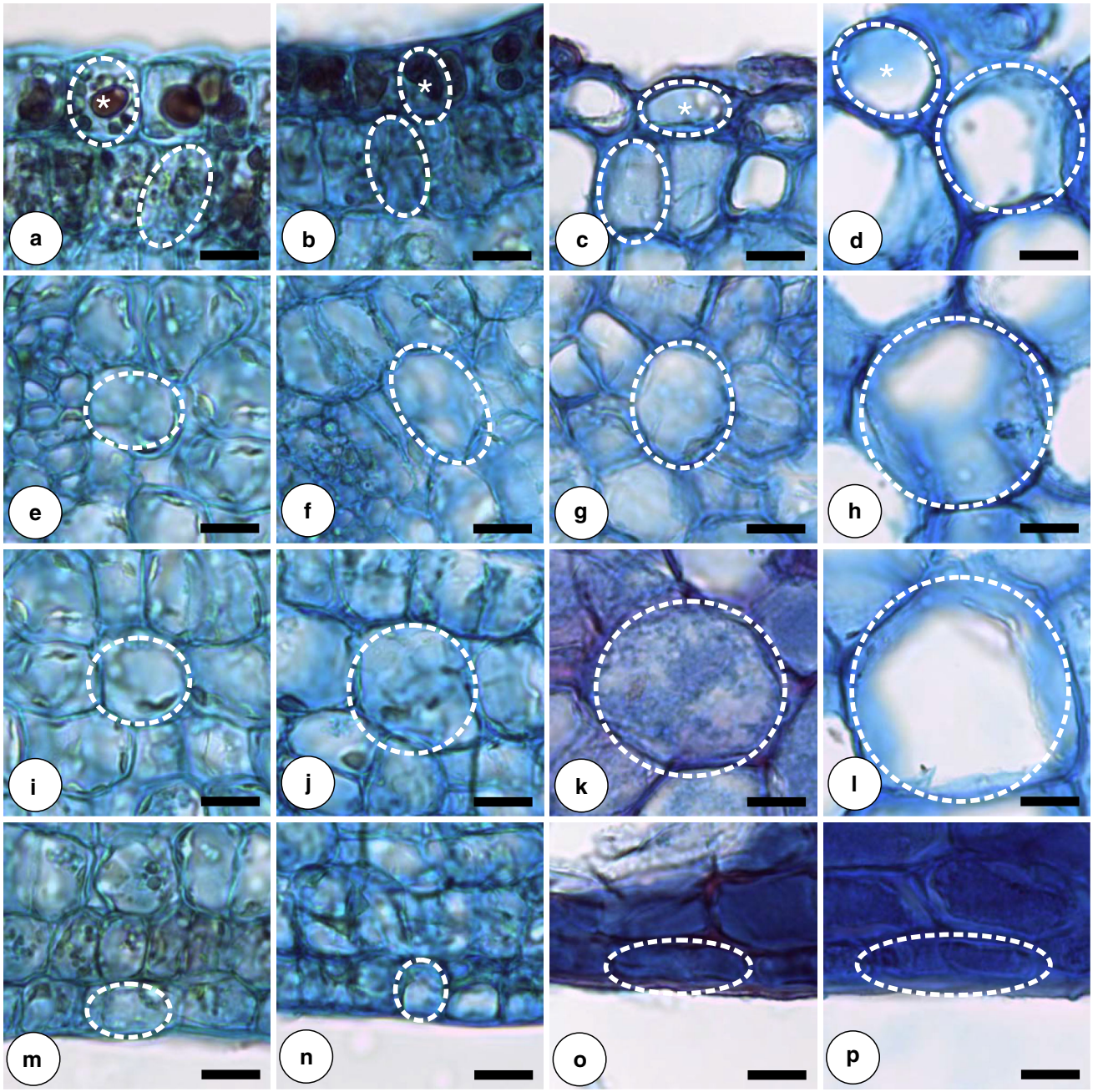


Fig. 5 Gradients of cell transformations and patterns of cell shape during the developmental anatomy of the galls induced by *Nothotrioza myrtoidis* on the leaves of *Psidium myrtoides*. **a**, **e**, **i**, **m** Young non-galled leaves; **b**, **f**, **j**, **n** Galls at induction phase; **c**, **g**, **k**, **o** Galls at growth and development phase; **d**, **h**, **l**, **p** Galls at maturation phase. **a**, **b**, **c**, **d** Inner epidermal (*asterisks*) and inner cortical cells; **e**, **f**, **g**, **h** median cortical cells; **i**, **j**, **k**, **l** outer cortical cells; **m**, **n**, **o**, **p** outer epidermal cells. A centrifugal gradient of cell hypertrophy is established. *Dotted lines* indicate the type of cell growth, which is either anisotropic (*flattened cells*) or isotropic (*rounded cells*) (sensu Baskin 2005). *Bars* 10 μm

when it is about 300 % thicker than the non-galled leaves (values followed by different letters are significantly different by the test of Kruskal–Wallis, followed by multiple Dunn's test. $\alpha = 0.05$). The senescent galls have necrotic and collapsed cells that could not be assessed by cytometric and histometric analyses.

Dynamics of cell wall epitopes during gall development

The distribution of pectin and protein epitopes is described for each tissue layer, and on the developmental anatomy basis. Such approach evidences the establishment of the functional gradients from the inner to the outer tissue

Table 1 Gradients of cell hypertrophy during the developmental anatomy of the galls induced by *Nothotrioza myrtoidis* on the leaves of *Psidium myrtoides* (mean cell area − μm^2 ± standard deviation)

Cell lineages	Developmental stage			
	Non-galled young leaf	Gall induction	Gall growth and development	Gall maturation
Adaxial epidermis/inner epidermis	110.6 ± 1.57*c*	130.6 ± 2.43*b*	132.8 ± 3.11*b*	306.6 ± 5.86*a*
Adaxial meristem/inner cortex	142.2 ± 2.48*d*	198.8 ± 3.65*c*	335.5 ± 7.15*b*	907.9 ± 15.63*a*
Median meristem/median cortex	185.1 ± 2.76*d*	237.1 ± 4.16*c*	445.9 ± 8.61*b*	1,340.0 ± 23.83*a*
Abaxial meristem/outer cortex	136.3 ± 2.44*d*	194.3 ± 3.49*c*	606.2 ± 8.18*b*	1,772.0 ± 27.45*a*
Abaxial epidermis/outer epidermis	67.37 ± 1.30*d*	93.28 ± 1.88*c*	127.8 ± 2.19*b*	199.5 ± 5.02*a*

Lines show ontogenetically correspondent tissues, and values followed by different letters in the lines are significantly different (Kruskal–Wallis, followed by multiple Dunn's test) ($\alpha = 0.05$) ($n = 150$; 5 cells of each tissue per image, 30 images per developmental stage)

layers, and the distribution of each epitope from gall induction until gall senescence.

The inner epidermis presented no detection for the investigated epitopes during the phase of gall induction. The high methylesterified HGAs and galactans were detectable at the phase of growth and development by the antibodies JIM7 (Fig. 6a) and LM5, respectively. The epitopes of high methylesterified HGAs were maintained in the cell walls of the inner epidermis during the phases of maturation (Fig. 6b) and senescence of the galls, but the epitopes of galactans were undetectable.

All the cortical layers had epitopes of high methylesterified HGAs labelled by JIM7 from the phase of growth and development until senescence (Fig. 6a–f), but the low methylesterified HGAs and AGPs were labelled by JIM5 and LM2 in the outer cortex only of the senescent galls (Fig. 6g, h). The epitopes of galactans bound by LM5 were detected in the median and outer cortices during the phase of growth and development (Fig. 6i, j), only in the inner cortex during the phase of maturation (Fig. 6k), and in all cortical layers during the phase of senescence (Fig. 6l, m). The epitopes of arabinans were labelled by the antibody LM6 exclusively in the outer cortex of the galls at the phases of maturation (Fig. 6n) and senescence.

In the vascular tissues of the galls, only the epitopes of high methylesterified HGAs were labelled by JIM7 in all phases of gall development (Fig. 6b, e, o). The epitopes of galactans were detected by LM5 in the vascular bundles of the galls only during the phase of growth and development (Fig. 6i). In the oil glands, no labelling was observed during the phase of gall induction. The higher diversity of epitopes was detected during the phase of growth and development, when the epitopes of high methylesterified HGAs, extensins, AGPs, galactans and arabinans were labelled by JIM7, LM1, LM2, LM5 and LM6, respectively (Fig. 6c, p, q, r). During the phases of gall maturation and senescence, only the epitopes of high methylesterified HGAs were detected by JIM7 in the oil glands.

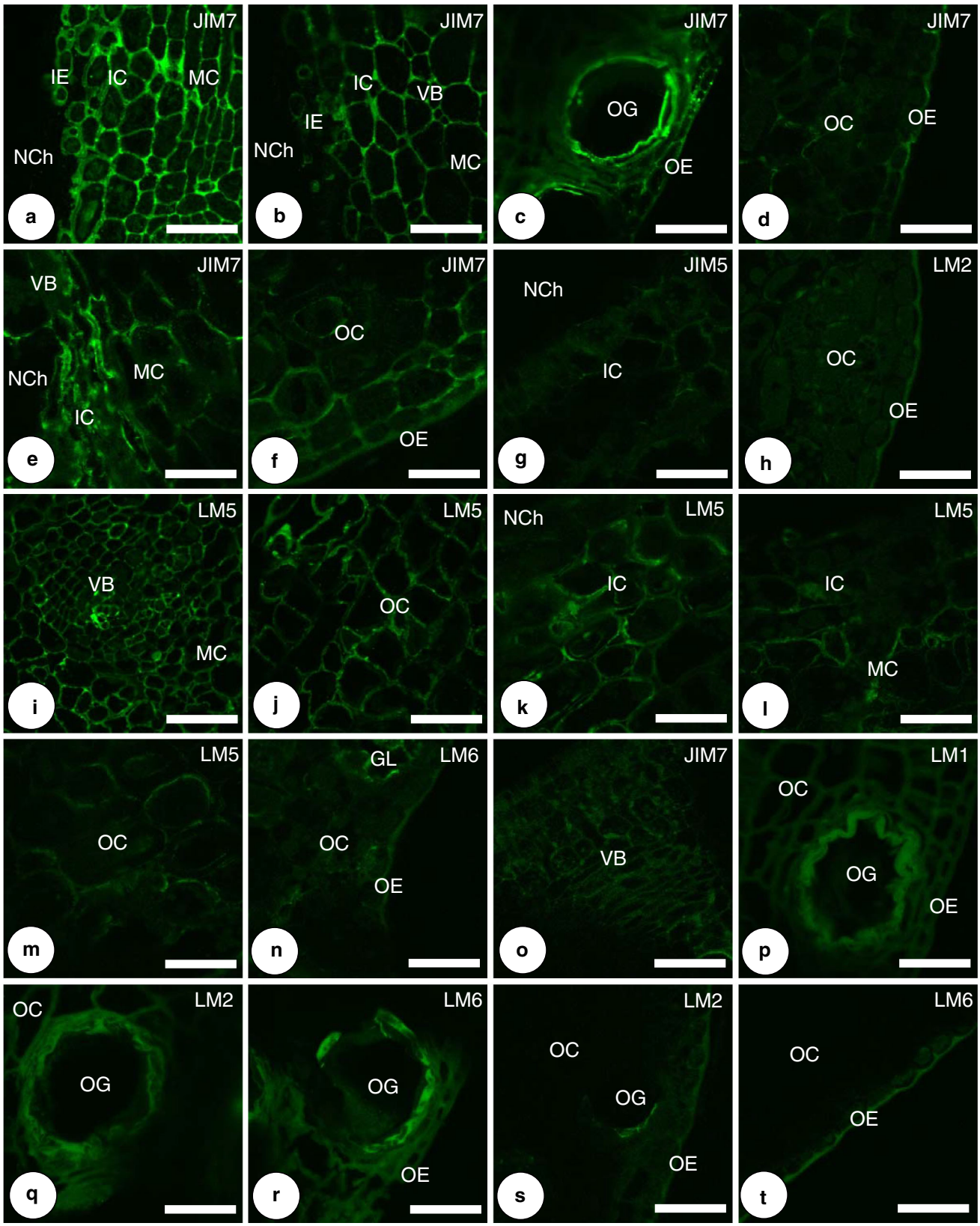
Fig. 6 Immunocytochemistry of the plant cell walls in the galls induced by *Nothotrioza myrtoidis* on the leaves of *Psidium myrtoides*. **o** Galls at induction phase; **a**, **c**, **i**, **j**, **p**–**r** Galls at growth and development phase; **b**, **d**, **k**, **n**, **s** Galls at maturation phase; **e**–**h**, **l**, **m**, **t** Galls at maturation phase. Fluorescence indicates positive binding of low methylesterified homogalacturonans by the JIM5 antibody, high methylesterified homogalacturonans by the JIM7 antibody, extensins by the LM1 antibody, arabinogalactan proteins by the LM2 antibody, and galactans and arabinans by the LM5 and LM6 antibodies. *IC* inner cortex, *IE* inner epidermis, *MC* medium cortex, *NCh* nymphal chamber, *OC* outer cortex; *OE* outer epidermis, *OG* oil gland, *VB* vascular bundle. *Bars* 50 μm

The outer epidermis presented increasing detection of cell wall epitopes from the phase of induction towards maturation of the galls. At the phase of induction there was no labelling of the investigated epitopes, but at the phase of growth and development, the epitopes of high methylesterified HGAs and extensins were labelled by JIM7 and LM1, respectively (Fig. 6c, p). In mature galls, the epitopes of low and high methylesterified HGAs, AGPs, galactans and arabinans were labelled by JIM5, JIM7, LM2, LM5 and LM6, respectively (Fig. 6d, n, s). The same cell wall epitopes were labelled during gall senescence, except for the epitopes of galactans (Fig. 6f, h, t).

Discussion

Patterns in gall development and the neo-ontogenesis of leaf tissues

The differentiation pattern of the primary tissues in the leaves of *P. myrtoides* is similar to that of other host plant of galling herbivores, such as *Lantana camara* (Moura et al. 2008), *Lonchocarpus muehlbergianus* (Isaias et al. 2011) and *Schinus polygamus* (Dias et al. 2013). The similar ontogenetical pattern of the host leaves is altered by the galling herbivores towards very different morphotypes (sensu Isaias et al. 2013). The feeding behaviour typical for the Psylloidea (Burckhardt 2005) assumed by the first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instar nymphs of *N. myrtoidis* causes a depression on the leaf lamina, with discrete sites of hyperplasia in the epidermis and adjacent tissues. Similar tissue responses were previously reported for other host plant–sucking insect systems, such as *L. camara–Aceria lantanae* (Moura et al. 2008) and *S. polygamus–Calophya duvauae* (Dias et al. 2013). The tissue layers of the galls induced by *N. myrtoidis* on *P. myrtoides* leaves are similar to other galls, such as the bivalve-shaped galls on *Lonchocarpus muelhbergianus* (Isaias et al. 2011), the midrib galls on *Copaifera langsdorffii* (Oliveira and Isaias 2010), and the globoid intralaminar galls on *Aspidosperma spruceanum* (Formiga et al. 2011). The four gall morphotypes have vascular bundles interspersed to a predominantly parenchymatic structure with a lignified zone in the outer cortex. Nevertheless, the specific association of the gall-inducing insects with their host plants promote alterations in the tissues towards adaptive phenotypes (sensu Stone and Schönrogge 2003), the diverse gall morphotypes (Isaias et al. 2013).

The second set of responses of *P. myrtoides* cells to *N. myrtoidis* activity leads to tissue hyperplasia mainly during the phase of growth and development, and then to cell hypertrophy at the phase of maturation. These two processes are common in the development of plant galls (Kraus et al. 2002; Moura et al. 2009; Oliveira and Isaias 2010; Dias et al. 2013; Ferreira and Isaias 2013; Magalhães et al. 2014), but present different dynamics which determine the variety of gall morphotypes. At the phase of maturation, the lignification of the outermost cell layers in the gall cortex is the most remarkable morphogenetic step in the galls of *N. myrtoidis.* Together with the enlarged oil glands, the lignified tissues play defensive roles in the structure of the gall (sensu Stone and Schönrogge 2003). Lignification may be triggered either by biotic or abiotic stresses (Lee et al. 2007), and is promoted at the same sites of reactive oxygen species (ROS) accumulation, as demonstrated by Carneiro et al. (2014) in the galls of *P. myrtoides*. The location of the additional oil glands confers chemical protection, by the production, storage and secretion of secondary metabolites (Apel et al. 2009; Carneiro et al. 2014), which can be toxic to pathogens, predators, and cecidophagous. The suppression of the differentiation of the oil glands in the inner cortex improves the quality of the gall nutritional sites for this triozid, as proposed by Nyman and Julkunen-Tiitto (2000) for galls of sawflies on willows. Consequently, it increases the adaptive value of the gall structure to the gall inducer, as described for *Solidago-Eurosta* galling systems (Weis and Abrahamson 1986). All the sets of cell and tissue responses cause the structure of this gall to be a unique defensive extended phenotype of *N. myrtoidis* (sensu Bailey et al. 2009).

In the *P. myrtoides–N. myrtoidis* system, the establishment of the galling insect determines a process of neoontogenesis, i.e., completely new ontogenetic steps on the host leaves which occur by coordinated cell redifferentiation (sensu Lev-Yadun 2003). The striking plasticity of plant cells is manipulated and, in the case of galls, is especially driven by the biotic stress imposed by the galling herbivores. This process allows the leaf cells, previously determined to differentiate into chlorophyllous parenchyma, to redifferentiate into functionally and structurally different tissues, namely homogenous parenchyma and sclerenchyma. During the developmental phases of the gall, tissue hyperplasia and cell hypertrophy occur at different regions of the cortex. The mechanisms that regulate such dynamics in galls are still poorly known, but recent investigations set lights on the influence of phenolics accumulation on the modulation of the activity of plant cell growth regulators (Bedetti et al. 2014). Hori (1992) firstly suggested the importance of phenolics on the inhibition of AIA oxidases and, thus, on the stimulation of cell growth by the increase of auxin levels. Bedetti et al. (2014) proved that phenolics and auxins accumulate at the same sites in the tissues of the galls on *Piptadaenia gonoacantha*, where auxin-(poly) phenol adducts are formed. Previous studies with the globoid galls of *P. myrtoides* have shown the establishment of a histochemical gradient of phenolics (Carneiro et al. 2014) that coincides with the gradients of cell transformations described herein. This suggests that the mechanisms of cell redifferentiation in galls occur by a synergistic and complex balance of cell division and expansion, and are most likely to be driven by specific gradients of metabolites accumulation.

Structural responses of plant cells and tissue functionalities

The tissue hyperplasia and prominent cell hypertrophy are dependent on the dynamic properties of the plant cell walls, and on the reorientation of cell division and expansion. The type of cell expansion in the cortex of the galls of *P. myrtoidis* shifted from the anisotropic to isotropic (sensu Baskin 2005), a tendency reported during the development of some plant galls (Oliveira and Isaias 2010; Isaias et al. 2011). Magalhães et al. (2014) were the first to elegantly demonstrate the rearrangement of cellulose microfibrils and the shifts on the direction of cell expansion during gall development. In the galls of *P. myrtoides*, the cortical cells hypertrophy isotropically in a centrifugal gradient from the inner towards the outer cortex, which determines the globoid shape of the galls. As for the kidney-shaped galls, the direction of elongation were altered from isotropic to anisotropic (Magalhães et al. 2014), we can infer that the types and sites of cell expansion should explain the uniqueness of each gall morphotype (Oliveira and Isaias 2010; Isaias et al. 2011; Ferreira and Isaias 2013).

The composition of pectins and glycoproteins varied along the development of the galls induced by *N. myrtoidis*, and should influence the functional roles of plant tissues (Dolan et al. 1997; Albersheim et al. 2011). Based on the investigated epitopes, a poor pectin composition of the cell walls is assumed for the sites of gall induction since only JIM7 labelled the high methylesterified HGAs in the cell walls of the vascular tissues, indicating juvenility. Cell wall pectin composition increased during the stage of gall growth and development for the JIM7 labelling of high methylesterified HGAs occurred in all tissue layers. Arabinans and galactans labelled by LM5 and LM6, respectively, were observed in specific tissue layers. The high methylesterified HGAs are involved with increased cell division, expansion and decreased adhesion (Xu et al. 2011), which are typical cell responses during gall growth. When associated with galactans and arabinans, the high methylesterified HGAs enhance cell wall porosity and flexibility (Verhertbruggen et al. 2009) by the depletion of calcium-mediated cross bonds between the side chains of HGAs. This peculiar cell wall composition is responsible for the establishment of a centrifugal gradient of cell hypertrophy from the inner towards the outer cortex in the galls of *P. myrtoides* (*cf.* cytometric analyses, Fig. 5). Formiga et al. (2013) related a similar cell wall composition of the galls on *Baccharis reticularia* to the mechanical stability of the fast-dividing cells.

The labelling of the high methylesterified HGAs associated with galactans, by JIM7 and LM5, at similar sites, is an additional support for the rigidity of the cell walls, as proposed by McCartney et al. (2000) and McCartney and Knox (2002). Even though the galactans are known to be one of the most flexible cell wall polymers (Ha et al. 1996), their labelling indicates that they are most likely to be found in the free form, instead of as side chains of pectins (Jones et al. 1997). Once detached from the pectin molecules, the remnant backbones cross link more effectively and form a coherent gel network (Hwang and Kokini 1991), thus turning the cell walls more rigid. The rigid galactan-rich cell walls hinder cell hypertrophy and galls grow preferably by tissue hyperplasia, as observed in the outer cortex during the stage of growth and development of the galls of *N. myrtoidis*. In fact, sites of increased cell division have been associated with the presence of galactans, while sites of cell hypertrophy are commonly associated with the presence of arabinans (Xu et al. 2011). The dynamics of these two pectin domains implies a functional shift of the cell wall, since the balance between arabinans and galactans modulates cell expansion (Jones et al. 1997), flexibility and adhesion (O'Donoghue and Sutherland 2012). The labelling of the epitopes of galactans by LM5 in the cell walls of the inner cortex indicates that they were stiffened, and the cells could not expand as the arabinan-rich cells of the outer cortex. A gradient of cell hypertrophy is established, determining the globoid shape of the galls on *P. myrtoides*.

Up to gall maturation, the low methylesterified HGAs were not labelled by the JIM5 antibody, but in senescent galls, such labelling occurred in the cells of the outer cortex. The demethylesterification demands the activity of enzymes, such as the PMEs, which seems to be activated when the gall inducer decreased its feeding activity. The demethylesterification of HGAs by PMEs was previously described by Willats et al. (2001), and during such process the carboxyl groups of de-esterified HGAs cross link to Ca^{2+}, resulting in the stiffening of the cell wall (Jiang et al. 2005; Albersheim et al. 2011; Liu et al. 2013). By senescence, this stiffening is crucial for cell wall rupturing (Carneiro et al. 2013). The dynamic composition of cell wall pectin on *P. myrtoides*, from non-galled tissues to gall induction towards maturation and senescence, indicate the timing of demethylesterification, possibly by the activity of PMEs, and determine variations on cell wall functions, as discussed by Liu et al. (2013) in *Gossypium* species and by Oliveira et al. (2014) in galls of *B. dracunculifolia*. Even though Formiga et al. (2013) suggested that HGAs demethylesterification could occur due to high oxidative stress, the galls of *N. myrtoidis* accumulate little reactive oxygen species (Carneiro et al. 2014), and are more likely to present demethylesterification mediated by the PMEs.

The association of galactans and arabinans labelled by LM5 and LM6, and AGPs labelled by LM2 indicates the control of cell expansion, and the effective prevention of premature cell death (Chaves et al. 2002; Guan and Nothnagel 2004). The AGPs are found at the plasma membrane, anchored by glycosylphosphatidylinositol terminals (Borner et al. 2002), which is a characteristic of signalling membrane proteins. AGPs may elicit the release of intercellular Ca^{2+} and the production of H_2O_2, which should trigger programmed cell death (PCD) (Chaves et al. 2002). Nevertheless, the absence of PCD in the globoid galls of *P. myrtoides* indicates that the production of intercellular calcium and H_2O_2 mediated by AGP may act as signalers for the morphogenesis of the galls and deserves more studies on the molecular basis. When the AGPs are absent, as observed in the galls at senescent phase, a gradient of cell degeneration is established from the innermost towards the outermost cell layers, and from the apex of the gall towards its base. During this process, nutrients and metabolites which are accumulated in these galls (*cf.* Carneiro et al. 2014) are believed to be reallocated back to the host leaves. At this point of gall development, instead of associating with AGPs, the arabinans and galactans occur at the same sites of HGAs, which indicates that cell wall polysaccharides are less intricately bound to each other, as the calcium-mediated cross bonds between HGAs

do not tend to occur (Willats et al. 2001; Lord and Mollet 2002). The functional consequence of such arrangement is an increase in cell wall porosity, which enables the reallocation of nutritional resources.

Within all developmental stages of the galls induced by *N. myrtoidis*, the oil glands were the structures which had the greatest alterations, with strong hypertrophy (about 170 %; *cf.* section "Developmental anatomy of leaves and galls"). In the cell walls of such structures, all the antibodies labelled their specific investigated epitopes except for the JIM5 and LM5. The labelling of the epitopes of high methylesterified HGAs together with that of extensins indicated an increase in cell wall rigidity, as proposed by Cassab (1998), but the labelling of the LM2 for the epitopes of AGPs, and of LM6 and LM5 for the epitopes of galactans and arabinans indicated the property of cell expansion (Jones et al. 1997). Such balance of pectin and protein on the composition of the cell walls determined the maintenance of the structure and functionality of the oil glands, despite the massive hypertrophy and hyperplasia of the secretory epithelium. The labelling of the epitopes of AGPs by the antibody LM2 demonstrated sites where premature cell death was prevented, as reported for the secretory ducts of galls on *B. reticularia* (Formiga et al. 2013), and for the mucilage cells in *Araucaria angustifolia* (Mastroberti and Mariath 2008).

Final considerations

The cellular transformations in the cortex of *P. myrtoides* galls depend particularly on the centrifugal gradient of cell hypertrophy, which is established in a temporal-spatial basis. The establishment of the gradient coincides with the dynamic distribution of pectin and protein epitopes in the cell walls, during the developmental stages of the galls. Variations in cell wall flexibility and rigidity are inferred by current literature on the labelling of distinct MAbs on cell layers along gall stages. The establishment of the cecidogenetic field determines tissue hyperplasia during gall growth and developmental stage on the cells with high methylesterified HGAs associated to galactans labelled by JIM7 and LM5, respectively. During gall maturation, cell hypertrophy takes place on the outer cortical cells, which demands cell wall flexibility conferred by high methylesterified HGAs associated to arabinans labelled by JIM7 and LM6, respectively. Current approaches on the developmental anatomy and immunocytochemistry of the galls induced by *N. myrtoidis* on *P. myrtoides* elegantly demonstrated the role of pectin and protein domains as part of the pool of cell responses necessary for the neo-ontogenesis of leaf tissues towards a complete new phenotype, the globoid gall morphotype.

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

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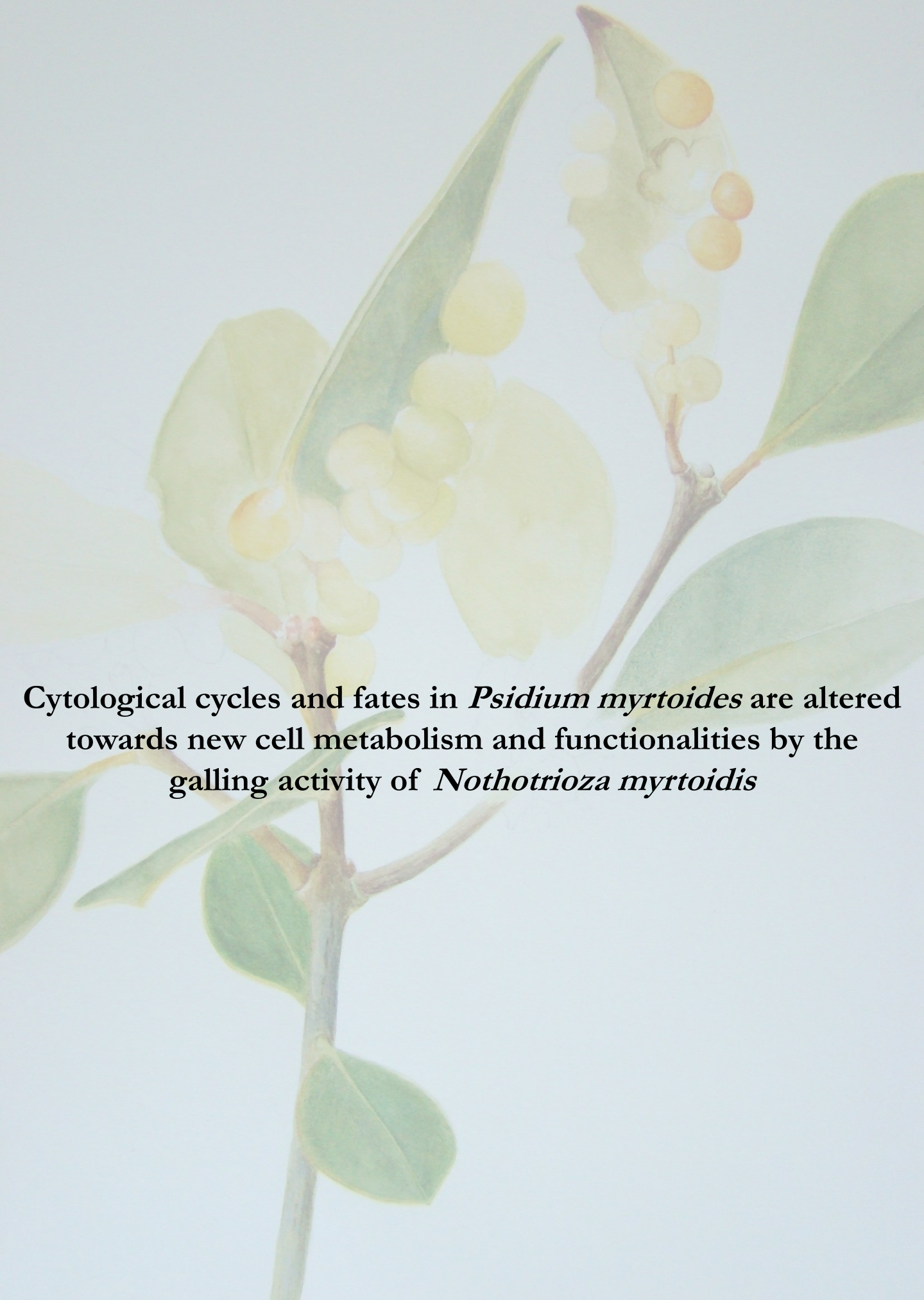

Cytological cycles and fates in *Psidium myrtoides* are altered towards new cell metabolism and functionalities by the galling activity of *Nothotrioza myrtoidis*

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ORIGINAL ARTICLE

Cytological cycles and fates in *Psidium myrtoides* are altered towards new cell metabolism and functionalities by the galling activity of *Nothotrioza myrtoidis*

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Abstract The morphogenesis of galls occurs by the redifferentiation of cells that assume new functions in the modified host plant organs. The redifferentiated cells in the galls of *Nothotrioza myrtoidis* on *Psidium myrtoides* have low complexity metabolism and are photosynthesis-deficient. These galls were studied in search for evidences of the establishment of new cell cycles and fates and cytological gradients that corroborate their metabolic profile. Young and mature leaves of *P. myrtoides* and leaf galls induced by *N. myrtoidis* at different developmental stages were collected along 24 months and analyzed under light and transmission electron microscopy. The leaves of *P. myrtoides* are long-lasting and did not senesce within the analyzed period, while the galls have a shorter cycle, and senesce within 1 year. A homogenous parenchyma is established by a "standby-redifferentiation" of the chlorophyllous tissues, and sclerenchyma cells redifferentiate from parenchyma cells in the outer cortex of the mature galls. The lack of organelles, the underdeveloped lamellation of chloroplasts, and the occurrence of few plastoglobules are related to the photosynthetic deficiency of the galls. No cytological gradients were observed, but the organelle-rich cells of the vascular and perivascular parenchymas are similar to those of the nutritive tissues of galls induced by other insect taxa. These cells nearest to the feeding sites of *N. myrtoidis* present higher metabolism and well-developed apparatus for the prevention of oxidative stress. The features herein described corroborate the low metabolic profile of the galls as the cell cycles and fates of *P. myrtoides* are manipulated for completely new functionalities.

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Introduction

The development of plant organs depends basically on three phenomena: growth, differentiation, and morphogenesis, with the differentiation being responsible for the determination of cell shapes and functions (Buvat 1989; Evert 2006). Even though plant cells are known to be plastic (Buvat 1989; Gianoli and Valladares 2012), the organogenesis in plants tends to be conservative and constant when subject to specific biotic and abiotic stimuli (Fahn 1990). Galling insects manipulate the differentiation of the plant cells from pre-established patterns towards new morphogenesis (Mani 1964; Rohfritsch 1992). By changing plant cell differentiation through very refined stimuli (Hori 1992), the galling insects directly affect plant morphogenesis and growth as new cell cycles and fates must be established.

The study of Neotropical insect galls has currently been the focus of fruitful discussions concerning plant cell biology and morphogenesis (Moura et al. 2009; Oliveira and Isaias 2009, 2010a; Formiga et al. 2011; Isaias et al. 2011; Castro et al. 2012a; Bedetti et al. 2013; Dias et al. 2013; Ferreira and Isaias 2013; Guimarães et al. 2013, 2014). The cytological characterization has been already carried out for Cynipid and Cecidomyiidae galls (Bronner 1992), which were revisited on the search for similar patterns and functionalities in the Neotropical galls (Oliveira and Isaias 2010b; Oliveira et al. 2010, 2011b; Vecchi et al. 2013). Some of these patterns were corroborated, while new cytological and histochemical gradients related to gall metabolism were reported in the galls of *Pseudophacopteron* sp. (Psyllidae), a sucking insect, on *Aspidosperma australe* (Apocynaceae) (Oliveira and Isaias 2010b). In this system, nutritive-like cells occur, and the

overdevelopment of plastoglobules was considered an ultrastructural response that helps overcoming the high oxidative stress within gall tissues. As these corpuscles recover the membrane systems of the thylakoids, the galls may photosynthesize at the same levels of their host leaves (Oliveira et al. 2011a).

The galls herein studied are induced by *Nothotrioza myrtoidis* Burck. (Triozidae), a sucking insect, on *Psidium myrtoides* O. Berg (Myrtaceae), an evergreen shrub or small tree. The whole cycle of the galls, from induction until senescence, lasts 1 year (Carneiro et al. 2013), and the galls have unique histochemical gradients. Also, they were found to be photosynthesis-deficient and are subject to low oxidative stress (Carneiro et al. 2014) when compared to other galls. By assessing the cytological development of the leaf galls on *P. myrtoides*, we expect to evidence the manipulation of cell cycles and the alterations of cell fates induced by *N. myrtoidis* and their relation with the neo-established functionalities of the plant tissues. Furthermore, we aim to verify the existence of cytological gradients that could corroborate the low metabolic profile of these galls, as well as the patterns previously described for other plant galls. The following questions are addressed: (1) how are the plant cell cycles and fates affected by the development of *N. myrtoidis* galls? And (2) are there cytological gradients that corroborate the low metabolic profile of these galls?

Material and methods

Sampling

The population of *P. myrtoides* O. Berg (Myrtaceae) with galls induced by *N. myrtoidis* Burckhardt is located at the Reserva Particular do Patrimônio Natural Serra do Caraça, municipality of Catas Altas, Minas Gerais state, Brazil (20° 06′ 22″ S–43° 29′ 42″ W). Non-galled leaves (young and mature, $n=5$ per developmental stage) and galls at the phases of induction, growth and development, maturation and senescence ($n=5$ per developmental stage) were collected during two complete life cycles of the galling insects, from October 2011 to October 2013. The material was fixed in 2.5 % glutaraldehyde (grade I) and 4.5 % formaldehyde in phosphate buffer (0.1 M; pH 7.2) (Karnovsky 1965).

Light microscopy

The fixed samples were dehydrated in *n*-butyl series (Johansen 1940), embedded in Paraplast® (Kraus and Arduin 1997), sectioned (12–14 μm) in a rotary microtome (Jung biocut), and stained with 9:1 (*v*/*v*) solution of Astra blue and safranin (Bukatsch 1972, modified to 0.5 %).

Transmission electron microscopy

The fixed samples were post-fixed in 1 % osmium tetroxide in phosphate buffer (0.1 M; pH 7.2), dehydrated in ethanol series (O'Brien and McCully 1981), and embedded in Spurr®. Ultrathin sections were obtained with a diamond knife in the ultramicrotome Reichert-Jung–Ultracut (Leica, Wetzlar, Germany), attached to copper nets, contrasted with uranyl acetate and lead citrate (Reynolds 1963), and analyzed under the transmission electron microscope Tecnai™ G2-12-SpiritBiotwin (FEI, Hillsboro, USA) at 120 kV, at the Centro de Microscopia of the Universidade Federal de Minas Gerais.

Results

Developmental anatomy

The young leaves of *P. myrtoides* have uniseriate epidermis, and homogenous chlorophyllous parenchyma, which is partially differentiated; vascular bundles differentiate from procambial strands located in the middle layers of the parenchyma, and oil glands differentiate from both adaxial and abaxial surfaces of the epidermis (Fig. 1a). The chlorophyllous parenchyma of mature leaves is differentiated into palisade and spongy parenchyma (Fig. 1b).

The galls of *N. myrtoidis* are induced by the first instar nymphs in the young leaves, and the cells respond to the stimuli by hypertrophying and dividing to enclose the insect inside the nymphal chamber (Fig. 1c). By late growth and developmental stage, the galls are broad chambered and thin walled (Fig. 1d). The galls are covered by uniseriate inner and outer epidermis, the cortex is composed by homogenous parenchyma, and vascular bundles redifferentiate in the inner cortex (Fig. 1, detail box).

Dynamics of cell cycles

The life cycle of the galls of *N. myrtoidis* on the leaves of *P. myrtoides* lasted 1 year (Fig. 2). Gall induction took place on young leaves in October 2011. By December 2011, galls had entered the phase of growth and development and leaves had grown towards maturation. The leaves matured by February 2012, but galls remained at the phase of growth and development until August 2012 (Fig. 2). Galls matured by September 2012 and senesced by October 2012, when another cycle of galls and leaves began. Leaves of the first cycle did not senesce until the end of the period of observation (Fig. 2).

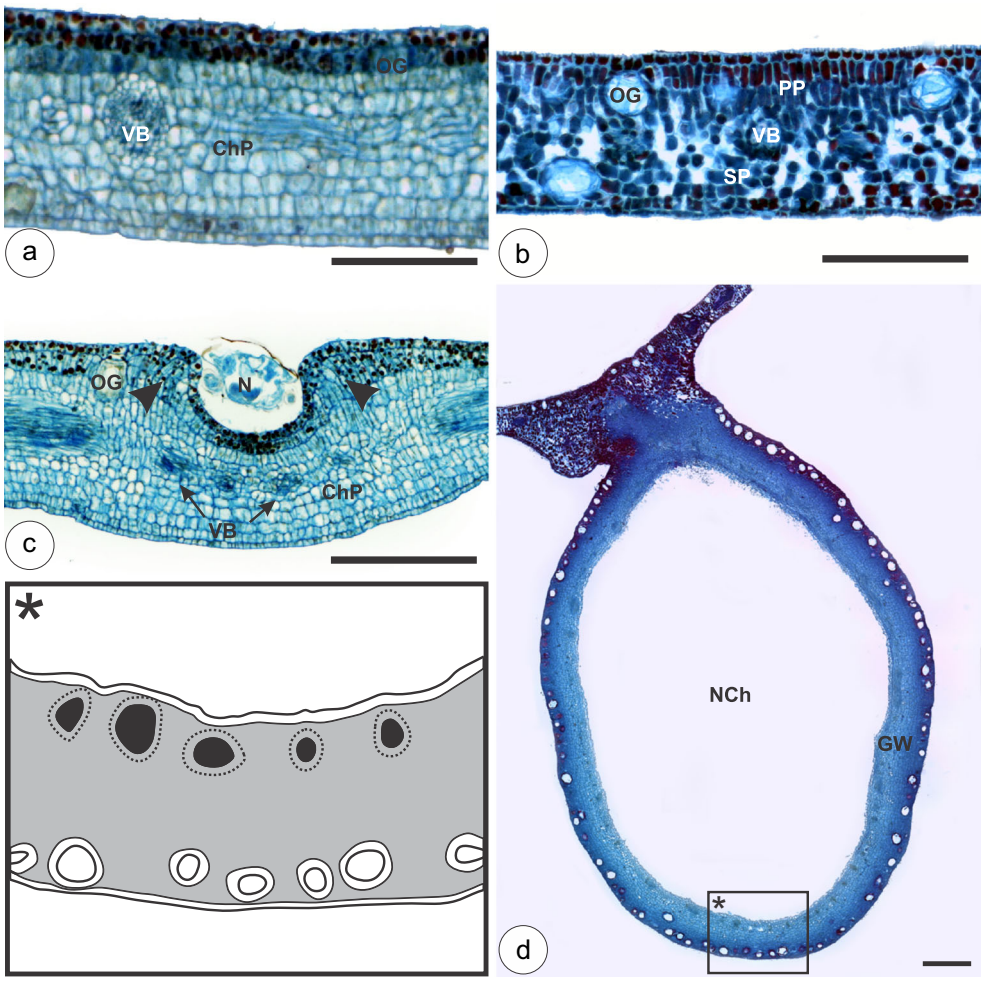

Fig. 1 Anatomy of the leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. **a** Young leaves with uniseriate epidermis, homogenous chlorophyllous parenchyma (*chp*), interspaced vascular bundles (*vb*), and subepidermal oil glands (*og*) undergoing differentiation. **b** Mature leaves with uniseriate epidermis, chlorophyllous parenchyma with differentiated palisade (*pp*) and spongy parenchyma (*sp*), interspaced vascular bundles, and subepidermal oil glands. **c**, **d** Galls. **c** Phase of induction with the first instar nymph (*n*) feeding on the leaf tissues. The chlorophyllous parenchyma responds to the feeding stimuli by elongating, dividing (*arrow heads*), and swelling. **d** Phase of growth and development, when the galls grow into a pocket-shaped structure with an ample nymphal chamber (*nch*). In detail (*asterisk box*), the gall wall (*gw*) composed by uniseriate epidermis and oil glands derived from the outer epidermis of the galls (*white*), multilayered homogenous parenchyma (*gray*), and vascular tissues near the nymphal chamber (*black*). *Dotted lines* indicate the location of perivascular parenchyma. *Bars* **a**=100 μm; **b**, **c**=200 μm; **d**=400 μm

Cell fates: from non-galled leaves to galls

Epidermal cells In the young leaves of *P. myrtoides*, epidermal cells are thin walled, with slightly thicker external periclinal walls, large hyaline vacuoles, few chloroplasts and mitochondria, and large nuclei with conspicuous nucleoli (Fig. 3a). In the mature leaves, the secondary periclinal cell walls of epidermal cells are thick, the vacuoles are large with phenolics, chloroplasts and mitochondria are rare, and the nuclei are small with peripheral heterochromatin (Fig. 2b). Gall induction takes place on young leaves; the cells remain with homogenous thin walls, and the protoplast is similar to that of young leaves. Heterogenous cuticle and collapsed cells occur on the epidermis lining the nymphal chamber (Fig. 3c). At the phase of growth and development, the inner epidermis is an intermittent layer of cells with homogenously thickened, polylamellate walls, and thin cuticle (Fig. 3d). The protoplast is poorly altered in comparison to the cells of the galls at the phase of induction; the nuclei have more electron dense euchromatin. At maturation, the protoplasts remain similar, but the secondary layers of the cell wall start to disaggregate (Fig. 3e). At senescence, the cells undergo autolysis and collapse.

Chlorophyllous parenchyma In the young leaves, mesophyll cells have thin and homogenous primary cell walls. The intercellular spaces are reduced. Vacuoles are mostly large and central, with granular inclusions, and sometimes form a vacuome around the nucleus. Nuclei are large and round, with conspicuous nucleoli (Fig. 4a). Chloroplasts are approximately round shaped, with different degrees of organization in the lamellae of grana, small starch grains, and plastoglobules are present. Mitochondria are associated with chloroplasts, and

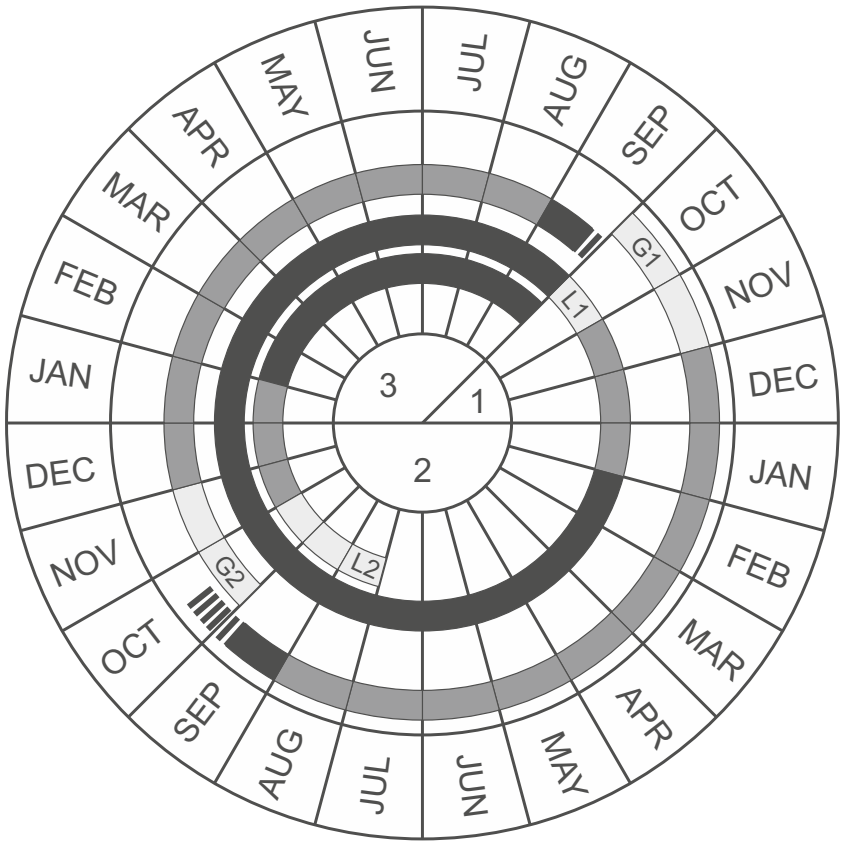

Fig. 2 Diagram indicating the time of cell differentiation during the developmental cycles of the leaves of *Psidium myrtoides* and of the galls of *Nothotrioza myrtoidis*. *Gray scale bars* indicate the degree of cell differentiation from low (*light gray*), to medium (*dark gray*) until high degree (*black*). *Discontinuous black bars* indicate cell death. *1* year of 2011, *2* year of 2012, *3* year of 2013. *G1* first cycle of galls, *L1* first cycle of non-galled leaves, *G2* second cycle of galls, *L2* second cycle of non-galled leaves

the Golgi apparatus is sometimes observed on the periphery of the cytoplasm (Fig. 4b). In mature leaves, cell walls have thin secondary layers and intercellular spaces are abundant. Vacuoles are large and central, filled with phenolic substances. Nuclei are relatively small, round, or elongated, with peripheral heterochromatin and conspicuous nucleoli. Chloroplasts are elongated, with well-organized lamellae. Starch grains are associated to many large plastoglobules and mitochondria (Fig. 4c).

At the phase of gall induction, the cells are hypertrophic and have thin walls, large vacuoles, and few intercellular spaces (Fig. 4d), which remain reduced throughout gall development. The cytoplasm is similar to that of young leaves, but multivesicular bodies and lamellar bodies with electron lucent matrix may occur (Fig. 4e, f). Chloroplasts have poor lamellate thylakoids and small electron dense plastoglobules (Fig. 4g). At the phase of growth and development, the cells are hypertrophic with polylamellate walls and the lumina are almost completely occupied by vacuoles with phenolic inclusions (Fig. 5a). The cytoplasm has electron dense matrix and few organelles. The structure of the chloroplasts and nuclei is similar when compared to that of young leaves and galls at the induction phase (Fig. 4b). At maturation, cells are larger and their vacuoles occupy the whole lumina of the cells. Different phenolic inclusions are frequent. Cytoplasm is reduced to a fine peripheral compartment, poor in organelles, except for the presence of occasional amyloplasts, degraded chloroplasts, and mitochondria (Fig. 5c). Sclerenchyma cells differentiate from the outermost parenchyma layers of the gall cortex and have lignified thickened walls with live protoplasts (Fig. 5d). At senescence, the cell walls are sinuous with degraded lamellae. Membrane systems are disorganized; the tonoplasts lose integrity and release the phenolic contents into the cytoplasm (Fig. 5e). Small autophagic vacuoles appear in the cells undergoing autolysis (Fig. 5f).

Vascular and perivascular tissues In the young leaves, the cells have fragmented vacuole, dense cytoplasm, large nuclei, lamellar bodies and associated multivesicular bodies, and little smooth endoplasmic reticulum (Fig. 6a, b). In mature leaves, the cells have large vacuoles or vacuomes with osmiophilic inclusions, large nuclei, chloroplasts, and many mitochondria; mature sieve tube elements and associated fibers are frequent (Fig. 6c). At the phase of gall induction, the cells are similar to the ones of young leaves, with large nuclei, conspicuous nucleoli, and dispersed heterochromatin; cytoplasm is rich in mitochondria, ribosomes, and rough endoplasmic reticulum (Fig. 6d). At the phase of growth and development, the cells have thin, polylamellate walls, relatively small vacuoles, and mitochondria-rich cytoplasm. Lomasomes, multivesicular bodies, and lamellar bodies are frequent. The nuclei are big, round shaped, with electron lucent euchromatin and dispersed

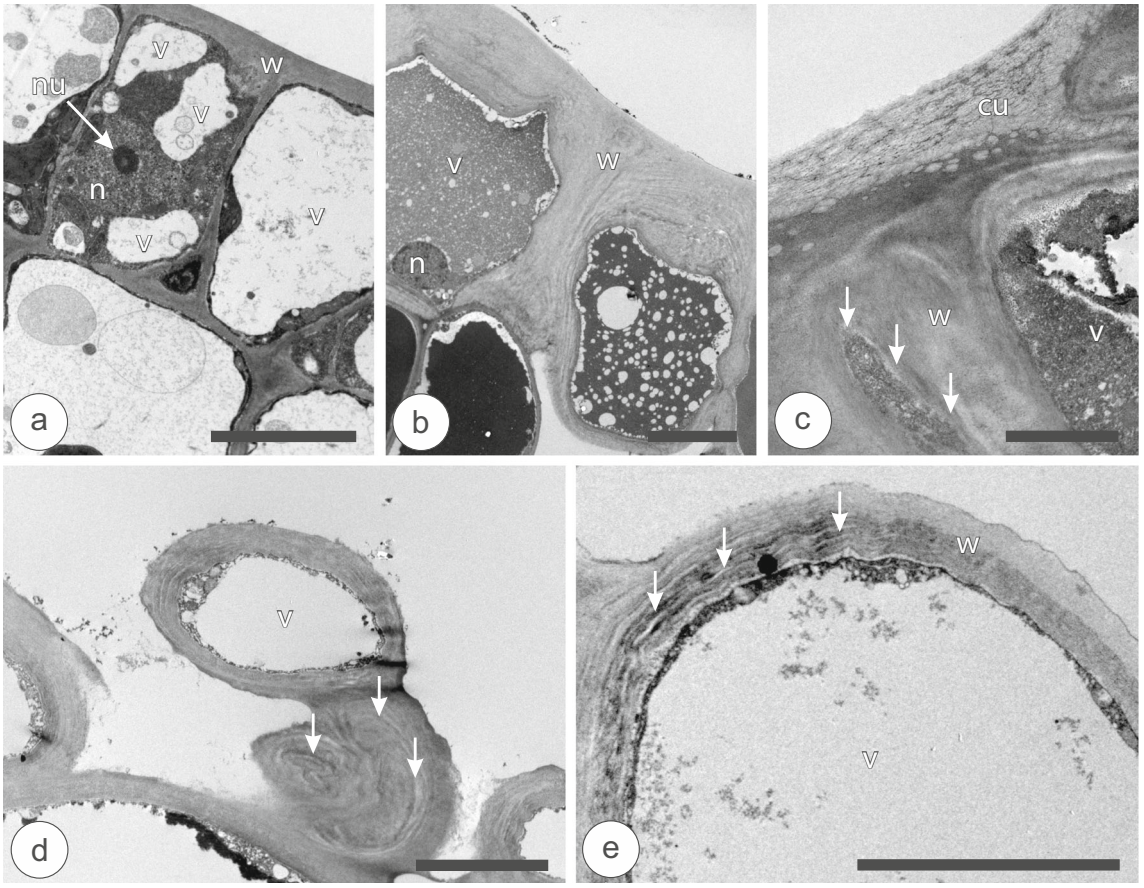

Fig. 3 Developmental cytology of the epidermal cells in the leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. **a** Young leaf cell with large nucleus (*n*) and evident nucleolus (*nu*), fragmented vacuole (*v*), and thin walls (*w*). **b** Cell of mature leaf with small nucleus and large central vacuole with phenolic inclusions. **c**–**e** Galls. **c** Phase of induction. Cells have large vacuoles, sometimes abnormally thickened cell walls compressing the protoplast (*arrows*) and heterogeneous cuticle (*cu*). **d** Phase of growth and development. Cells with large vacuoles form an intermittent layer and collapsed cells may occur (*arrows*). **e** Phase of maturation. Cells with large vacuoles, with secondary walls, which present disaggregation of lamellae (*arrows*). *Bars* **a**, **b**, **d**, **e**=5 μm; **c**=2 μm

microdomains of heterochromatin. Smooth endoplasmic reticulum is discrete (Fig. 6e). At maturation, galls remain with metabolically active cells with mitochondria, smooth endoplasmic reticulum, lamellar bodies, multivesicular bodies, lomasomes, poorly lamellate chloroplasts, and large nuclei with dispersed heterochromatin and inconspicuous nucleoli (Fig. 6f). At senescence, the cells are similar to those of the previous stage. Nevertheless, they have more abundant smooth endoplasmic reticulum, ribosomes, autophagic vacuoles, lomasomes, and fragmented vacuoles and their nuclei have greater amount of heterochromatin (Fig. 6g, h).

Discussion

New cell cycles and fates at the sites of gall induction

The characteristics of the cells, primarily destined to originate palisade and spongy chlorophyllous parenchyma, are structurally and functionally altered at gall sites towards a homogenous non-chlorophyllous parenchyma. These alterations are induced by *N. myrtoidis* on young leaves, interrupting the already initiated course of tissue differentiation (Carneiro et al. 2013). Herein we refer to such alterations as a product of cell redifferentiation (sensu Lev-Yadun 2003).

The induction phase and the early stages of the phase of growth and development are crucial for the establishment of the gall. Cells with thin walls and reduced intercellular spaces are characteristic of young, fast-growing tissues and were already reported for the development of other galls (Kraus 2009; Castro et al. 2012b; Vecchi et al. 2013). It is well known that gall development depends on the continuous stimuli of the galling larva and that the structural complexity of galls is somewhat determined by the duration of such stimuli, i.e., the amount of time spent during gall morphogenesis (Rohfritsch 1992). Even though the galls of *N. myrtoidis* have a 1-year life cycle (Carneiro et al. 2013), they are cytologically simple and do not fit the ultrastructural patterns found for other long-lasting Neotropical galls (Oliveira et al. 2010, 2011b). This distinctive

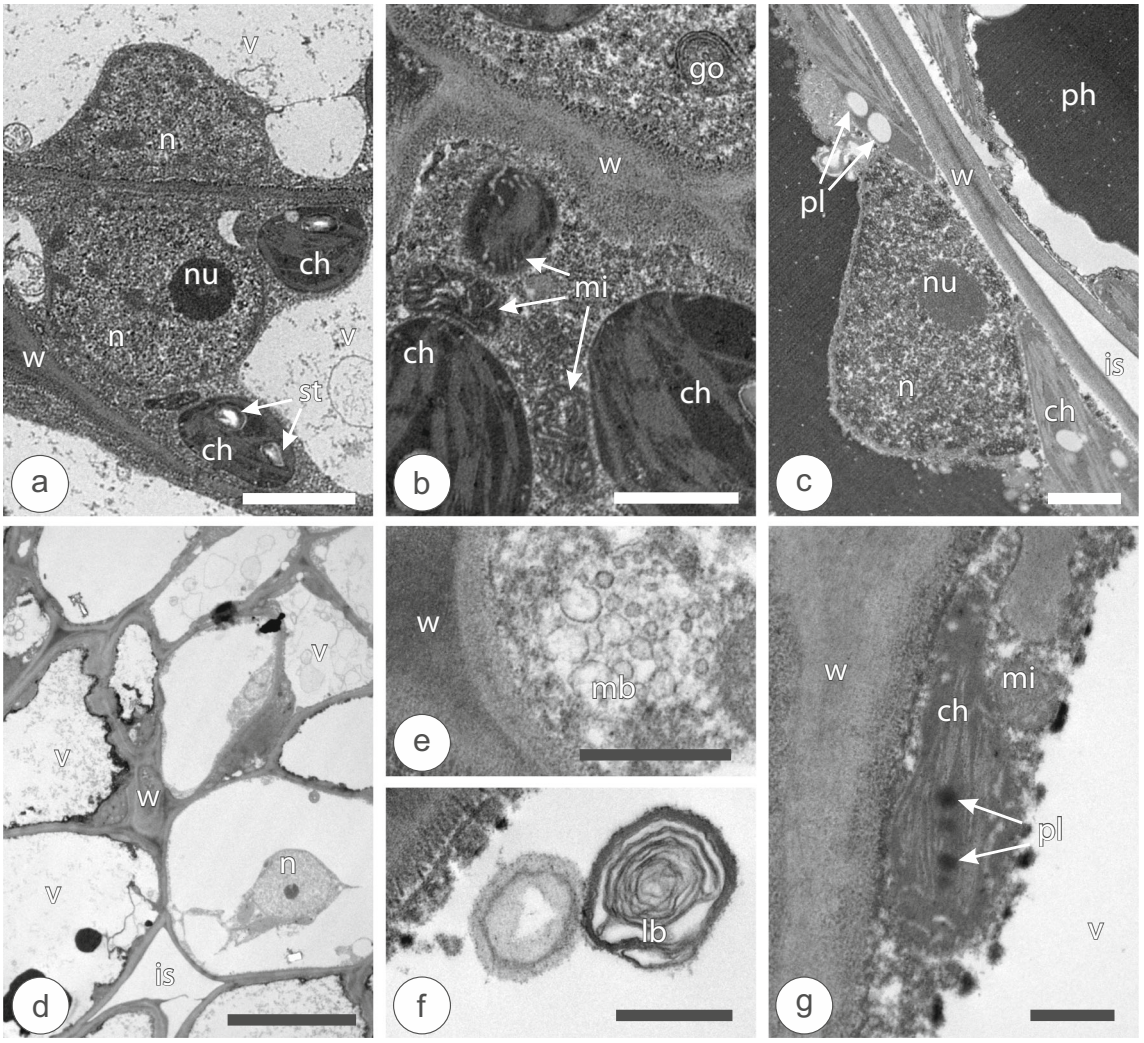

Fig. 4 Developmental cytology of mesophyll cells in the leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. **a**, **b** Young leaves. **a** Cells with thin walls (*w*), large nuclei (*n*) and conspicuous nucleoli (*nu*), fragmented vacuole (*v*), and round-shaped chloroplasts (*ch*) with starch grains (*st*). **b** Detail of cells with thin walls, underdeveloped Golgi apparatus (*go*), and chloroplasts associated with mitochondria (*mi*). **c** Mature leaves. Cells have thin walls, intercellular spaces (*is*), large vacuoles with phenolics (*ph*), large nucleus with conspicuous nucleolus, and well lamellate chloroplasts with large plastoglobules (*pl*). **d**–**g** Galls. **d** Phase of induction. Cells are thin walled, with large vacuoles, sometimes with central nucleus and reduced intercellular spaces. **e**–**g** Phase of growth and development. **e** Detail of multivesicular body (*mb*) near the plasma membrane. **f** Detail of lamellar body (*lb*) near the plasma membrane. **g** Detail of chloroplast with small plastoglobules, associated with mitochondria (*mi*). *Bars* **a**=2 μm; **b**, **c**=1 μm; **d**=10 μm; **e**–**g**=500 ηm

feature should be determined by the relatively low impact exerted by *N. myrtoidis*, a sucking insect with widespread feeding sides inside an ample nymphal chamber (Carneiro et al. 2014). Not only is the chlorophyllous parenchyma structurally affected by gall development, but also is the adaxial epidermis. The reduced thickness of the epidermis and cuticle at the sites of gall development facilitates the access of *N. myrtoidis* to its feeding sites, the vascular bundles. This epidermal feature sets a clear example of how the galling insects effectively manipulate plant morphogenesis and how these new cell fates increase the adaptive value of the gall structure to the galling herbivore (sensu Stone and Schönrogge 2003).

The timing of plant cell cycles is manipulated by the galling insects and synchronized to their own life cycles. From induction to the phase of growth and development, the galls of *P. myrtoidis* present few ultrastructural changes, differently from the non-galled leaves, whose cells mature and differentiate into a typical photosynthesizing chlorophyllous parenchyma during the same period. At the phase of gall maturation, a schlerenchymatic mechanical tissue, common in mature galls of psyllids (Meyer 1987) and of other insect taxa (Rohfritsch 1992; Stone and Schönrogge 2003), redifferentiates within a relative brief period of time. This sclerenchyma differentiates at the same sites of ROS accumulation in the galls of *N. myrtoidis* (Carneiro et al. 2014), corroborating the involvement of H_2O_2 in the synthesis of lignins as proposed by Olson and Varner (1993). Even though the sclerenchyma is often described to be a dead tissue at

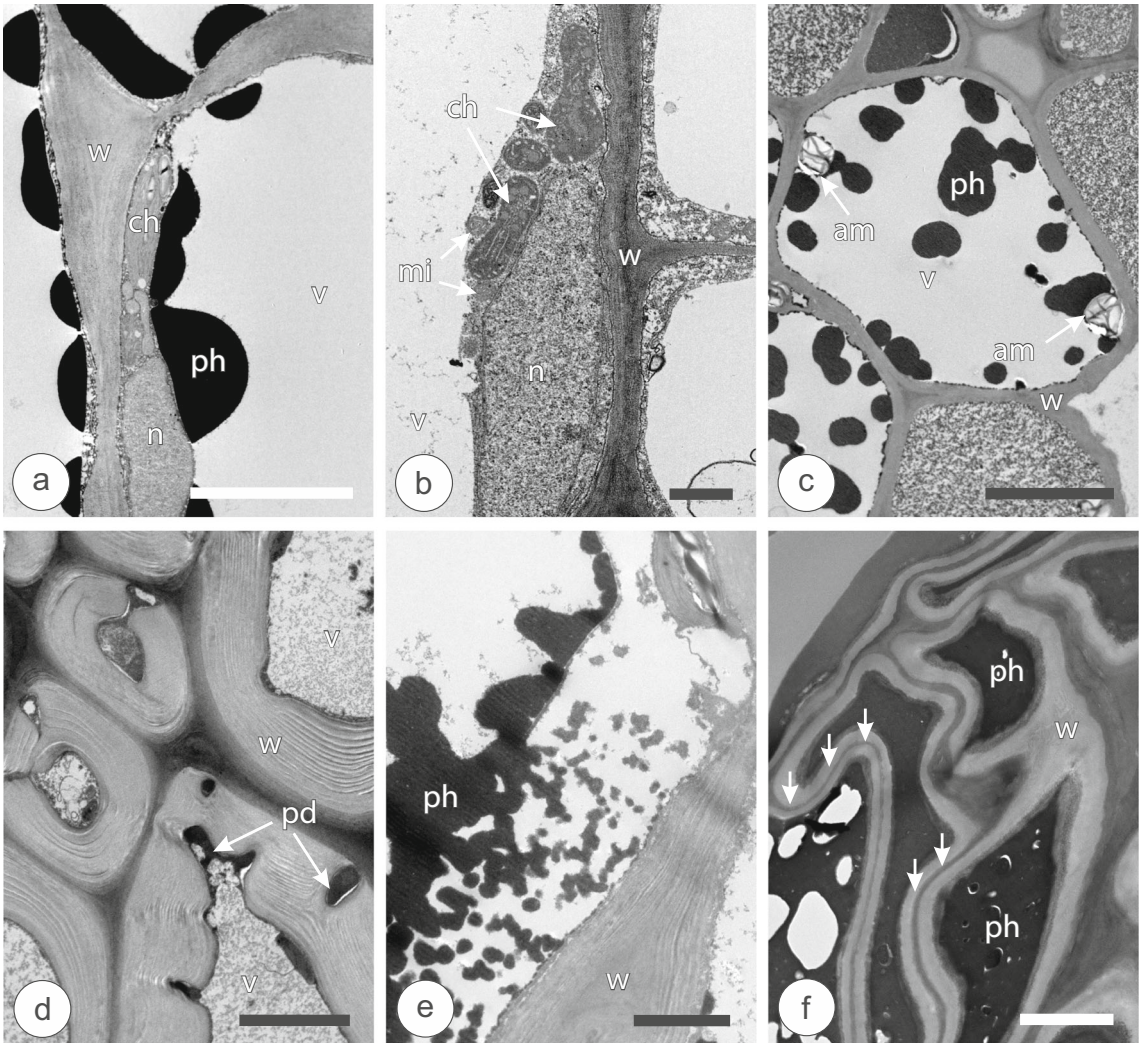

Fig. 5 Developmental cytology of mesophyll cells in the galls of *Nothotrioza myrtoidis*. **a**–**c** Phase of maturation. **a** Cells with large central vacuoles (*v*) and peripheral phenolic (*ph*) inclusions, small nucleus (*n*), and underdeveloped chloroplast (*ch*). **b** Detail of the small nucleus with unconspicuous nucleolus, underdeveloped chloroplasts associated with small mitochondria (*mi*). **c** Cells with large vacuoles with phenolic inclusions and peripheral amyloplasts (*am*). **d**–**f** Phase of senescence. **d** Cells of the outer cortex with thick secondary walls (*w*) and many plasmodesmata (*pd*). Protoplasts are live and have large central vacuoles. **e** Detail of cell with thickened wall and disrupted tonoplast and consequent leakage of phenolics into the cytoplasm. **f** Senescent cells with abundant phenolic inclusions and abnormally sinuous walls (*arrows*). *Bars* **a**, **d**=5 μm; **b**=1 μm; **c**=10 μm; **e**, **f**=2 μm

maturity (Evert 2006), the redifferentiated sclerenchyma cells of the galls of *N. myrtoidis* remain alive and are able to respond to the stimuli of the galling insects until the phase of senescence.

The developmental cytology revealed the manipulation of the host cell cycles of *P. myrtoides* by *N. myrtoidis*. At first, the redifferentiation of host plant cells occur by the confluence of the vacuoles, cell growth, and shifting of the nucleus and cytoplasm to the periphery of the cell, as proposed by Buvat (1989) for overall cell differentiation. The first morphogenetic step of the galls is similar to the establishment of a secondary meristem, whose source cells are somewhat differentiated and originate new tissues (Mauseth 1988; Buvat 1989; Fahn 1990; Evert 2006). The phase of growth and development is characterized by a "standby-redifferentiation," when cells remain unaltered during a long period of time. These cells undergo abrupt modifications during maturation and senescence. This dynamics of cell alterations suggests that the fine mechanisms of cell cycle regulation in plants (Jager et al. 2005) are somehow manipulated by the galling insects. For instance, specific sites of accumulation of auxin may be involved in the regulation of cell cycles, as demonstrated by Bedetti et al. (2014) in the galls of *Piptadenia gonoacantha*.

Cytological assessment of metabolic features

The metabolic-related features of the leaves of *P. myrtoides* and of the galls of *N. myrtoidis* were evidenced mainly in the

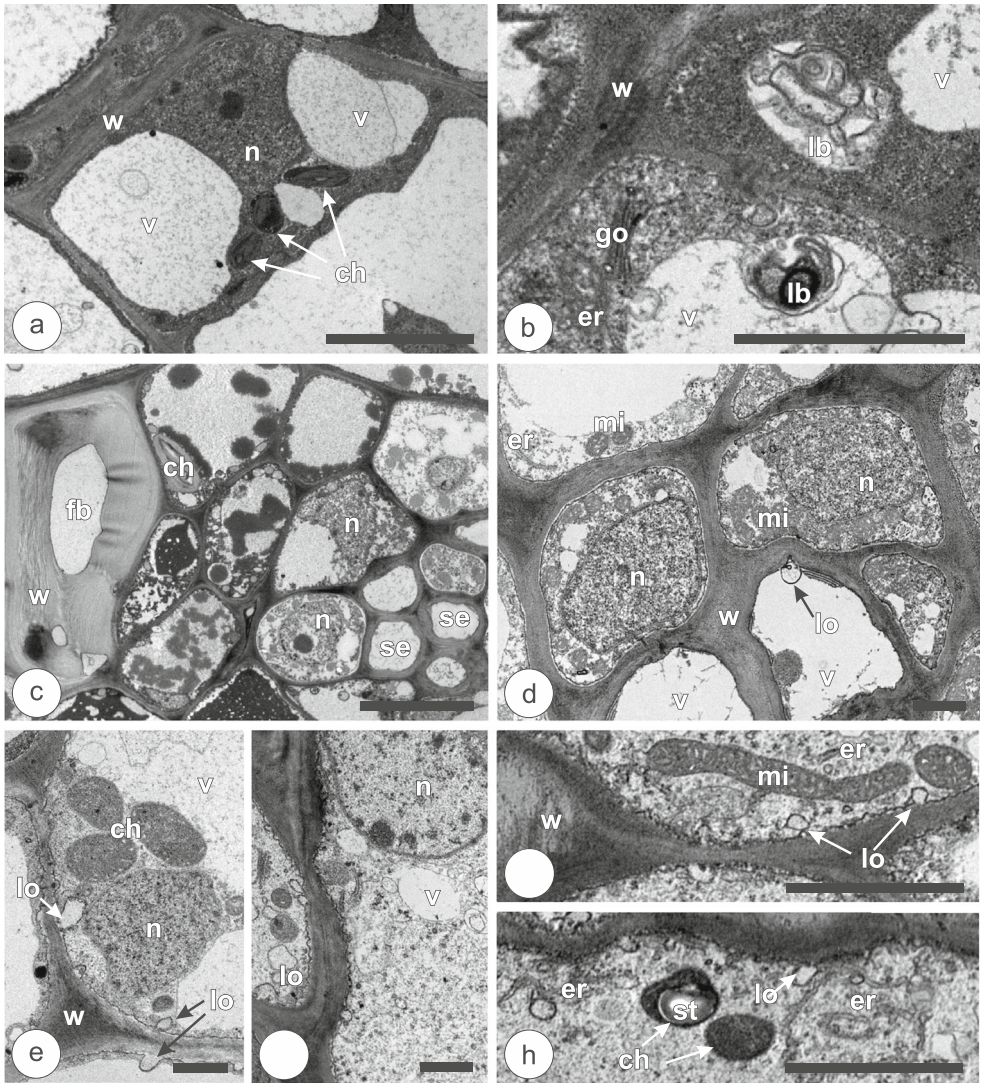

Fig. 6 Developmental cytology of vascular and perivascular cells in the leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. **a**, **b** Young leaves. **a** Cell with thin walls (*w*), large nucleus (*n*) and conspicuous nucleolus, fragmented vacuole (*v*), and underdeveloped chloroplasts (*ch*). Detail of the dense cytoplasm with rough endoplasmic reticulum (*er*), Golgi apparatus (*go*), and lamellar bodies (*lb*) near the plasma membrane. **c** Mature leaves. Vascular and perivascular parenchyma present metabolically active cells with large nuclei and conspicuous nucleoli, chloroplasts, and phenolic inclusions in the vacuoles. Mature fibers (*fb*) and mature sieve tube elements (*se*) can be observed. **d**–**h** Galls. **d** Phase of induction. Cells with many mitochondria (*mi*) and nuclei, lomasomes (*lo*), and rough endoplasmic reticulum near the cell wall. **e** Phase of growth and development. Cells with large nuclei, underdeveloped chloroplasts, and many lomasomes. **f** Phase of maturation. Cell with large nucleus, dense cytoplasm, and small vacuole, with many lomasomes near the cell wall. **g**, **h** Phase of senescence. Cells with large mitochondria, rough endoplasmic reticulum, degraded chloroplast with starch grains (*st*), and many lomasomes. *Bars* **a**, **c**=5 μm; **b**, **g**, **h**=2 μm; **d**–**f**=1 μm

cells of the mesophyll. The lack of intercellular spaces indicated the occurrence of little gas exchange (Kraus 2009) and consequent reduced photosynthetic metabolism. This configures the *N. myrtoidis* galls on *P. myrtoides* as sinks of photoassimilates, similarly to the horn-shaped galls of *Copaifera langsdorffii,* which also had homogenous parenchyma with low chlorophyll content (Castro et al. 2012b). Moreover, the ultrastructure of the chloroplasts in galls also point out to the photosynthetic deficiency when compared to the leaves of *P. myrtoides*, whose chloroplasts have low stack grana with few electron lucent thylakoids, similar to the sun-type chloroplasts described by Lichtenthaler et al. (1981). On the contrary, the chloroplasts in the galls have little lamellation, and the grana, when present, are similar to those of the shade-type leaves, i.e., they have high stack grana with many electron dense thylakoids (Lichtenthaler et al. 1981). In mature galls, chloroplasts differentiate into amyloplasts and lose the photosynthetic function. This indicates that galls photosynthesize at lower levels when compared to the leaves as suggested by Carneiro et al. (2014). Such photosynthetic deficiency is also indicated by the lack of plastoglobules in the chloroplasts of galls when compared to the leaves.

The plastoglobules are commonly found in tissues subjected to high oxidative stress, and their presence minimizes the damages caused by reactive oxygen species (ROS) to the photosynthetic apparatus (Asada 2006; Moller et al. 2007). ROS are produced by photosynthesizing cells (Moller et al. 2007), but in galls, where the biotic stress imposed by the galling insect should even enhance the production of ROS. In fact, recent studies have shown higher accumulation of ROS in galled than in non-galled tissues of galls induced by *Pseudophacopteron* sp., a sucking insect, on *A. australe* (Oliveira et al. 2011b; Isaias et al. 2011). In this system, the plastoglobules develop exclusively in the galls because of the stress imposed by the galling herbivore and allow the galls to photosynthesize at the same levels of the non-galled leaves (Oliveira et al. 2011a). Even though *N. myrtoidis* is also a sucking insect, their galls accumulate little ROS in the mesophyll (Carneiro et al. 2014), and plastoglobules development is blocked, in contrast to the photosynthesizing leaves. Altogether, these cytological features corroborate the low metabolism of the cells in the mesophyll and the hypothesis that the galls of *N. myrtoidis* are indeed photosynthesis-deficient (Carneiro et al. 2014).

Differently from the cells of the mesophyll, the cytological characteristics of the vascular and perivascular parenchyma indicate their high metabolism. From the phase of gall induction until senescence, these cells present large active nuclei, many mitochondria, endoplasmic reticulum, lomasomes, multivesicular bodies, and lamellar bodies, which were already reported for metabolically active cells in the nutritive tissues of galls induced by Thysanoptera (Raman and Ananthakrishnan 1983). Even though true nutritive tissues are not reported for the galls of Psylloidea (Meyer 1987), the tissues near the feeding sites of *N. myrtoidis*, i.e., the vascular bundles and neighboring cells, regard some similarities with the nutritive tissues of galls induced by Hymenoptera, Diptera, and Lepidoptera (Bronner 1992; Oliveira et al. 2011b; Vecchi et al. 2013). Nevertheless, they do not form any gradient as the ones described by Bronner (1992) and Oliveira and Isaias (2010b). The fact that sap-sucking insects like *N. myrtoidis* feed preferably on the vascular bundles (Prado and Tjallingii 1994) explains the higher accumulation of ROS at these sites (Carneiro et al. 2014). The consequent oxidative stress of such accumulation should be compensated by the activity of the lomasomes, lamellar bodies, and multivesicular bodies. These structures are believed to continuously recycle the endomembrane systems together with the endoplasmic reticulum (Staehelin 1997) and, in the galls, may play a role which is analogous to that of the plastoglobules in the chloroplasts of the non-galled leaves, i.e., repairing oxidative damage.

During the cytological development of *N. myrtoidis* galls, the cell cycles are fastened, as the life cycle of the galls is much shorter than that of the host non-galled leaves. The fates of the mesophyll cells are changed by "standby-redifferentiation" during the phase of growth and development, followed by abrupt changes during the redifferentiation of a mechanical tissue at the phases of gall maturation and senescence, followed by tissue necrosis. This sequence of cytological events is a new record for the development of insect galls. No cytological gradients, such as the ones classically described for galls of Cynipids and Cecidomyiids of the temperate region (Bronner 1992), and for the Neotropical galls of cecidomyiids (Oliveira et al. 2010, 2011b) and psyllids (Oliveira and Isaias 2010b), were evidenced. Nevertheless, the features found herein corroborate the low metabolism of the photosynthesis-deficient mesophyll of these galls (Carneiro et al. 2014) which does not accumulate as much ROS as some other Neotropical galls. Also, a new cytological association involves the perivascular parenchyma and the true nutritive tissues of other insect-induced galls (Bronner 1992; Oliveira et al. 2010, 2011b), which share cytological similarities. As expected, the metabolism assessed by cytological features is in accordance with the histochemical profile previously described for the *N. myrtoidis* galls (Carneiro et al. 2014).

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Could the extended phenotype extend to the cellular and subcellular levels in insect-induced galls?

Could the extended phenotype extend to the cellular and subcellular levels in insect-induced galls?

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Abstract

Neo-ontogenesis of plant galls involves redifferentiation of host plant tissues to express new phenotypes, when new cell properties are established via structural-functional remodeling. Herein, *Psidium cattleianum* leaves and *Nothotrioza cattleiani* galls are analyzed by developmental anatomy, cytometry and immunocytochemistry of cell walls. We address hypothesis-driven questions concerning the organogenesis of globoid galls in the association of *P. cattleianum* - *N. cattleianum*, and *P. myrtoides* - *N. myrtoidis*. These double co-generic systems represent good models for comparing final gall shapes and cell lineages functionalities under the perspective of convergent plant-dependent or divergent insect-induced characteristics. Gall induction and growth and development are similar in both galls, but homologous cell lineages exhibit divergent degrees of cell hypertrophy and directions of elongation. Median cortical cells in *P. cattleianum* galls hypertrophy the most, while in *P. myrtoides* galls there is a centrifugal gradient of cell hypertrophy. Cortical cells in *P. cattleianum* galls tend to anisotropy, while *P. myrtoidis* galls have isotropically hypertrophied cells. Immunocytochemistry evidences the chemical identity and functional traits of cell lineages: epidermal cells walls have HGAs and galactans, which confer rigidity to sites of enhanced cell division, oil gland cell walls have AGPs that help avoiding cell death, and parenchyma cell walls have HGAs, galactans and arabinans, which confer porosity. Variations on such chemical identities are related to specific sites of hypertrophy. Even though the double co-generic models have the same macroscopic phenotype, the globoid morphotype, current analyses evidence that the extended phenotype *N. cattleiani* is substantiated by cellular and subcellular specificities.

Introduction

The ontogenesis of plant organs occurs via conservative cellular mechanisms that act synergistically for the determination of the variable forms and functions observed in nature [1]. Plant galls represent the neo-ontogenesis [2] of their host organs towards a new phenotype, i. e., the gall morphotypes [3]. For the generation of these gall morphotypes, plant tissues and cells respond to the stimuli of gall-inducing herbivores by redifferentiating [4] new cell types. In the context of gall structure, such cells have adaptive significance for the gall inducers as far as their protection and nourishment are concerned [5, 6].

Neotropical gall morphotypes have been studied on developmental anatomy basis, focusing on the responses of tissue hyperplasia and cell hypertrophy, the degree of isotropy and/or anisotropy of cell expansion [7], and the structural-functional traits derived from these responses [8, 9, 10, 11, 12]. More recently, the immunocytochemistry of cell walls in gall tissues have been studied [13, 14], and helped elucidating the functionalities of the cell walls, and their roles in gall development. Under the perspective of the developmental anatomy and immunocytochemistry of plant cell walls, Carneiro et al. [2] provided an interesting insight about the organogenesis of a globoid leaf gall on *Psidium myrtoides* (Myrtaceae) induced by *Nothotrioza myrtoidis* (Triozidae). The composition of cell walls during the development of this gall influences dynamic properties of cell lineages in terms of rigidity, flexibility, porosity, and adhesion, as described for plant organs in general [15, 16, 17]. Such properties affected the mechanisms of cell growth, i. e., division and/or expansion, and

determined the establishment of a centrifugal gradient of cell hypertrophy with isotropic growth in the cortex of *N. myrtoidis* galls [2].

Current model of study, the interaction between the host plant *Psidium cattleianum* Sabine (Myrtaceae) and the gall-inducing herbivore *Nothotrioza cattleiani* Burckhardt (Triozidae) results in the morphogenesis of globoid galls, very similar to those of the double co-generic system, *P. myrtoides-N. myrtoidis* [2]. The galls on *P. cattleianum* and *P. myrtoides* are both globoid [3], protrude to the abaxial surface of the leaf lamina, and have univoltine cycles [18, 19]. To the extent of ecological and macro-morphological aspects, the phenotypic expression of the genes from the two species of *Nothotrioza* exerts biochemical influence on the cells of two species of *Psidium.* Unexpectedly, they generate the same extended phenotype [20], the globoid gall morphotype. As the gall structure is adaptive for their inducers [21], and varies according to the phylogeny of galling insects [22], we hypothesize that *Nothotrioza* spp. galls on *Psidium* spp. are unique entities, i. e., true extended phenotypes with species-specific traits at the cellular and subcellular levels. The following questions are addressed: (1) Are there divergent patterns on the way *Nothotrioza* spp. manipulate the standard leaf morphogenesis of *Psidium* spp. towards the ontogenesis of globoid galls? (2) Should the gradients of cell transformations be quantitatively divergent on the co-generic systems? (3) Is the distribution of pectins and proteins a conservative trait of the cell lineages within and between the *Nothotrioza* spp. galls?

Material and methods

Study area

The studied population of *P. cattleianum* is located at the Parque Estadual Pico do Marumbi, municipality of Piraquara, state of Paraná, Brazil. Individuals (n = 5) with galled leaves were marked and accompanied during 2012 and 2013.

Ethics statement

The authors declare that the studied species are not protected and/or threatened. The access to the protected area of the Parque Estadual Pico do Marumbi, and the permission for field sampling were granted by the Instituto Ambiental do Paraná – IAP (document number 34.14), and by the Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio (document number 33424-4).

Structural analyses

Samples of young and mature leaves, and galls at the stages of induction, growth and development, maturation and senescence [19] (n = 5 per developmental stage) were collected from different individuals, and fixed in Karnovsky's solution in 0.1 M phosphate buffer (pH 7.2) [23]. The material was dehydrated in ethanol series [24], embedded in glycolmethacrylate (Leica®), sectioned (6-10 µm) with a rotary microtome Hyrax (Zeiss®), stained with 0.05% Toluidine O blue, pH 4.6 [25]. Part of the material was dehydrated in n-butyl series [24], embedded in Paraplast® [26], sectioned (12-14 µm) with a rotary microtome (Jung biocut) and stained with 9:1 (v/v) Astra blue

and safranin [27] (modified to 0.5%). Histological slides were observed and photographed under light microscope (Leica® DM500) coupled with digital camera (Leica® ICC50 HD).

Cytometry and histometry

Digital images were obtained with an optical photomicroscope Leica® DM500, and analyzed with the AxioVision® software, Zeiss Imaging Systems, version 4.7.2 [28]. Cells from the adaxial and abaxial protoderm, the adaxial, median and abaxial layers of the ground meristem of non-galled leaves, as well as their redifferentiated gall tissues had their areas individually measured (n = 50; 5 cells of each tissue per image, 10 images per developmental stage). Also, the major axes of these cells – anticlinal and periclinal – were measured in each tissue lineage and the ratio between them was indicative of the degree of isotropy/anisotropy. The thickness of the mesophyll was measured at different developmental stages.

Immunocytochemistry of plant cell wall epitopes

Unstained material (n = 3; from different individuals) fixed in Karnovsky's solution in 0.1 M phosphate buffer (pH 7.2) [23], dehydrated in ethanol series [24], and embedded in glycolmethacrylate (Leica®) was incubated with the monoclonal antibodies JIM5, JIM7, LM1, LM2, LM5, LM6, LM19 and LM20 (Centre for Plant Sciences, University of Leeds, UK). These antibodies specifically bind epitopes of low methylesterified homogalacturonans (HGAs) (JIM5) [15, 16, 29, 30], medium- high methylesterified HGAs (JIM7) [15, 16, 30], extensins (LM1) [31, 32, 33, 34], arabinogalactan proteins (APGs) (LM2) [35], galactans (LM5) [36], arabinans (LM6) [37], unesterified HGAs (LM19), and high methylesterified HGAs (LM20) [38]. The sections were immersed in blocking solution with 3% (w/v) powdered milk in PBS for 30 min to avoid cross labelling, and incubated with primary antibodies in PBS for 2 h at room temperature. For the control tests, the primary antibodies were suppressed. Sections were washed in PBS, and incubated in the secondary antibody anti-rat IgG – FITC (Sigma) in PBS for 2 h in the dark. After washing in PBS, the sections were mounted in 50% glycerin and analyzed under a Confocal Zeiss 510 META microscope, with excitation wavelength of 488 ηm and 505–530 ηm emission filter.

Statistical analyses

Statistical analyses of the cytometric variables were performed using the software Past 3 [39]. Normal data (Shapiro-Wilk test) were compared by parametric tests of ANOVA followed by t-test or multiple tests of Tukey. Non-normal data were compared using the non-parametric tests of Kruskal-Wallis followed by Dunn's multiple tests. All tests used $\alpha = 0.05$.

Results

Ontogeny of non-galled leaves and galls

Non-galled leaves

The leaves of *P. cattleianum* are simple, opposite, and emerge in pairs from the apical meristem. The uniseriate protoderm is derived from the divisions of the marginal initials, and the multilayered ground meristem is derived from the submarginal initials. At the early stages of leaf development, the ground meristem is divided into three regions, i.e. adaxial, median and abaxial layers. The cells of such layers divide anticlinally causing the elongation of the limb, and periclinally, increasing mesophyll thickness. Initially, the ground meristem has two adaxial layers, two median layers and two abaxial layers. Procambial strands differentiate from the median layers (Fig. 1a). As the leaves develop, periclinal divisions add one cell layer both to the adaxial and abaxial layers, which end up with three layers each, and have phenolic inclusions. The median layers, whose cells have hyaline protoplasts, divide periclinally, reaching six cell layers (Fig. 1b). Mature leaves have uniseriate epidermis, 12-layered mesophyll with one-layered hypodermis derived from the uppermost cell layer of the ground meristem. The palisade parenchyma is derived from the adaxial and the uppermost cell layers of the median ground meristem. The spongy parenchyma is 8-9-layered and is derived from both the median and abaxial layers of the ground meristem (Fig. 1c). Vascular bundles are collateral and often surrounded by a sclerenchymatic bundle sheath.

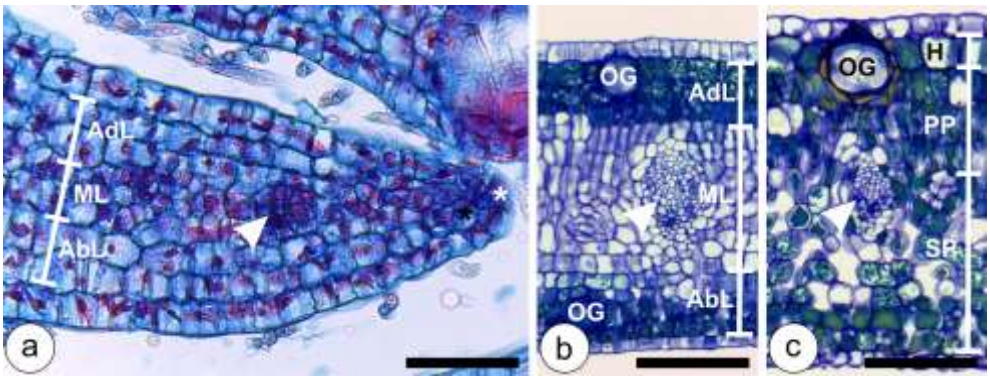

Fig. 1. **Developmental anatomy of *Psidium cattleianum* leaves.** (**a**) Early developmental stage of the leaf, with uniseriate epidermis derived from marginal initial (white asterisk), ground meristem derived from submarginal initial (black asterisk) divided into two adaxial layers (AdL), two median layers (ML), and two abaxial layers (AbL). Procambium (arrow head) differentiates from the median layers of the ground meristem. (**b**) Young leaf with uniseriate epidermis, three-layered AdL, six-layered ML, three-layered AbL, and collateral vascular bundles (arrow head). Developing oil glands (OG) differentiate both from the adaxial and abaxial epidermal surfaces. (**c**) Mature leaves with uniseriate epidermis, one-layered hypodermis (H), three-layered palisade parenchyma (PP) and eight-layered spongy parenchyma (SP). Mature vascular bundles (arrow head) and oil glands (OG) are observed. Bars: (**a**) 50 µm; (**b, c**) 100 µm.

Galls

N. cattleiani induces galls on young leaves of *P. cattleianum*, which maintain its tissue zonation (Fig. 2a). The inner and the outer gall epidermises are derived from the adaxial and abaxial leaf epidermises, respectively. Oil glands differentiate exclusively from the gall outer epidermal cells and are localized among the outermost cortical cell layers. The cortex has three zones: inner, median and outer layers, which redifferentiate from the adaxial, median and abaxial layers of the ground meristem of the young leaves, respectively.

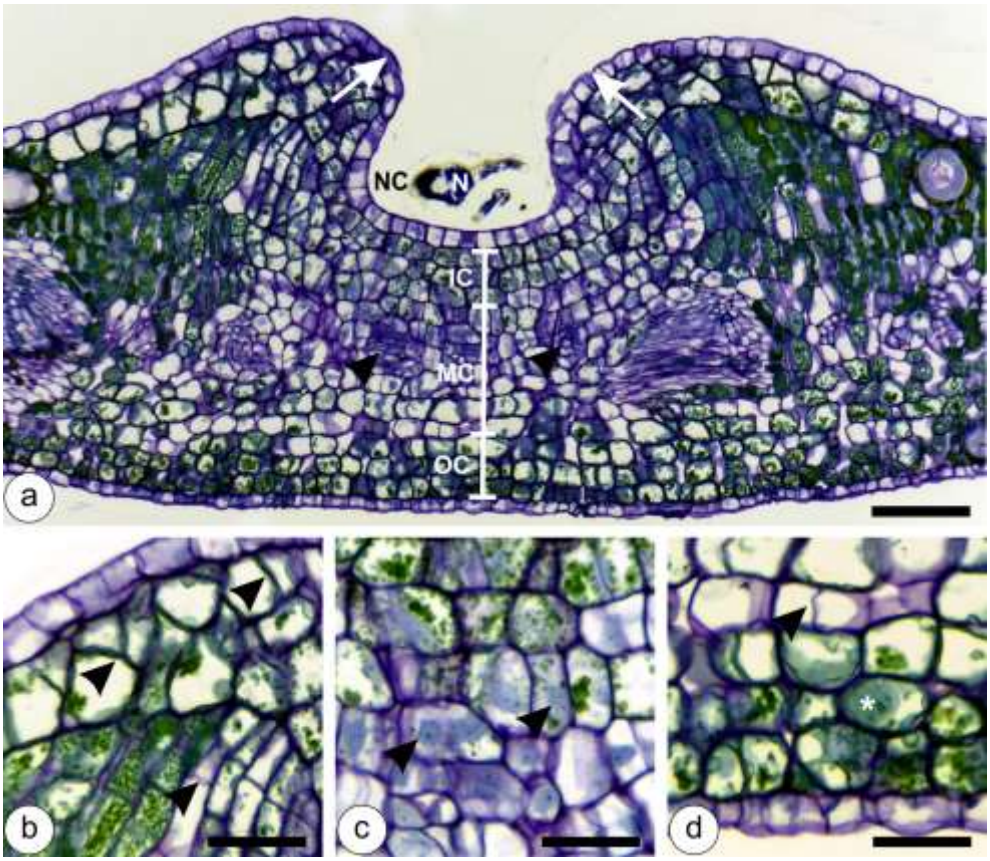

Fig. 2. **Developmental anatomy of *Nothotrioza cattleiani* galls at the stage of induction.** (**a**) General view, showing the growth of projections (arrows) over the first instar nymph (N) to form the nymphal chamber (NC). Gall cortex has three-layered inner cortex (IC), six-layered median cortex (MC) and three-layered outer cortex (OC), and redifferentiated procambium strands (arrow heads). (**b**) Detail of the projections with sites of tissue hyperplasia (arrow heads) in the hypodermis (H) and palisade parenchyma (PP). (**c**) Detail of median cortical cells with hyaline protoplasts and conspicuous nuclei and nucleoli. (**d**) Detail of outer cortical cells with slightly hypertrophied cells, anticlinal cell divisions (arrow head) and phenolic inclusions in the protoplast (asterisk). Bars: (**a**) 100 µm; (**b-d**) 50 µm.

Gall induction occurs via the stimuli of the first instar nymphs, which establish on the adaxial leaf surface, and start feeding preferably on the vascular bundles. Projections on the adaxial leaf surface grow around the insect body, and constitute the first set of anatomical alterations towards the development of the galls (Fig. 2a, b). The epidermal cells divide anticlinally and elongate periclinally, while the cells of the adaxial layers divide both periclinally and anticlinally for the morphogenesis of the projections (Fig. 2b). Such emergencies grow over the insect, towards one another to form an occluded ostiole; they enclose the insect within a nymphal chamber, determining the endophytic habitus of *N. cattleiani*. Right below the nymphal chamber, the gall cortex has slightly hypertrophied cells that divide anticlinally (Fig. 2c, d; arrows). The median cortical layers have hyaline protoplasts, large nuclei and conspicuous nucleoli (Fig. 2c). The inner and outer layers have inconspicuous nuclei and phenolic inclusions in the vacuoles (Fig. 2c, d). The outer gall epidermis remains uniseriate, as its cells divide anticlinally (Fig. 2d).

During the stage of growth and development, when the second, third and fourth instar nymphs are inside the galls, the second set of anatomical alterations takes place. At this stage, the gall cortex enlarges together with the nymphal chamber for the determination of the gall globoid shape (Fig. 3a). The inner cortical layers divide mainly anticlinally, so the number of cell layers does not increase. The median and outer cortical layers, on the other hand, divide both anticlinally and periclinally, and are responsible for increasing the number of gall cortical cell layers. Vascular bundles redifferentiate from the middle layers of the gall cortex, close to the nymphal chamber (Fig. 3b). The inner and the outer epidermal layers remain uniseriate, and divide anticlinally to accompany the growth of the developing gall. The inner epidermal cells divide sparsely and elongate periclinally, while the outer epidermal cells divide intensely, and do not elongate (Fig. 3c, e). Cells of the inner and the outer cortical layers remain with phenolic inclusions (Fig. 3c, e), while the cells of the median cortical layers have hyaline protoplasts (Fig. 3c, d). Vascular bundles are collateral, with few tracheal elements, and abundant phloem and parenchyma (Fig. 3c). The galls increase in size until the stage of maturation, which begins when the nymphs reach the fifth instar, and lasts until they molt into adults, and are ready to leave the galls. The structural organization in the cortex of mature galls is similar to that of the previous developmental stage, with hypertrophied cells throughout the cortex (Fig. 4a-d). The inner epidermis remains uniseriate; the inner cortical cells have phenolic inclusions, and the vascular bundles develop into a collateral arrangement, with equal portions of xylem and phloem (Fig. 4a). In the transition between the median cortical cells, which have hyaline protoplasts, and the outer cortical cells with phenolic inclusions, it is possible to observe anticlinally elongated cells (Fig. 4b). Sclerenchyma cells (5-6 layers) differentiate from the outer cortical cells, and constitute the most remarkable anatomical feature of mature galls (Fig. 4c). The outermost cortical cells located between the sclerenchyma layers and the outer epidermis are vacuolated and periclinally elongated (Fig. 4d).

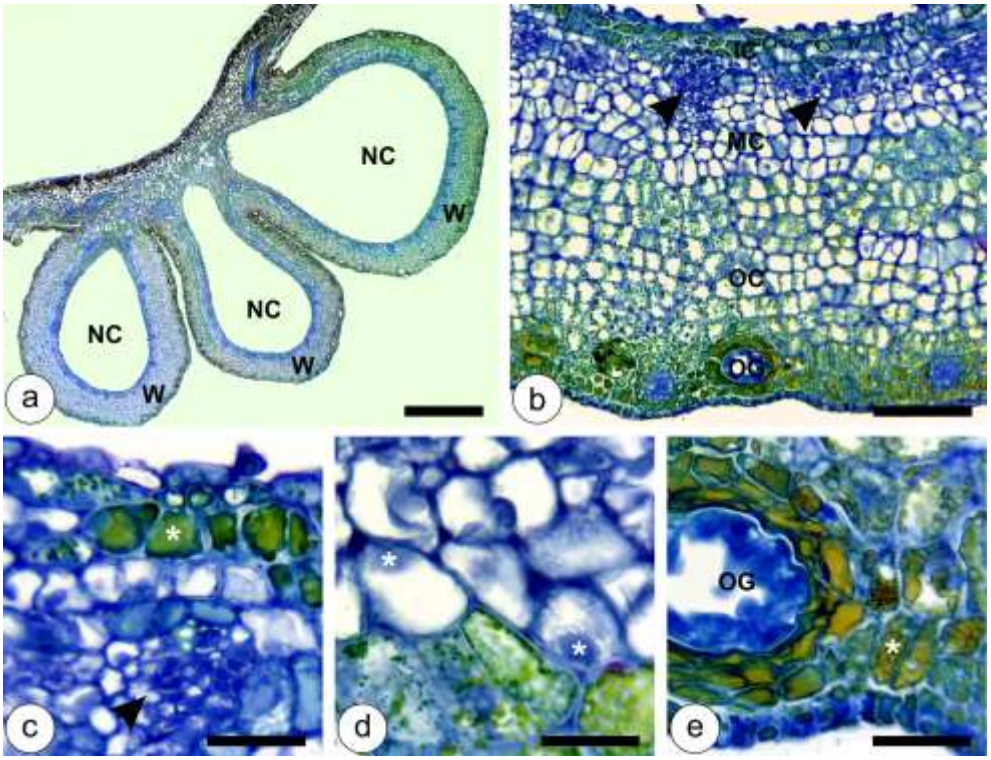

Fig. 3. **Developmental anatomy of *Nothotrioza cattleiani* galls at the stage of growth and development.** (**a**) General view of three galls, with thin walls (W) and ample nymphal chamber (NC). (**b**) Detail of gall cortex with reduced inner cortex (IC), and hyperplasic median (MC) and outer (OC) cortices. Vascular bundles (arrow heads) are distributed closest to the nymphal chamber in the MC, and the oil glands (OG) differentiate exclusively from to outer epidermis in the OC. (**c**) Detail of the uniseriate inner epidermis, inner cortical cells with phenolic inclusions (asterisk), and vascular bundles with few tracheal elements, and abundant phloem and parenchyma (arrow head). (**d**) Detail of the median cortical cells with hyaline protoplasts and conspicuous nuclei (asterisks). (**e**) Detail of outer cortical cells with phenolic inclusions (asterisk). Oil glands are fully developed. Bars: (**a**) 2 mm; (**b**) 200 µm; (**c-e**) 50 µm.

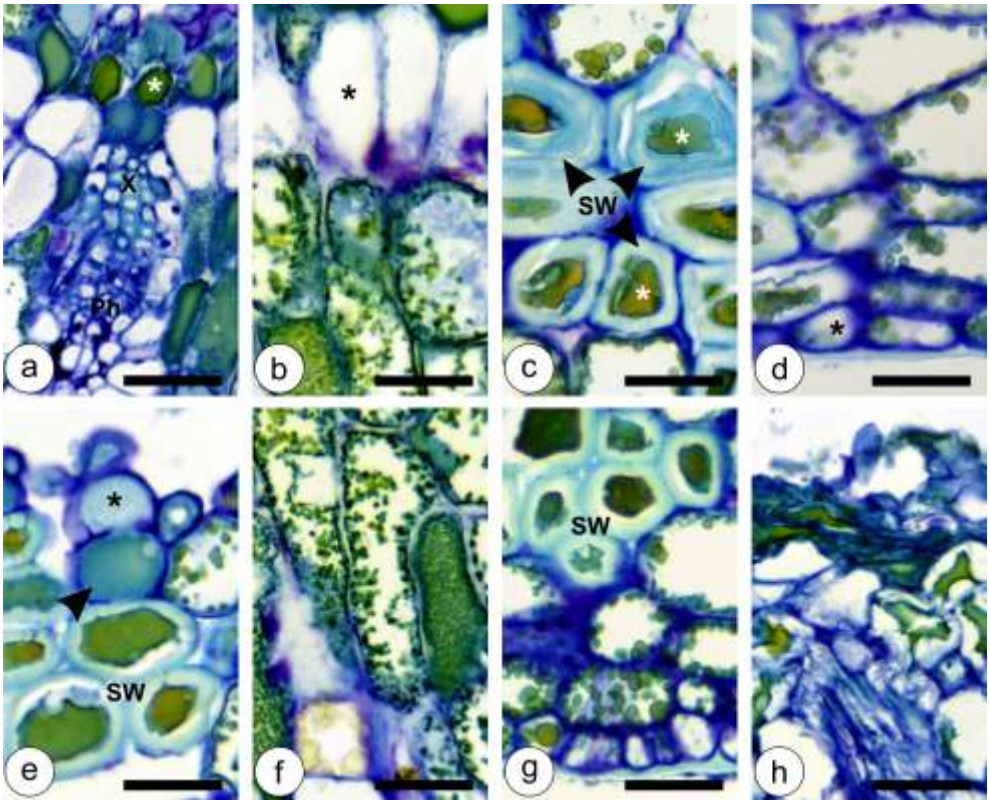

Fig. 4. **Developmental anatomy of *Nothotrioza cattleiani* galls**. (**a-c**) Stage of maturation. (**d-f**) Stage of senescence. (**a**) Detail of hypertrophied inner epidermal cells, inner cortical cells with phenolic inclusions (asterisk) and collateral vascular bundles with equal portions of xylem (X) and phloem (Ph). (**b**) Detail of hypertrophied and anticlinally elongated median cortical cells with hyaline protoplasts (asterisks), and the outer cortical cells with phenolic inclusions. (**c**) Detail of live sclerenchyma cells at the outer cortex, with phenolic inclusions and thick secondary walls (SW). (**d**) Detail of the periclinally elongated outermost cortical cell layers adjacent to the outer epidermis (asterisk). (**e**) Detail of inner epidermal (asterisk) and inner cortical cells (arrow head); median cortical cells with secondary lignified cell walls (SW) and phenolic inclusions. (**f**) Detail of phenolic substances accumulated in the anticlinally elongated median cortical cells (asterisk). (**g**) Detail of the outer cortical cells and outer epidermis with secondary walls (SW) and vacuolated non-lignified cells. (**h**) Detail of necrotic sites in the gall apex that lead to gall dehiscence. Bars: 50 µm.

At their final developmental stage, senescent galls present anatomical alterations, which lead to a dehiscence mechanism by which the galls open. Median cortical cells lignify, but remain live with phenolic inclusions within their protoplasts (Fig. 4e). Phenolic substances accumulate gradually in the median cortical cells, which are elongated (Fig. 4f). The outer cortical cells and outer epidermis remain similar to the previous developmental stage, with sclerenchyma cells and vacuolated non-lignified cells (Fig. 4g). Ultimately, necrotic sites develop from the apex towards gall base, and from the innermost towards the outermost cortical cell layers (Fig. 4h). The necrotic tissues crack due to the tension exerted by the adjacent live and turgid cells, thus rupturing the gall wall, and allowing the scape of the adult insect.

Dynamics of cell sizes and shapes

During the maturation of the non-galled leaves of *P. cattleianum*, cells from all tissues grow. Epidermal cells on the adaxial and abaxial surfaces of mature leaves increase in area approximately 100% and 90% in relation to the young leaves, respectively. Chlorophyllous parenchyma cells from the adaxial, median and abaxial layers of the young leaves grow approximately 130% towards the stage of leaf maturation (Table1). As the galls are induced on young leaves, the tissues undergo different dynamics of cell size and shape. The inner epidermal cells derived from the adaxial epidermis of the leaves grow 25% towards the stage of gall induction, 120% towards the stage of growth and development, 72% towards the stage of maturation, and 20% towards the stage of senescence. Parenchyma cells of the inner cortical layers derived from the adaxial cell layers of leaves grow 17% towards the stage of gall induction, 180% towards the stage of growth and development, 60% towards the stage of maturation, and 80% towards the stage of senescence. The median cortical layers derived from the median layers of the leaves grow 24% towards the stage of gall induction, 395% towards the stage of growth and development, 44% towards the stage of maturation, and 23% towards the stage of senescence. The outer cortical cells derived from the abaxial cell layers of the leaves grow 69% towards the stage of gall induction, 148% towards the stage of growth and development, 138% towards the stage of maturation, and decrease their size in about 12% towards the stage of senescence. The outer epidermal cells derived from the abaxial epidermis of the leaves grow 36% towards the stage of gall induction, 100% towards the stage of growth and development, 6% towards the stage of maturation, and decrease their size in about 22% towards the stage of senescence (Table1). The thickness of the mesophyll of young leaves (345.06 ± 12.55 µm) increases towards mature leaves (447.64 ± 5.77 µm). The mesophyll is slightly thinner in the galls at the induction stage (334.19 ± 12.22 µm) when compared to young leaves. As the galls develop, mesophyll thickness increases massively at the stages of growth and development (873.14 ± 82.50 µm), maturation (1086.42 ± 31.75 µm) and senescence (1170.59 ± 43.92 µm).

Table 1. Dynamics of cell hypertrophy during the ontogenesis of *Psidium cattleianum* leaves and *Nothotrioza cattleiani* galls (mean cell area - µm² ± standard deviation).

Developmental stages	Cell lineages				
	Adaxial/inner epidermis	Adaxial meristem/ inner cortex	Median meristem/ median cortex	Abaxial meristem/ outer cortex	Abaxial/outer epidermis
Young non-galled leaf	254.96 ± 10.18*f*	324.57 ± 12.94*d*	336.53 ± 13.32*e*	236.95 ± 8.70*d*	149.22 ± 6.45*d*
Mature non-galled leaf	510.63 ± 15.64*ed*	768.37 ± 26.97*c*	764.61 ± 28.49*d*	549.10 ± 25.07*d*	288.57 ± 10.42*c*
Gall induction	319.01 ± 20.37*d*	381.00 ± 27.56*d*	417.99 ± 24.38*de*	399.03 ± 31.09*d*	203.51 ± 12.45*cd*
Gall growth and development	705.05 ± 46.88*ce*	1077.00 ± 65.12*c*	2066.94 ± 122.63*c*	993.39 ± 78.59*c*	406.62 ± 34.59*b*
Gall maturation	1216.55 ± 67.23*b*	1735.63 ± 84.40*b*	2993.51 ± 119.72*b*	2372.67 ± 152.36*a*	434.92 ± 22.46*a*
Gall senescence	1469.29 ± 115.32*a*	3135.36 ± 159.55*a*	3695.63 ± 161.63*a*	2091.96 ± 98.98*b*	340.91 ± 19.52*bc*

Values followed by different italic letters in the columns indicate significant difference between the cell areas from the different lineages at different developmental stages. (ANOVA followed by t-test or multiple tests of Tukey; Kruskal-Wallis followed by Dunn's multiple tests. $\alpha = 0.05$).

The adaxial epidermal cells are anticlinally elongated in the young leaves, and elongate periclinally in mature leaves. During gall development on young leaves, the adaxial epidermal cells originate the anticlinally elongated inner epidermal cells at gall induction. Such cells elongate periclinally at the stages of growth and development and maturation, and tend to grow isotropically at gall senescence (Table 2). The cells of the adaxial meristem in the young leaves are slightly anticlinally elongated. During the differentiation of palisade parenchyma, this pattern is maintained during leaf maturation. The inner cortical cells derived from the adaxial meristem are anticlinally elongated, tending to grow isotropically in the galls at growth and developmental stage. During gall maturation and senescence, these cells elongate anticlinally (Table 2). The median and abaxial meristem cells in the young leaves are slightly periclinally elongated, and originate the cells of the spongy parenchyma in the mature leaves, which tend to grow isotropically. In the galls, the median cortical cells elongate anticlinally at induction, while the outer cortical cells tend to isotropy. At the stage of growth and development, both cell lineages tend to grow isotropically, but at the stages of gall maturation and senescence, the median cortical cells elongate anticlinally, while the outer cortical cells elongate periclinally (Table 2). The abaxial epidermal cells grow isotropically in the young leaves, but as they mature, these cells elongate periclinally. In the galls at the stages of induction and growth and development, the outer epidermal cells grow isotropically, but during gall maturation and senescence, the growth is anisotropic with periclinal elongation (Table 2).

Table 2. Directions of cell expansion and degree of isotropy/anisotropy during the ontogenesis of *Psidium cattleianum* leaves and *Nothotrioza cattleiani* galls (µm ± standard deviation).

Developmental stages		Cell lineages				
		Adaxial/inner epidermis	Adaxial meristem/ inner cortex	Median meristem/ median cortex	Abaxial meristem/ outer cortex	Abaxial/outer epidermis
Young non-galled leaf	A	20.86 ± 0.39*d*	21.26 ± 0.33*f*	22.67 ± 0.77*d*	19.21 ± 0.36*d*	14.30 ± 0.40*c*
	P	14.00 ± 0.44**d**	17.85 ± 0.69**d**	18.24 ± 0.43**e**	15.42 ± 0.53**d**	13.10 ± 0.55**d**
	R	1.49	1.19	1.24	1.24	1.09
Mature non-galled leaf	A	21.63 ± 0.37*dc*	45.30 ± 0.92*d*	30.09 ± 0.83*d*	24.83 ± 0.59*c*	14.30 ± 0.25*c*
	P	28.06 ± 0.70**e**	20.06 ± 0.76**d**	32.30 ± 1.02**d**	27.47 ± 1.01**c**	24.77 ± 0.78**ab**
	R	0.77	2.25	0.93	0.90	0.57
Gall induction	A	20.91 ± 0.66*d*	25.72 ± 1.19*ef*	25.64 ± 1.10*d*	22.67 ± 0.68*cd*	14.62 ± 0.47*c*
	P	17.74 ± 0.79**d**	17.08 ± 0.70**d**	19.78 ± 0.87**e**	21.17 ± 1.16**cd**	16.10 ± 0.73**d**
	R	1.17	1.50	1.29	1.07	0.90
Gall growth and development	A	25.16 ± 1.10*c*	36.19 ± 1.30*c*	51.86 ± 1.92*c*	35.38 ± 1.39*b*	21.52 ± 0.81*b*
	P	33.77 ± 1.29**c**	35.46 ± 1.21**c**	47.06 ± 1.91**c**	32.59 ± 1.75**b**	20.97 ± 1.14**c**
	R	0.74	1.02	1.10	1.08	1.02
Gall maturation	A	35.16 ± 1.10*b*	52.36 ± 2.35*bd*	71.52 ± 2.51*b*	48.23 ± 2.03*a*	21.93 ± 0.59*b*
	P	42.16 ± 1.60**b**	41.54 ± 1.44**b**	53.03 ± 1.37**b**	61.25 ± 2.83**a**	24.95 ± 0.84**a**
	R	0.83	1.26	1.34	0.78	0.87
Gall senescence	A	41.70 ± 1.79*a*	84.48 ± 4.56*a*	80.75 ± 2.79*a*	48.30 ± 1.61*a*	19.09 ± 0.69*a*
	P	43.82 ± 2.03**a**	50.07 ± 1.63**a**	59.47 ± 1.59**a**	55.10 ± 1.75**a**	21.70 ± 0.74**bc**
	R	0.95	1.68	1.35	0.87	0.87

Lines labelled "A" and "P" respectively correspond to anticlinal and periclinal axes of the cells from different cell lineages at different developmental stages. Lines labelled "R" correspond to the ratio A/P from the same cell lineage at different developmental stages. Values lower than 0.9 indicate tendency to anisotropic growth with periclinal elongation; values between 0.9 and 1.1 indicate tendency to isotropic growth, and values higher than 1.1 indicate tendency to anisotropic growth with anticlinal elongation. Values followed by different italic and boldface letters in the columns indicate

significant difference between anticlinal and periclinal axes of cells (ANOVA followed by t-test or multiple tests of Tukey; Kruskal-Wallis followed by Dunn's multiple tests. $\alpha = 0.05$).

Immunocytochemistry of plant cell walls

Non-galled leaves

In the adaxial epidermis of young leaves, epitopes of medium-high methylesterified homogalacturonans (HGAs), extensins, arabinogalactan proteins (AGPs) and galactans are bound by the antibodies JIM7, LM1, LM2 and LM5, respectively (Fig. 5a). In the abaxial surface of the epidermis, the same epitopes, except for the extensins, are bound. The cells of the oil glands have epitopes of medium-high methylesterified HGAs bound by JIM7, unesterified and high methylesterified HGAs bound by LM19 and LM20, and AGPs, galactans and arabinans bound by LM2, LM5 and LM6, respectively. In all cell layers of the chlorophyllous parenchyma, the walls have epitopes of low methylesterified HGAs bound by JIM5, high methylesterified HGAs bound both by JIM7 and LM20, and galactans and arabinans bound by LM5 and LM6, respectively. AGPs bound by LM2 are labeled strictly in the abaxial layers of the chlorophyllous parenchyma. Cells of the vascular tissues have epitopes of medium-high methylesterified HGAs, extensins, AGPs and galactans, respectively bound by JIM7, LM1, LM2 and LM5 (Fig. 5a). At this developmental stage, low methylesterified HGAs (Fig. 6a) widespread in the leaf tissues, extensins and AGPs in the cells of the vascular tissues are detected in microdomains of the cell walls, generating discontinuous signals. High methylesterified HGAs, AGPs, galactans (Fig. 6b), arabinans, and extensins had more ubiquitous epitopes, and generated continuous signal in epidermal cells.

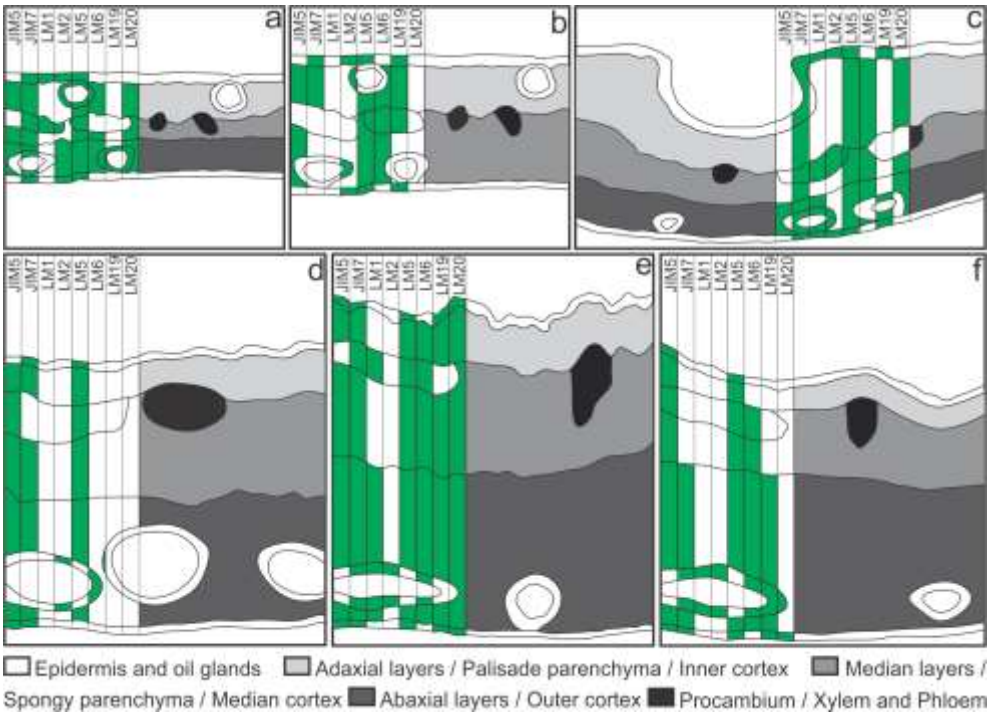

Fig. 5. Development-dependent immunolabelling of plant cell wall pectins and proteins during the development of *Psidium cattleianum* leaves and *Nothotrioza cattleiani* galls. Gray scale labels indicate different ontogenetic tissues and the filling in the perpendicular bars indicates positive

binding of low methylesterified homogalacturonans by the JIM5 antibody, medium-high methylesterified homogalacturonans by the JIM 7 antibody, extensins by the LM1 antibody, arabinogalactan proteins by the LM2 antibody, galactans and arabinans by the LM5 and LM6 antibodies, and unesterified HGAs and high methylesterified HGAs by the LM19 and LM20 antibodies. (**a**) Young leaves. (**b**) Mature leaves. (**c-f**) Galls. (**c**) Induction. (**d**) Growth and development. (**e**) Maturation. (**f**) Senescence.

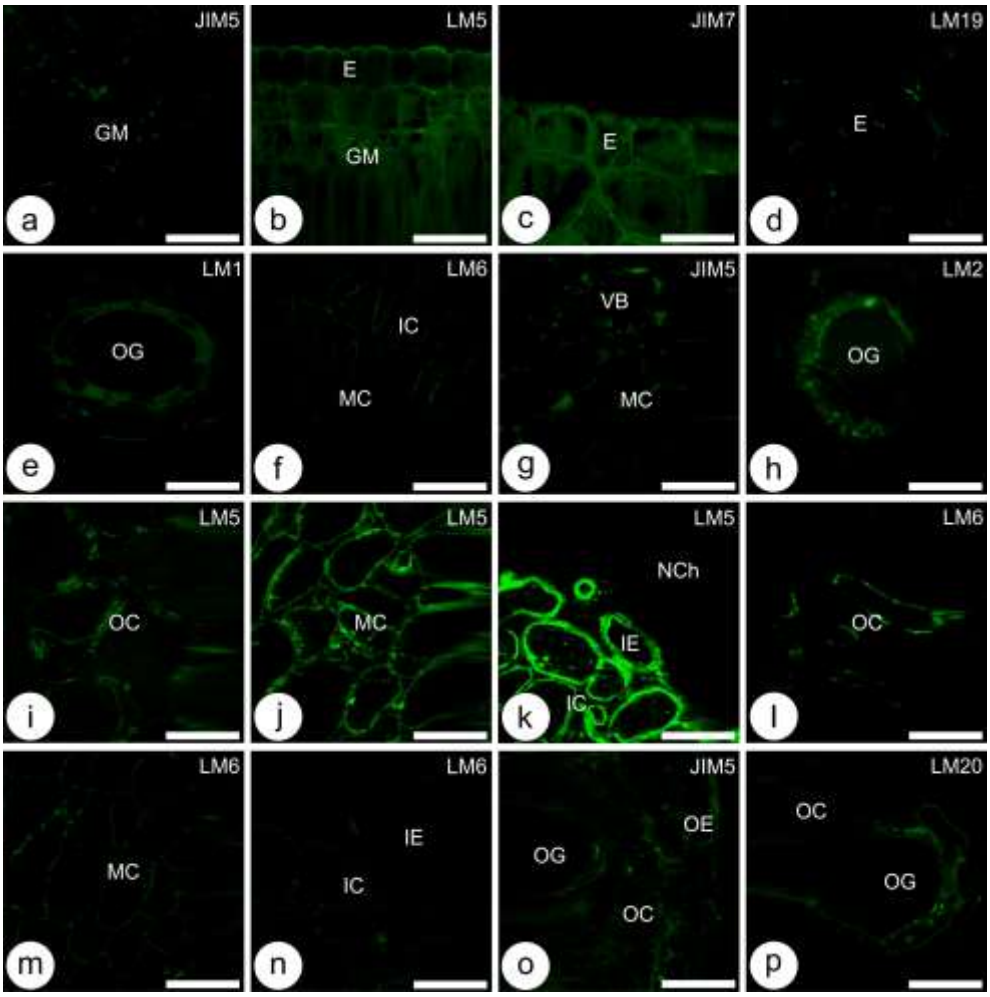

Fig. 6. **Immunocytochemistry of plant cell wall pectins and proteins during the development of non-galled leaves of *Psidium cattleianum* and galls induced by *Nothotrioza cattleiani*.** (**a, b**) Young leaves. (**c, d**) Mature leaves. (**e-p**) Galls. (**e, f**) Induction. (**g, h**) Growth and development. (**i-n**) Maturation. (**o, p**) Senescence. (**a, c, f, h-n, p**) **C**ontinuous signal of the labelling of ubiquitous epitopes. (**b, d, e, g, o**) Discontinuous signal of the labelling of microdomains of epitopes. (**a, g, o**) Low methylesterified homogalacturonans (HGAs). (**b, i-k**) Galactans. (**c**) Medium-high HGAs. (**d**) Unesterified HGAs. (**e**) Extensins. (**f, l-n**) Arabinans. (**h**) Arabinogalactan proteins. (**p**) High methylesterified HGAs. E: epidermis; GM: ground meristem; IC: inner cortex; IE: inner epidermis; MC: median cortex; NCh: nymphal chamber; OC: outer cortex; OE: Outer epidermis; OG: oil gland; VB: vascular bundle. Bars: 50 µm.

In mature leaves, the epidermis in the adaxial surface have epitopes of unesterified and medium-high methylesterified HGAs bound by LM19 and JIM7, and epitopes of AGPs and

galactans bound by LM2 and LM5, respectively (Fig. 5b). In the abaxial surface, epitopes of AGPs, galactans and unesterified HGAs are bound by LM2, LM5 and LM19, respectively. In the cells of the oil glands, only the epitopes of AGPs are bound by LM2. Cells of the chlorophyllous parenchyma have epitopes of low methylesterified HGAs, galactans and arabinans respectively bound by JIM5, LM5 and LM6. Epitopes of unesterified and medium-high methylesterified HGAs bound by LM19 and JIM7 are strictly labeled in the palisade parenchyma. In the vascular tissues, phloem and parenchyma cells have epitopes of galactans and arabinans bound by LM5 and LM6, and unesterified HGAs bound by LM19 (Fig. 5b). Cells of mature leaves have ubiquitous epitopes of low and high methylesterified HGAs (Fig. 6c), galactans, and arabinans in the parenchyma and vascular tissues, which generated continuous signals. On the other hand, epitopes of AGPs, unesterified HGAs (Fig. 6d), and galactans in the epidermis are more scarce and generated discontinuous signals.

Galls

In the galls at the stage of induction, epidermal cells of the inner and outer surfaces have epitopes of unesterified and medium-high methylesterified HGAs bound by LM19 and JIM7, extensins bound by LM1, and AGPs and galactans bound respectively by LM2 and LM5 (Fig. 5c). Cells of the oil glands have the same epitopes as the epidermis, except for the absence of galactans. Epitopes of medium-high methylesterified HGAs bound by JIM7, galactans and arabinans bound respectively by LM5 and LM6, and high methylesterified HGAs bound by LM20 are detected in all gall cortical layers. Low methylesterified HGAs bound by JIM5 and extensins bound by LM1 are detected specifically in the outer cortical cell layers. Cells of the vascular bundles have epitopes of medium-high methylesterified HGAs bound by JIM7, extensins bound by LM1, and galactans bound by LM5 (Fig. 5c). Cells in the galls at the stage of induction have epitopes of low methylesterified HGAs, extensins (Fig. 6e), and AGPs distributed in microdomains, which generated discontinuous signals. Epitopes of high methylesterified HGAs, extensins, galactans and arabinans, and AGPs in the oil glands (Fig. 6f) are more abundant and generate continuous signals.

During the stage of growth and development, the inner epidermal cells have epitopes of medium-high methylesterified HGAs bound by JIM7, and galactans bound by LM5 (Fig. 5d). The outer epidermal cells have epitopes of low and medium-high methylesterified HGAs bound respectively by JIM5 and JIM7. In the cells of the oil glands, epitopes of AGPs and arabinans are bound by LM2 and LM6, respectively. All cortical cells have epitopes of low and medium-high methylesterified HGAs bound respectively by JIM5 and JIM7, and galactans bound by LM5. The epitopes bound in the cells of vascular tissues and cortices are similar, except for the absence of the epitopes of high methylesterified HGAs in the latter (Fig. 5d). At this developmental stage, epitopes of low methylesterified HGAs are distributed in microdomains, which generated discontinuous signals (Fig. 6g). The epitopes of medium-high methylesterified HGAs, AGPs (Fig. 6h), galactans and arabinans are more abundant and generated continuous signals.

The gall inner and outer epidermal cells at the phase of maturation have epitopes of unesterified, low and high methylesterified HGAs bound by LM19, JIM5 and LM20, medium-high methylesterified HGAs bound by JIM7, and galactans bound by LM5 (Fig. 5e). Cells of the oil glands have epitopes of medium-high methylesterified HGAs bound by JIM7, and epitopes of extensins, AGPs and arabinans are respectively bound by LM1, LM2 and LM6. Cells from all

cortical layers have epitopes of unesterified, low, medium-high and high methylesterified HGAs bound by LM19, JIM5, JIM7 and LM20, and galactans and arabinans bound respectively by LM5 and LM6. Epitopes of extensins are labeled only in the cells from the outer cortical layers. In the cells of the vascular tissues, epitopes of medium-high methylesterified HGAs bound by JIM7, and epitopes of extensins, galactans and arabinans are respectively bound by LM1, LM5 and LM6 (Fig. 5e). At the phase of maturation, the observed epitopes of unesterified HGAs and extensins were distributed in microdomains, which generated discontinuous signals. The epitopes of low and medium-high methylesterified HGAs, AGPs, galactans, and arabinans are more ubiquitous, and generated continuous signals. The galactans and arabinans are distributed in centripetal (Fig. 6i-k) and centrifugal (Fig. 6l-n) gradients in the cortical cells, respectively.

During the stage of senescence, inner epidermal cells have epitopes of low methylesterified HGAs bound by JIM5, and galactans bound by LM5 (Fig. 5f). The outer epidermal cells have epitopes of unesterified, low, medium-high and high methylesterified HGAs bound by LM19, JIM5, JIM7 and LM20, and galactans bound by LM5. Epitopes of low methylesterified HGAs bound by JIM5 and galactans bound by LM5 are detected in all layers of the gall cortex. Epitopes of unesterified and medium-high methylesterified HGAs bound by LM19 and JIM7 are detected strictly in the outermost layers of the gall cortex. Epitopes of arabinans are detected by LM6 in the cells of the median and outer layers of the gall cortex. In the vascular tissues, phloem and parenchyma cells have epitopes of low methylesterified HGAs bound by JIM5, and galactans and arabinans bound respectively by LM5 and LM6 (Fig. 5f). At the final stage of gall development, the epitopes of low and medium-high methylesterified HGAs are distributed in microdomains, which generate discontinuous signals (Fig. 6o). Extensins, AGPs, galactans, arabinans, and unesterified HGAs (Fig. 6p) are more abundant, which generate continuous signals.

Discussion

Ontogenetic changes in leaves lead to new cell fates in galls

The development of the simple leaves of *Psidium cattleianum* follows a common pattern described by Hara [40] for dicotyledons, in which the ground meristem precociously exhibit differentiated tissue layers, and the procambium differentiates from the median layers. This pattern has been reported for Neotropical host plants, which interact with different gall inducers [2, 10, 41, 42]. Even though such similarity is conserved across the non-galled leaves, different gall morphotypes develop, namely, the pocket gall induced by *Aceria lantanae* on *Lantana camara* [41], the bivalve-shaped gall induced by *Euphalerus ostreoides* on *Lonchocarpus muehlbergianus* [10], and the globoid galls induced by *Calophya duvauae* on *Schinus polygamus* [42] and by *Nothotrioza myrtoidis* on *Psidium myrtoides* [2].

The globoid morphotype is the most common gall shape found in nature [3], and anatomical studies on the development of such structures can elucidate the processes that lead to their morphogenesis. In fact, it has been widely reported that different taxa of insects induce morphologically distinct galls on the same host plants [10, 19, 41, 43, 44, 45, 46, 47, 48], which reinforce the hypothesis that galls are the extended phenotypes of the insects [20]. From this

perspective, the association of *Nothotrioza* spp. with *Psidium* spp. recently described in the Neotropics [19] represents a good model to compare if the final shapes of the galls are determined by convergent plant-dependent or divergent insect-induced characteristics. The galls induced by *N. myrtoidis* on *P. myrtoides* previously studied by Carneiro et al. [2] are globoid and extremely similar to the ones induced by *N. cattleiani* on *P. cattleianum* studied herein. Galls of *P. cattleianum* are induced on young leaves [18], which have reactive tissues that respond to gall induction by cell hypertrophy and determined sites of hyperplasia, leading to the formation of emergencies growing over the insect body. A similar mechanism was described for the induction of the leaf galls on *P. myrtoides* [2], *Piptadenia gonoacantha* [49], and *Schinus polygamus* [42], which indicates a conservative trait of galls induced on leaf surface. Cell hypertrophy and tissue hyperplasia in the median and outer cortical layers were also observed during the stage of gall growth and development. Together, such characteristics have constituted the most widespread features of insect-induced galls [50]. The stage of gall growth and development is characterized by the great increase in biomass [51], which in the case of the galls of *N. cattleiani* on *P. cattleianum* occurs via pronounced hyperplasia of the median and outer cortical cell layers, as observed for the galls of *N. myrtoidis* on *P. myrtoides* [2].

By the end of the stage of growth and development and at the beginning of maturation, the galls on *P. cattleianum* assume a structure commonly observed in many galls, i. e., neo-formed vascular bundles interspaced in parenchyma cells, and sclerenchyma in the outer cortical cell layers. This pattern of gall organization has been reported in the Neotropical region for the midrib galls on *Copaifera langsdorffii* [9], the bivalve-shaped gall on *Lanchocarpus muehlbergianus* [10], the intralaminar globoid galls on *Aspidosperma spruceanum* [52], and the extralaminar globoid galls on *Psidium myrtoides* [2]. Nevertheless, the discontinuous groups of sclereids found in the outer cortical layers of mature galls on *P. cattleianum* differ from the arrangement of a sclerenchymatic ring described by Rohfritsch [51] as pattern. The role of lignified tissues in galls is widely regarded as a defensive strategy for the gall inducers [53], which increase the adaptive value of the gall [5]. Recent studies have related developmental processes, such as lignification, to the balance of reactive oxygen species (ROS) in gall tissues [9, 10, 13, 14, 54, 55, 56]. Such subcellular signalers are known to take part in plant responses to the attack of parasites and/or herbivores [57], and the oxidative stress triggered by their accumulation may affect the deposition of lignins at specific sites, as proposed by Carneiro et al. [2, 55]. The lignification of gall cortical cells by the end of the maturation stage implies the occurrence of an oxidative burst [56], which besides affecting gall structure, may constitute a signaler for the beginning of gall senescence.

At senescence, *N. cattleiani* galls exhibit spontaneous dehiscence for the scape of adults, with great similarities to the mechanism described for *N. myrtoidis* galls [2]. As far as we are concerned, these are the only anatomical descriptions of spontaneous mechanisms of dehiscence for insect galls. Generally, gall-inducing insects have different strategies for leaving the galls, which include actively digging a scape tunnel by the feeding larva [49, 51], when the gall tissues are completely fused. In other cases, the galls are permanently open and the inducers simply leave their galls through the pre-existing aperture, as it is observed in the leaf galls of *Pseudotectococcus rollinae* on *Rollinia laurifolia* [58], and *Callophya duvauae* on *Schinus polygamus* [42]. In the galls induced by *N. cattleiani* on *P. cattleianum*, the massive hypertrophy of the inner cortical cells during the transition

from gall maturation towards senescence exerts tension on peripheral necrotic cells, which end up rupturing, setting the adult insects free. Even though the expressed phenotype is the same, i. e., the gall wall rupturing, the underlying anatomical dynamics involve peculiar and distinct cell responses in *P. cattleianum* galls when compared to those of *P. myrtoides* [2]. The growth of targeted cell lineages at very specific moments is most likely to be driven by hormones, like indol-acetic acid (IAA). Such hormone was histochemically localized in the cortical cells of the galls on *Piptadenia gonoacantha*, where phenolic substances were also accumulated [59]. This co-localization suggest that phenolics may influence cell growth due to the inhibition of the IAA oxidases [60], thus increasing the bioavailability of active IAA in the cells. The observation of phenolic inclusions in specific cell types which grow differently, both in the galls of *P. myrtoides* [2, 55] and *P. cattleianum* corroborate this hypothesis. From the anatomical point of view, the double co-generic systems *Psidium – Nothotrioza* have conservative morphogenetical traits, but specific cell dynamics for the neo-ontogenesis [2] of flat leaves towards globoid galls.

Dynamics of cell elongation for the neo-ontogenesis of the globoid shape

The dynamics of tissue hyperplasia and cell hypertrophy, as well as the occurrence of isotropic and/or anisotropic types of cell expansion [7] depend on the structure and functionality of cell walls [17]. Neotropical galls have been increasingly analyzed by the quantitative perspective of cell hypertrophy, with special attention to the directions of cell elongation [2, 9, 10, 11, 12]. The tendency of shifting from isotropic to anisotropic cell elongation on *P. cattleianum* galls is similar to that of *Baccharis dracunculifolia* [12]. Some other galls have opposite dynamics, with cells changing from anisotropic to isotropic type of cell growth along the development, as reported for *C. langsdorffii* [9], *L. muehlbergianus* [10], and *P. myrtoides* galls [2]. These results suggest that the types of cell elongation in different galls are not strictly dependent on their final shapes.

The cell transformations described for the cortical parenchyma of *N. myrtoidis* galls ultimately generate the globoid gall morphotype by the establishment of a centrifugal gradient of cell hypertrophy [2]. Despite the macro-morphological similarity with *N. myrtoidis* galls, the galls induced by *N. cattleiani* on *P. cattleianum* do not have such gradient, as the median layers of the cortex are the most hypertrophic, followed by the inner and outer cortical layers. Similar patterns of cell hypertrophy were reported for the midrib galls on *C. langsdorffii* [9], and in the fusiform stem galls on *Marcetia taxifolia* [11]. The hypothesis of the similarity in the anatomical development of *Nothotrioza* spp. galls in *Psidium* spp. leaves is partially corroborated, as both galls exhibit time-based occurrence of tissue hyperplasia and cell hypertrophy along gall developmental stages. Nevertheless, both the sites of cell hypertrophy and the dynamics of cell elongation in the different tissue layers constitute divergent patterns between such co-generic systems.

Immunocytochemical identity of cell lineages and cell wall functionalities

The dynamic properties of cell walls and tissue lineages regulate developmental processes in plants [17]. In the case of the galls induced by *Nothotrioza* spp. on *Psidium* spp., which are both

globoid but somewhat anatomically different, the pectin and protein composition of the cell walls vary according to the cell lineages. Epidermal cells of *P. cattleianum* leaves and *N. cattleiani* galls remain uniseriate during their ontogenesis, and exhibit conservative cell wall composition. The association of homogalacturonans (HGAs) and galactans seems to be especially important for the identity of epidermal cells in this gall, since their epitopes are constant throughout leaf and gall development. The detection of galactans, alone or associated to HGAs, is an indicative of cell wall rigidity [61, 62], and has been associated to sites of hyperplasia [63]. In fact, galactans and HGAs detection in epidermal cell walls of leaves and galls confer them stability and rigidity. These two properties are reinforced by the detection of extensins [64, 65] in the epidermal cells of young non-galled leaves and galls of *P. cattleianum* at the induction stage. The reinforcement of young epidermal cells by the extensins reflects the expected pattern of epidermal cells differentiation, which divide anticlinally to accompany the growth of either leaves or galls, without hypertrophying or losing the uniseriate organization. Current results corroborate the functional roles of HGAs and galactans in fast-dividing cells as observed by Xu et al. [63] in *Musa* tissues.

Oil glands, which are specialized epidermal cells, have different chemical composition in their cell walls along the development of leaves and galls. In addition to HGAs, arabinans, galactans, and extensins, oil glands have arabinogalactan proteins (AGPs), which are believed to play a role in the prevention of programmed cell death [66, 67]. Also, they modulate cell division and expansion in all developmental stages, as previously reported by Carneiro et al. [2] in the galls induced by *N. myrtoidis* on *P. myrtoides*. The AGPs seem to be markers of the secretory cells identity, as they were also detected in the mucilage cells of *Araucaria angustifolia* [68], in the secretory ducts of galls on *B. reticularia* [13]. The modulation of oil gland cell properties relies on the balance among flexibility, rigidity and porosity. The flexibility is conferred by arabinans to expanding cells [63], while the rigidity is the result of the stabilization by the cross bonds established by extensins among cell wall polymers [33, 64, 65]. Porosity is conferred by the association of arabinans and HGAs due to the depletion of calcium-mediated cross links [38]. The chemical composition in the walls of ordinary epidermal cells and oil gland cells are maintained for the effective mediation of the gall – environment interface, as far as protection of gall tissues and gall inducer is concerned. Both the chemical composition of the cell walls and the localization of the oil glands reveal a similar defensive phenotype of galls [2] in the double co-generic systems *Psidium – Nothotrioza*.

The chemical identity of almost all cortical parenchyma cell walls retains some similarity to that of epidermal cells in terms of the presence of HGAs and galactans. Nevertheless, the association of these epitopes with arabinans enhances the flexibility and porosity of their cell walls [38] due to the alignment of HGAs, as previously proposed by Foster et al. [69]. These functional aspects of parenchyma cell walls guarantee the establishment of metabolic gradients in the cortex, as histochemically detected for other galls [9, 54, 55]. Porosity seems to be a key property of the host plant cell walls, which is conservative for galls. In current model, it is especially useful for parenchyma cells, which demand higher cell to cell flux of molecules. This property was reported by Oliveira et al. [56] in *Baccharis dracunculifolia* galls and by Carneiro et al. [2] in *Psidium myrtoides* galls, where the porosity is related to the reallocation of molecules drained from the senescent galls towards the leaves. Besides porosity, the co-occurrence of a centripetal gradient of galactans and a centrifugal gradient of arabinans in mature galls should maintain the inner cortical cells more rigid

[61, 62] and the outer cortical cells more flexible [63]. Nevertheless, the enhanced rigidity of the outer cortex is guaranteed by lignin deposition in the thick secondary cell walls, instead of by the presence of galactans. In fact, histometry (*cf.* Table 1) validates that the flexible arabinan-rich cells of the outer cortex, prior to lignifying, hypertrophy more than the rigid galactan-rich cells of the inner cortex, corroborating the functionality of such epitopes.

Changes on the cellular identities of epidermal and parenchyma lineages are ultimately related to variations on the degree of methylesterification of the HGAs, the major pectins of the cell walls. Demethylesterified HGAs cross link to calcium [70], a well-known cellular signaler that integrates the symplast and apoplast for many cell responses [71], and rigidify cell walls. Current analyses reveal that cycles of high methylesterified HGAs production are interposed by cycles of demethylesterification by Pectin methylesterases (PMEs) to create a balance between such forms. Up to gall maturation, the degree of HGAs methylesterification does not corroborate the gradient of rigidity assumed for the gall cortical layers. At gall senescence, however there is a tendency of increased rigidity due to lower degree of pectin methylesterification, more pronouncedly in the outer cortex. In fact, the action of PMEs during the morphogenesis of galls has been assumed to occur at the late stages of gall development, when the tissues are more rigid and the degree of cell hypertrophy is the highest [2, 14]. In *N. cattleiani* galls, cell hypertrophy continues to occur until senescence in the median and inner cortices. This is in accordance with the balance on the degree of pectin methylesterification, and plays crucial role in the cellular mechanism of gall dehiscence.

Similar fluctuations on the epitopes related to cell wall rigidity were observed in the vascular system, and in its less plastic tissues. Structural integrity was maintained in the vascular bundles for the effective nutrition of the gall inducer, which is a phloem feeder [72]. Furthermore, the vascular tissues of senescent galls have cell wall epitopes similar to that of mature leaves, which is the standard morphogenetical pattern. For all other cell lineages, the comparison of cell wall epitopes in mature leaves and mature galls corroborate that galls represent the neo-ontogenesis of leaf tissues [2], i. e., divergent cell fates. The neo-ontogenesis is most evident in the cells of the ground system, which undergo hypertrophy and hyperplasia, the core processes by which galls are anatomically defined. The fine regulation of such processes, either related to convergent or divergent final forms of the galls, are peculiarities of each system. Herein, the immunocytochemical analyses in the galls of the double co-generic systems *Psidium – Nothotrioza* reveal completely different set of cell wall epitopes. These differences reveal fine regulation of cell processes towards unique functional-structural aspects required for gall development. Current comparative approach corroborates the hypothesis that the extended phenotype concept [20] can be validated in the cellular and subcellular levels, which are true insect-induced unique characteristics.

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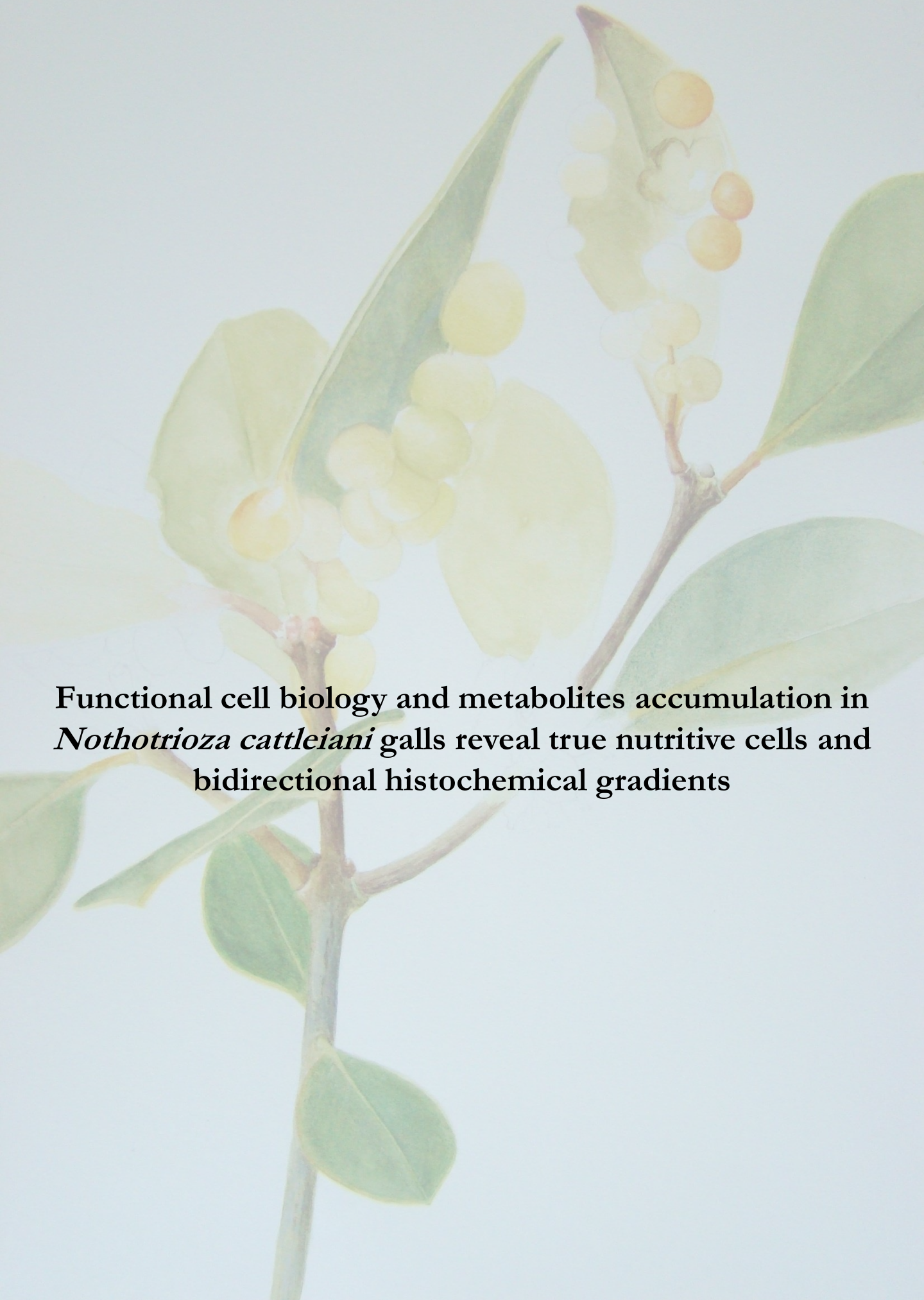

Functional cell biology and metabolites accumulation in *Nothotrioza cattleiani* galls reveal true nutritive cells and bidirectional histochemical gradients

RESEARCH ARTICLE

Functional cell biology and metabolites accumulation in *Nothotrioza cattleiani* galls reveal true nutritive cells and bidirectional histochemical gradients

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ABSTRACT

Plant cells respond to abiotic and biotic stimuli, which generate adaptive phenotypes in plant organs. In the case of plant galls, cell phenotypes are adaptive for the gall inducer, and assume characteristics mainly linked to its protection and nutrition. Herein, the cytological development and histochemistry of the sucking-insect gall of *Nothotrioza cattleiani* on the leaves of *Psidium cattleianum* is compared to those of other galls, especially *N. myrtoidis galls*, searching for conservative and divergent alterations in cell fates and cycles. Leaf cell fates are completely changed towards galls, except for epidermal cells, but the comparison between *Nothotrioza* spp. galls show conservative fates. Nevertheless, time-based cytological development of *N. cattleiani* galls is different from the standy-redifferentiation of *N. myrtoidis* galls. Bidirectional gradients of metabolites accumulation and true nutritive cells associated to vascular tissues are herein reported for the first time in insect galls. The globoid galls of *N. cattleiani*, though macro-morphologically similar to the galls of *N. myrtoidis*, are unique extended phenotypes, as far as the cellular, subcellular and histochemical traits are concerned.

KEYWORDS: carbohydrate-related enzyme activity; cytology; development; metabolic gradients; primary and secondary metabolites; *Psidium cattleianum*.

INTRODUCTION

The capability of insects to induce galls depends on properties intrinsic to plant cells, which are ultimately defined by the biotic and abiotic stimuli from the surrounding environment, and leads to different cell phenotypes (Gianoli and Valladares 2012). Plant cells under the influence of gall-inducing insects redifferentiate (*sensu* Lev-Yadun 2003), and assume new cycles and fates for the neo-ontogenesis of plant galls (*sensu* Carneiro et al. 2014*a*). Even though specific galling insect taxa are classically known to induce conservative, morphologically different galls (Mani 1964; Rohfritsch 1992), recent studies documented extremely diverse aspects of cell biology in Neotropical galls (Moura et al. 2009; Oliveira and Isaias 2009, 2010*a*; Formiga et al. 2011; Isaias et al. 2011; Castro et al. 2012*a*; Bedetti et al. 2013; Dias et al. 2013; Ferreira and Isaias 2013; Guimarães et al. 2013, 2014).

The metabolism of Neotropical galls has been assessed using cytological and histochemical methods (Oliveira and Isaias 2010*b*; Oliveira et al. 2010, 2011*a*, *b*; Vecchi et al. 2013) searching for gradients already described for nutritive tissues in Cynipid and Cecidomyiidae galls (Bronner 1992). In the Neotropical galls with true nutritive tissues, some of the metabolic gradients proposed by Bronner (1992) are corroborated. Unexpectedly, cytological and histochemical gradients are also reported for the galls induced by the sucking insect, Pseudophacopteron sp. (Psyllidae), on *Aspidosperma australe* (Oliveira and Isaias 2010*b*), which have nutritive-like parenchyma cells. Despite being regarded as structurally simple and devoid of nutritive cells (Meyer 1987), galls of sucking-insects, namely *Euphalerus ostreoides* (Isaias et al. 2011) and *Nothotrioza myrtoidis* (Carneiro and Isaias 2014, Carneiro et al. 2014*b*), also have cytological and histochemical characteristics of metabolically active cells. These cells are evidenced in the vascular tissues and parenchyma near the nymphal chamber. *N. myrtoidis* galls have gradients of primary and secondary metabolites, discrete accumulation of reactive oxygen species, and are photosynthesis-deficient (Carneiro et al. 2014*b*). Moreover, the completion of cell cycles through standby redifferentiation is ensured by the chemical (Carneiro et al. 2014*b*), and cytological ROS-scavenging apparatus (Carneiro and Isaias 2014).

Herein, we assess the cytology and histochemistry of the galls induced by *Nothotrioza cattleianum* Burckhardt (Triozidae) (Carneiro et al. 2013) on *Psidium cattleiani* Sabine (Myrtaceae). The structural-functional aspects concerned to cell biology in *P. cattleianum* galls are matched against those of other galls, especially the co-generic

system, *N. myrtoidis* – *P. myrtoides*. The galls of *Nothotrioza* spp. on *Psidium* spp. are both globoid (sensu Isaias et al. 2013), and have similar phenology (Butignol and Pedrosa-Macedo 2003, Carneiro et al. 2013). We expect that the developmental cytology and histochemical profile of *P. cattleianum* galls should reveal conservative traits concerned to the sucking feeding habit of *N. cattleiani*, but also distinctive ones of a unique extended phenotype (*sensu* Bailey et al. 2009). The following questions are addressed: (1) Do the cells of *N. cattleiani* galls have conservative cell fates in relation to the non-galled leaves, and in comparison to *N. myrtoidis* galls? (2) Do the cytological and histochemical profiles of *Nothotrioza* spp. galls indicate unique neo-established tissue functionalities?

METHODS

Sampling

The population of *P. cattleianum* Sabine (Myrtaceae) with galls induced by *N. cattleiani* Burckhardt is located at the Parque Estadual Pico do Marumbi, municipality of Piraquara, Paraná state, Brazil. Non-galled leaves (young and mature, n = 5 per developmental stage), and galls at the phases of induction, growth and development, maturation and senescence (n = 5 per developmental stage) were collected during the years of 2012 and 2013. The samples were fixed in 2.5% glutaraldehyde (grade I) and 4.5% formaldehyde in phosphate buffer (0.1 M; pH 7.2) (Karnovsky 1965).

Light microscopy

Fixed samples were dehydrated in ethanol series (Johansen 1940), embedded in glycolmethacrylate (Leica®), sectioned (6-10 μm) with a rotary microtome Hyrax (Zeiss®), stained with 0.05% Toluidine O blue (pH 4.6) (O'Brien et al. 1964). Histological slides were observed and photographed under light microscope (Leica® DM500) coupled with digital camera (Leica® ICC50 HD).

Histochemical analyses

Histochemical tests for primary and secondary plant metabolites, reactive oxygen species (ROS), and the activity of enzymes related to carbohydrate metabolism were performed using fresh samples of mature leaves and galls. Control tests were performed according to the references, and also by comparison with blank sections. Samples were submitted to the tests/reagents, following the methods described subsequently.

Fehling's reagent (Reducing sugars): Equal parts of "A" (II copper sulphate 6.93% w:v) and "B" (sodium potassium tartrate 34.6% and 12% sodium hydroxide m:m:v) solutions and heat to pre-boiling temperature (Sass 1951).

Lugol's reagent (Starch): 1% Potassium iodine-iodide solution for 5 min (Johansen 1940).

Sudan Red B (Total lipids): Saturated solution of Sudan red B in 70° GL ethanol for 5 min (Brundett et al. 1991).

Coomassie blue (Total proteins): 0.25% Coomassie blue solution for 5 min (Dunn 1993).

Ferric chloride (Phenolics): 1% Ferric chloride solution for 5 min (Johansen 1940).

DMACA (Proanthocyanidins): Fixation in 0.5% Caffeine sodium benzoate in 90% buthanol for 1-2 h. Reaction in 1% *p*- dimethylaminocinnamaldehyde (Feucht et al. 1986).

NADI (Terpenoids): 1% α-naphtol, 1% dimethyl-*p*-phenylenediamine in 0.01 M phosphate buffer (pH 7.2) for up to 30 min (David and Carde 1964).

Wiesner reagent (Lignins): 2% phloroglucinol in acidified solution for 5 min (Johansen 1940).

Acid Phosphatase activity: Incubation in 0.012% lead nitrate and 0.1 M potassium sodium glycerophosphate in 0.5 M acetate buffer (pH 4.5) for 24 h, at room temperature. Reaction in 1% ammonium sulphate for 5 min, after washing in distilled water (Gomori 1956).

Glucose-6-phosphatase activity: Incubation in 20 mg of potassium glucose-6-phosphate in 125 ml of 0.2 M Tris–maleate buffer (pH 6.7), 3 ml of 2% lead nitrate in 7 ml of distilled water for 15 min to 2 h, at 37°C. Reaction in 1% ammonium sulphate for 5 min, after washing in distilled water (Jensen 1962).

Phosphorylase activity: Incubation in 1% glucose-1-phosphate in 0.1M acetate buffer (pH 6.0) for 2 h at room temperature. Reaction in Lugol's reagent for 5 min (Jensen 1962).

Sucrose synthase activity: Fixation in 2% paraformaldehyde with 2% polyvinylpyrrolidone and 0.005 M dithiothreitol for 30 min. Incubation in 5 ml of 150 mM NADH, 5 ml (1 U) of phosphoglucomutase, 5 ml of 3 mM glucose-1,6-biphosphate, 5 ml (1 U) of glucose-6-phosphate dehydrogenase, 5 ml (1 U) of UDPG–pyrophosphorylase, 280 ml of 0.07% aqueous nitro-blue tetrazolium (NBT), 350 ml of buffer, and 50 ml of substrate. Buffer is composed by 100 mM HEPES, 10 mM MgCl2, 2 mM EDTA, 0.2% BSA, 2 mM EGTA at pH 7.4. Substrate is composed by 0.75 M sucrose, 15 mM UDP, and 15 mM pyrophosphate (Wittich and Vreugdenhil 1998).

Invertases activity: Incubation in 0.38 mM sodium phosphate (pH 7.5), 0.024% NBT, 0.014% phenazin metasulphate, 30 U of glucose oxidase, 30 mM of sucrose at room temperature for 3 h (Zrenner et al. 1995, Doehlert and Felker 1987).

Treated sections were mounted on glass slides with Kaiser's glycerol gelatin (Kraus and Arduin 1997), observed and photographed under light microscope (Leica® DM500) coupled with digital camera (Leica® ICC50 HD).

Transmission electron microscopy

The fixed samples were post-fixed in 1% osmium tetroxide in phosphate buffer (0.1 M; pH 7.2), dehydrated in ethanol series (O'Brien & McCully 1981), and embedded in Spurr®. Ultrathin sections were obtained with a diamond knife in the ultramicrotome Reichert-Jung – Ultracut (Leica, Wetzlar, Germany), attached to copper nets, contrasted with uranyl acetate and lead citrate (Reynolds 1963). The sections were analyzed under the transmission electron microscope Tecnai™ G2-12 - SpiritBiotwin (FEI, Hillsboro, USA) at 120 kV, at the Centro de Microscopia of the Universidade Federal de Minas Gerais (CM – UFMG).

RESULTS

Morphology and development of leaves and galls

The galls of *Nothotrioza cattleiani* are globoid and protruded to the abaxial surface of the leaves of *Psidium cattleianum* (Fig. 1A). The young leaves of *P. cattleianum* have uniseriate epidermis, homogenous chlorophyllous parenchyma with interspaced vascular bundles undergoing differentiation. As they mature, epidermis remains uniseriate; hypodermis differentiates under the adaxial surface of the epidermis, and dorsiventral chlorophyllous parenchyma, interspaced with collateral vascular bundles, is observed (Fig. 1B). The galls of *N. cattleiani* are induced on the young leaves, whose cells redifferentiate to form a depression on the leaf lamina, and ultimately generate a globoid gall with an ample chamber and relatively thin wall. In mature galls, such walls have uniseriate inner and outer epidermis, hyperplasic and hypertrophied homogenous parenchyma, and collateral vascular bundles near the nymphal chamber (Fig. 1C). The galls have one-year life cycle, with four developmental stages: induction, growth and development, maturation and senescence.

Cytological development of cell lineages

Epidermis: In the young leaves of *Psidium cattleianum*, epidermal cells have thin anticlinal walls, fragmented hyaline vacuoles, few chloroplasts and mitochondria, and large central nuclei with conspicuous nucleoli (Fig. 2A). In the mature leaves, the walls

are thicker, and the vacuoles have confluenced into a big central vacuole with phenolics; chloroplasts and mitochondria are rare, and the nuclei are small with peripheral heterochromatin (Fig. 2B, C). Galls are induced on young leaves, and exhibit epidermal cells with homogenously thin walls, with large hyaline vacuoles. Nuclei are peripheral and the electron-dense cytoplasm is reduced to a fine region near the cell walls (Fig. 2D). Epidermal cells in the galls at the stage of growth and development have heterogeneous thick cuticle, with protoplast similar to that of previous stage. At the phase of maturation, the inner epidermis is intermittent; cells exhibit heterogeneously thickened and polylamellate walls, and thin cuticle (Fig. 2E). The protoplast is poorly altered in comparison to the cells of the previous stage. At senescence, the cells have vacuoles with phenolic inclusions and walls with disaggregated lamellae, and end up undergoing autolysis.

Chlorophyllous parenchyma: In the young leaves, mesophyll cells have thin, homogenous primary cell walls, and reduced intercellular spaces. Their vacuoles are large, sometimes fragmented around the nucleus, with electron-dense granular inclusions. Nuclei are large, with electron-dense euchromatin and conspicuous nucleoli (Fig. 3A). Chloroplasts are elliptical, with organized grana, small starch grains, and scarce electron-dense plastoglobules. Small round mitochondria are in association with chloroplasts, and rough endoplasmic reticulum (RER) is sometimes observed on the periphery of the cytoplasm (Fig. 3B). In mature leaves, the palisade parenchyma cells have large phenolic-rich vacuoles, many chloroplasts with well-organized thylakoid lamellae, and starch grains; nuclei are peripheral, with inconspicuous nucleoli (Fig. 3C). Chloroplasts with large and numerous plastoglobules are frequent (Fig. 3D). Spongy parenchyma cells have large hyaline vacuoles, peripheral nuclei with inconspicuous nucleoli, and chloroplasts with well-organized thylakoid lamellae, starch grains and rare plastoglobules (Fig. 3E, F).

Galls at induction stage have hypertrophic and hyperplasic cells, with thin primary walls, sometimes fragmented vacuoles, with phenolic inclusions. They have large nuclei with conspicuous nucleoli, small mitochondria associated to chloroplasts with well-organized thylakoid lamellae and few small plastoglobules (Fig. 4A). Polysomes, Golgi apparatus and multivesicular bodies are sometimes visualized in the periphery of the cells (Fig. 4B, C). At the phase of growth and development, the cells have secondary polylamellated walls, phenolic-rich vacuoles, and electron-lucent cytoplasm poor in organelles. Nuclei are small, with little heterochromatin at the periphery; mitochondria are small, associated to chloroplasts with well-organized thylakoid lamellae, starch grains

and few small plastoglobules (Fig. 4D, E). Cells of mature galls are large, with big phenolic or hyaline vacuoles, reduced cytoplasm, sometimes with periplasmic spaces. Cell walls are secondary, either homogeneously thickened and polylamellated, or irregularly thickened, sometimes polylamellated, with sites of different electron-density (Fig. 4F, G). Organelles are scarce; lamellar bodies, degraded nuclei and small chloroplasts with underdeveloped thylakoid lamellae and plastoglobules are observed at the periphery of the cells (Fig. 4G, H). At gall senescence, the outer cortical cells exhibit thickened, polylamellated secondary walls, and remain live, with phenolic-rich vacuoles. Inner cortical cells have signs of degradation, with large periplasmic spaces, disrupted tonoplast, and chloroplasts with vestigial thylakoid grana and plastoglobules (Fig. 4I, J).

Vascular and perivascular parenchyma: In the young leaves, the cells have primary thin walls, sometimes sinuous, and with many plasmodesmata; nuclei are large, with inconspicuous nucleoli and dispersed heterochromatin (Fig. 5A). They have large, sometimes fragmented hyaline vacuoles, dense cytoplasm with round mitochondria, small underdeveloped chloroplasts, with few plastoglobules, little rough endoplasmic reticulum (RER), and lamellar bodies continuous to the plasma membrane (Fig. 5B, C). In mature leaves, cells have primary thin walls; cytoplasm is hyaline, with many mitochondria, scarce RER, and large nuclei with dispersed heterochromatin (Fig. 5D). Phenolic-rich vacuoles and periplasmic spaces are frequent. In the galls at induction phase, cells are thin walled, with electron-dense cytoplasm, large nuclei, abundant polysomes, sometimes evident Golgi apparatus, large mitochondria, abundant plasmodesmata with evident microtubules (Fig. 5E, F). Lamellar and multivesicular bodies are frequent (Fig. 5G). At the phase of growth and development, cells have irregularly thickened walls with sites of different electron-density, and fragmented hyaline vacuoles. Cytoplasm is electron-lucent, and organelle-rich, with abundant mitochondria and polysomes, Golgi apparatus, RER, large nuclei sometimes with conspicuous nucleoli and dispersed heterochromatin (Fig. 5H). Lomasomes and multivesicular bodies are frequent (Fig. 5I). Cells of mature galls have thickened and somewhat sinuous secondary walls, large vacuoles either hyaline or phenolic-rich, and abundant mitochondria. Cytoplasm is electron-dense, and periplasmic spaces may be observed (Fig. 5J). At gall senescence, cells are metabolically active, with organelle-rich cytoplasm. They have abundant mitochondria, polysomes and RER; nuclei are large, with peripheral heterochromatin. Lomasomes and lamellar bodies are rarely seen (Fig. 5K).

Histochemical profile of leaves and galls

Primary metabolites: In the leaves, reducing sugars and primary starch grains are respectively localized by Fehling's and Lugol's reagents throughout the chlorophyllous parenchyma, and more rarely in the hypodermis. Reducing sugars are also localized in the phloem of vascular bundles. Lipid droplets and protein precipitates respectively stained by Sudan red B and Coomassie blue are observed more intensely in the chlorophyllous parenchyma and vascular bundles, and more scarcely in the epidermal cells and oil glands. The cuticle on both epidermal surfaces and the secretion of oil glands are also stained by Sudan redB. In the galls, reducing sugars are strongly stained by Fehling's reagent, forming a centripetal gradient of sugar accumulation from the outermost cell layers towards the inner cortex (Fig. 6A, 8). Primary starch grains are rarely stained, but may form a discrete centrifugal gradient evidenced by the reaction to Lugol's reagent (Fig. 6B, 8). Protein precipitates are stained by Coomassie blue in the vascular bundles, perivascular parenchyma, and outermost cortical cell layers, forming a bidirectional gradient from the median cortex towards the inner and outer cortices (Fig. 6C, 8). The cuticle of the outer epidermal cells and the secretion of oil glands were stained by Sudan red B (Fig. 6D).

Enzyme activity: Leaves have detectable activity of acid phosphatase exclusively in the cells of the spongy parenchyma. Galls have detectable activity of glucose-6-phosphatase and invertases in the vascular bundles, and perivascular parenchyma of the median and inner cortices (Fig. 6E, F; 8).

Secondary metabolites: Phenolic substances and terpenoids are respectively evidenced by ferric chloride and NADI solutions intensely in the palisade parenchyma of the leaves, and more scarcely in the epidermis, hypodermis, phloem and spongy parenchyma. Terpenoids are also stained in the epithelium of the oil glands. Proanthocyanidins are detected by DMACA more intensely in the adaxial epidermis and palisade parenchyma, and more weakly in the spongy parenchyma. Lignins react with Wiesner's reagent in the cell walls of xylem and pericilic fibers. Galls exhibit bidirectional gradients of phenolic substances, terpenoids and proanthocyanidins from the median cortex towards the inner and outer cortices (Fig. 7A-D, 8). Lignins are detected in the secondary walls of the outer cortical cells and in the xylem, forming a centrifugal gradient (Fig. 7E, 8).

Reactive oxygen species (ROS): ROS are detected intensely by DAB solution in the chlorophyllous parenchyma of leaves, and also in the phloem and vascular parenchyma. In the galls, ROS are detected weakly in a bidirectional gradient from the median cortex towards the inner and outer cortices. The most intense reactions are observed in the

vascular bundles and perivascular parenchyma, and in the epithelium of the oil glands (Fig. 7F, G; 8).

DISCUSSION

Structural-functional implications of cell fates

Cell redifferentiation (*sensu* Lev-Yadun 2003) is a crucial process by which gall-inducing insects coordinate cell reprogramming in plant organs towards gall morphogenesis. In the galls of *Nothotrioza cattleiani*, such process takes place in the young leaves during the induction phase (*sensu* Carneiro et al. 2013), similarly to *N. myrtoidis* galls (Carneiro et al. 2014*a*, 2014*b*, Carneiro and Isaias 2014). The induction of galls may occur both on young and/or mature plant organs, as observed for the leaf galls induced by *Calophya duvauae* on *Schinus polygamus* (Dias et al. 2013). The selection of different induction sites affects the patterning of cell fates in gall structure, possibly influencing its adaptive value to the gall inducers (Oliveira and Isaias 2009). Nevertheless, the selective pressures that act over gall morphology seem to be conservative across different gall systems, as convergent types of tissue organization and cell fates, i. e., vascular bundles interspersed to homogenous parenchyma cells, surrounded by sclerenchyma cells, are widely reported in literature (Meyer 1987, Rohfritsch 1992, Stone and Schönrogge 2003, Oliveira and Isaias 2009, Formiga et al. 2011, Carneiro et al. 2014*a*, Carneiro and Isaias 2014).

Despite structural similarities, most cell lineages in *Nothotrioza cattleiani* galls on *Psidium cattleianum* have developmental dynamics different from those of *N. myrtoidis* galls on *P. myrtoides*. Epidermal cells are the exception to this tendency, as they have conservative cytological traits in both systems, namely vacuolated cells that are well organized in the outer surface of the galls, but intermittent in the inner surface (Carneiro and Isaias 2014). Chlorophyllous parenchyma cells in *Nothotrioza* spp. galls have different time-based development. *N. myrtoidis* galls have parenchyma cells developing by standby-differentiation (*sensu* Carneiro and Isaias 2014), in which cell structure remains nearly unaltered for a long period of time, and undergo major changes from the stage of maturation to senescence. Conversely, the cytology of *N. cattleiani* galls reveals that parenchyma cells gradually, and constantly change from induction phase towards senescence. In both *Nothotrioza* spp. galls, the cell walls thicken and lignify in the outermost layers of the cortex, while the photosynthetic and respiratory apparatus of the cells are impaired. Gall structures are assumed to play defensive roles against natural enemies of the galling insects (*sensu* Stone and Schönrogge 2003). The changes on cell

297 fates in the chlorophyllous parenchyma, which have thickened and lignified walls in the
298 galls on *Psidium* spp., suggest the investment in a structure-based defense strategy that
299 should increase the adaptive values of the galls to the *Nothotrioza* spp.
300 Contrary to the new cell fates of chlorophyllous parenchyma cells in *N. cattelaini*
301 galls, vascular and perivascular parenchyma are the tissues with less structural alterations.
302 The fates of these cells are not altered from non-galled leaves to galls, and maintain well-
303 developed metabolic apparatus, namely large nuclei, and dense cytoplasm with abundant
304 polysomes and mitochondria, throughout gall development both in *N. myrtoidis* (Carneiro
305 and Isaias 2014) and in *N. cattleiani* galls. The maintenance of structural-functional
306 characteristics of vascular tissues indicates that their primordial role in plants, i. e.,
307 conducting water and solutes (Buvat 1989), is maintained in galls. Such strategy is
308 adaptive (*sensu* Stone and Schönrogge 2003) for the gall inducers as far as their nutrition
309 is concerned, since *Nothotrioza* spp. are sap feeders (Burckhardt 2005, Carneiro et al.
310 2013), thus depending on the intake of water and solutes by vascular tissues. Altogether,
311 the cellular characteristics of epidermis, chlorophyllous parenchyma, and vascular and
312 perivascular parenchyma corroborate the adaptive nature of the gall structure (*sensu*
313 Stone and Schönrogge 2003), as they directly relate to the maintenance of an adequate
314 microenvironment, protection against natural enemies, and enhancement of nutritional
315 options for the gall inducers, as classically hypothesized by Price et al. (1987) for galls in
316 general.
317 In relation to the non-galled leaves, cell fates are completely altered in galls,
318 except for the outer epidermis, whose cell cycles are intensified but the fates are
319 maintained. In relation to *N. myrtoidis* galls, cell fates are roughly the same, but the
320 standby-redifferentiation observed in the chlorophyllous parenchyma cells during the
321 stage of growth and development (Carneiro and Isaias 2014) does not occur in *N.*
322 *cattleiani* galls.
323 **Roles of cell metabolism in gall biology**
324 The functional aspects of plant tissues in the context of gall structure have been
325 related to the accumulation of defensive and nutritive metabolites (Hartley 1998), and to
326 the establishment of histochemical and cytological gradients in Cynipid (Bronner 1992),
327 Cecidomyiidae (Bronner 1992, Oliveira et al. 2010, 2011*a*), and Pseudophacopteronidae
328 (Oliveira and Isaias 2010*b*) galls. In the galls of *N. myrtoidis*, such gradients are not
329 observed in gall cortex due to its low structural complexity and metabolism (Carneiro and
330 Isaias 2014). The galls of *N. cattleiani* also lack cytological gradients, as the parenchyma

cells are metabolically impaired, with low protein synthesis, scarce and underdeveloped mitochondria and chloroplasts. Nevertheless, the vascular and perivascular parenchyma cells exhibit increasingly metabolic apparatus during gall morphogenesis, i. e., large nuclei, abundant rough endoplasmic reticulum, mitochondria, ribosomes, and polysomes. Such cell types have also been observed in the vascular and perivascular tissues in galls of *N. myrtoidis* (Carneiro and Isaias 2014), and share great similarities to the nutritive cells of cynipid (Bronner 1992), and cecidomyiid galls (Bronner 1992, Oliveira et al. 2010, 2011*a*).

Nutritive tissues have the most specialized cell types within gall structure, and they are constantly impacted by the gall inducers during their feeding (Bronner 1992). Both the impact of the feeding activity of the galling insects and the high metabolism intrinsic to these cell types, often lead to the accumulation of reactive oxygen species (ROS). In fact, ROS were histochemically localized in the nutritive tissues of galls induced by Cecidomyiidae on *Aspidosperma spruceanum* (Oliveira et al. 2010), and on *Copaifera langsdorffii* (Oliveira et al. 2011*a*). Furthermore, ROS have been localized in the nutritive-like ground parenchyma around the nymphal chamber in the galls of Pseudophacopteronidae on *Aspidosperma australe* (Oliveira and Isaias 2010*b*), and in the vascular bundles of *Nothotrioza myrtoidis* (Carneiro et al. 2014*a*) and *Euphalerus ostreoides* galls (Isaias et al. 2011). Vascular and perivascular parenchyma cells are metabolically active in the galls on *Psidium myrtoides,* with well-developed ROS-scavenging apparatus, i. e., lomasomes, lamellar and multivesicular bodies (Carneiro and Isaias 2014). Such structures were previously described in the nutritive cells of galls induced by Thysanoptera (Raman and Ananthakrishnan 1983), in the fast-dividing nutritive cells of lepidoptera galls on *Marcetia taxifolia* (Ferreira et al. 2015), and also herein for the galls on *P. cattleianum*. Lomasomes, lamellar and multivesicular bodies act together with the endoplasmic reticulum to recycle membrane systems (Staehelin 1997) to ensure the functionality of cells subjected to high oxidative stress.

In addition to ROS-scavenging apparatus, high protein synthesis seems to be characteristic of nutrition-related tissues in galls, i. e., true nutritive tissues in galls of cynipids (Bronner 1992), cecidomyiids (Oliveira et al. 2010, 2011*a*, Ferreira and Isaias 2014), and lepidoptera (Vecchi et al. 2013), and of nutritive-like parenchyma cells around the nymphal chamber of Psylloidea (Oliveira and Isaias 2010*b*, Isaias et al. 2011). In fact, the histochemical detection of proteins in the galls of *N. cattleiani* corroborates such premise, and reveals another similarity among vascular and perivascular parenchyma

365 cells in the galls of *P. myrtoides* (Carneiro et al. 2014*b*) and *P. cattleianum,* and those of
366 true nutritive tissues. The well-developed ROS-scavenging apparatus and high protein
367 synthesis seem to be a widespread trait of nutrition-related cells across different galls.
368 More than simply feeding on the vascular bundle cells, *N. cattleiani* induces the
369 redifferentiation of true nutritive cells in and around the vascular system, which is herein
370 reported for the first time in insect galls, as far as we are concerned.
371 Other cell types, such as those of the chlorophyllous parenchyma, may also be
372 subjected to high oxidative stress due to photosynthesis and respiration, which are ROS-
373 generating processes (Møller et al. 2007). Chloroplasts subjected to high oxidative stress
374 may develop plastoglobules, which help minimizing oxidative damage to the
375 photosynthetic apparatus (Asada 2006; Møller et al. 2007). In fact, the chlorophyllous
376 parenchyma cells of non-galled leaves of *P. myrtoides* (Carneiro and Isaias 2014), and of
377 *P. cattelainum* accumulate ROS, and have conspicuous and numerous plastoglobules.
378 Also, they have low-stack grana with electron-lucent thylakoids, which is characteristic of
379 sun-type chloroplasts previously described by Lichtenthaler et al. (1981), which
380 photosynthesize at high levels. Galls that accumulate high amounts of ROS, such as the
381 ones induced by the sucking-insect *Pseudophacopteron* sp. on *A. australe* (Oliveira et al.
382 2011*b*), have plastoglobules in the chloroplasts. In this system, the stress imposed by the
383 galling herbivore stimulates the differentiation of plastoglobules, which are not observed
384 in non-galled leaves, and both galls and leaves photosynthesize at the same levels
385 (Oliveira et al. 2011*b*). In the galls of *N. cattleiani*, the differentiation of plastoglobules is
386 blocked, similarly to the galls of *N. myrtoidis* (Carneiro and Isaias 2014), which are
387 photosynthesis-deficient (Carneiro et al. 2014*b*). In both *Nothotrioza* spp. galls, the
388 chloroplasts have less lamellation throughout gall development, and high-stack grana
389 with electron-dense thylakoids, as described for shade-type leaves (Lichtenthaler et al.
390 1981), which photosynthesize at low levels. The cytological features of chloroplasts in the
391 chlorophyllous parenchyma of *N. cattleiani* galls indicate low cell metabolism, and
392 corroborate the hypothesis that these galls may be photosynthesis-deficient, such as the
393 ones of the co-generic system, *N. myrtoidis* (Carneiro et al. 2014*b*, Carneiro and Isaias
394 2014).
395 Despite the low metabolism of chlorophyllous parenchyma cells in the galls of *N.*
396 *cattleiani*, secondary and primary metabolites are histochemically localized in the cortex
397 of mature galls, forming gradients somewhat similar to those reported for *N. myrtoidis*
398 galls (Carneiro et al. 2014*b*). In both *Nothotrioza* spp. galls, the scarcity of lipid droplets

399 and essential oils is attributed to the characteristics of the host plants, *P. myrtoides* and *P.*
400 *cattleianum.* These compounds are common in Myrtaceae (Ramos et al. 2010) and
401 represent a metabolic trait that is not changed due to gall formation, as observed for galls
402 of *Aceria lantanae* on *Lantana camara* (Verbenaceae) (Moura et al. 2008). The
403 centripetal gradients of reducing sugars, on the other hand, evidence the manipulation of
404 plant cell metabolism in galls, which conspicuously accumulate sugars. Sugars are not
405 synthesized at gall sites, but are drained from non-galled organs (Burstein et al. 1994,
406 Raman et al. 2006, Álvarez et al. 2009, Castro et al. 2012*b*, 2013). Herein, such premise
407 is confirmed for the galls of *N. cattleiani*, which have detectable activity of glucose-6-
408 phosphatase and invertases in the vascular bundles and surrounding tissues, next to the
409 larval chamber. These sites of enzymes activity are similar to those described in the galls
410 on A*spidosperma australe* (Oliveira and Isaias 2010*b*), *A. spruceanum* (Oliveira et al.
411 2010), and *Lonchocarpus muehlbergianus* (Isaias et al. 2011). Glucose-6-phosphatase is
412 involved in the formation of sucrose after starch breakdown (Baroja-Fernandez et al.
413 2003), and invertases catalyze the irreversible conversion of sucrose into glucose and
414 fructose (Koch 2004). The centrifugal histochemical gradient of starch in the galls of *P.*
415 *cattleianum* indicates that starch is converted into soluble sugars in the cells surrounding
416 the larval chamber, where enzyme activity is detected. A similar gradient of starch and
417 related enzyme activity is described for the galls on *Lonchocarpus muehlbergianus*
418 (Isaias et al. 2011), which reinforces the enzyme-mediated mobilization of starch, as a
419 pattern for inner cortical cells of galls. The activities of enzymes related to carbohydrate
420 metabolism determine the establishment of physiological sinks in plants (Koch 1996,
421 Koch and Zeng 2002), and are involved in tissue development and cell expansion (Rehill
422 and Schultz 2003), crucial for gall development. In fact, the galls on *Psidium* spp. act as
423 physiological sinks, but diverge in terms of the detectable carbohydrate related enzymes.
424 The activity of acid phosphatase is exclusive of *P. myrtoidis* galls (Carneiro et al. 2014*b*),
425 while the activity of glucose-6-phosphatase and invertases is just detected in *P.*
426 *cattleianum* galls.

427 Besides accumulating carbohydrates, the galls on *P. cattleianum* also have
428 gradients of total phenolics and proanthocyanidins, similar to the galls on *P. myrtoides*
429 (Carneiro et al. 2014*b*). The localization of such compounds in gall tissues is considered a
430 defensive chemical strategy of gall inducers against natural enemies (Bronner 1992) for
431 their unpalatablility. In both galls, phenolic compounds are believed to control cell
432 expansion and division by the modulation of indol-acetic acid (IAA) levels (Hori 1992).

In fact, recent studies localized phenolics and IAA at the same sites in *Piptadenia gonoacantha* galls (Bedetti et al. 2014), thus confirming the developmental role of phenolics in galls. Also, phenolics mediate the morphogenesis of vascular tissues (Aloni 2001), and should be involved in the neoformation of vascular bundles near the nymphal chamber. Neoformed vascular bundles contribute to the establishment of the gall as a sink of photoassimilates and to the nutrition of the gall inducer, which contradicts the classical role of phenolics as plant-defensive compounds in galls, as widely attributed in literature (Hartley 1998, Nyman and Julkunen-Tiitto 2000; Formiga et al. 2009). Another group of phenolic substances, the proanthocyanidins, accumulates both in the outermost and innermost cell layers of the *Psidium* spp. galls, with decreasing gradients towards the median cortex. Such substances act as antioxidants (Simmonds 2003, Bouaziz et al. 2005), and their distribution along the gradient of ROS concentration possibly prevent oxidative stress in gall tissues, keeping cell alterations to a minimum.

CONCLUSIONS

The similar phenotypes of the globoid galls of the double co-generic systems, *Nothotrioza cattleiani* - *Psidium cattleianum* and *Nothotrioza myrtoidis* - *Psidium myrtoides*, are not extended to the cytological and histochemical levels. Nevertheless, the lack of cytological gradients and the formation of somewhat conservative histochemical profiles are common to both galls, with the centripetal accumulation of sugars and centrifugal detection of lignins, as conservative traits possibly linked to the taxonomical proximity of the involved species.

The bidirectional gradients of metabolites accumulation described for *N. cattleiani* galls, together with the redifferentiation of true nutritive cells associated to vascular bundles are unique features. Also, cytological development in *N. cattleiani* galls is gradual from induction towards senescence, differently from the standby-redifferentiation of *N. myrtoidis* galls, which reinforce the exclusive subcellular aspects of distinctive extended phenotypes.

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CONTRIBUTIONS BY AUTHORS

Both authors substantially contributed to the preparation of manuscript and the research presented; have seen and agreed to submit the manuscript.

CONFLICTS OF INTEREST

No conflicts of interest.

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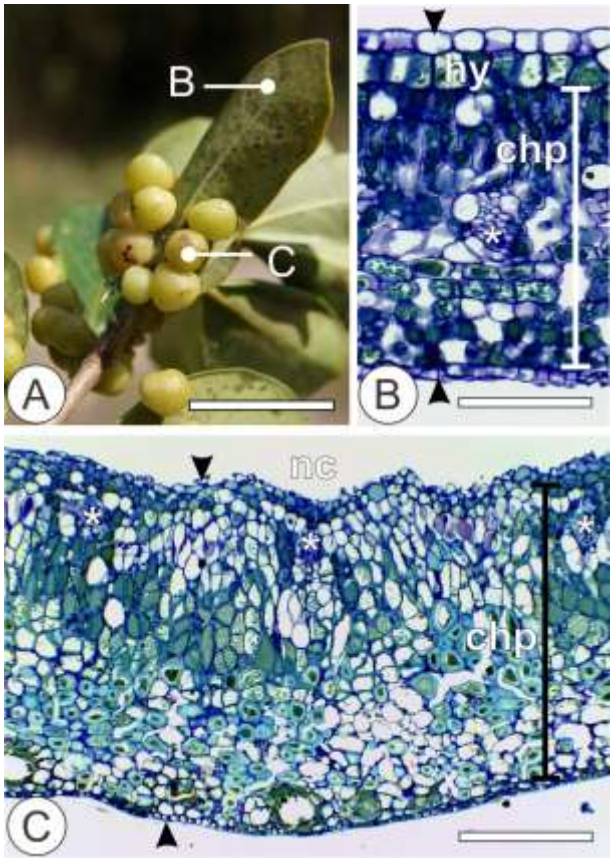
699 **FIGURE LEGENDS**



700

701 **Figure 1.** Morphology and anatomy of *Psidium cattleianum* leaves and *Nothotrioza*
702 *myrtoidis* galls. A. Detail of a simple leaf with globoid galls protruded to the abaxial
703 surface. B. Cross section of mature leaf with uniseriate epidermis on both surfaces (arrow
704 heads), hypodermis (hy), and vascular bundles (asterisks) interspaced to the dorsiventral
705 chlorophyllous parenchyma (chp). C. Cross section of mature gall with uniseriate
706 epidermis on both surfaces (arrow heads), and vascular bundles (asterisks) near the
707 nymphal chamber (nc) interspaced to the homogenous chlorophyllous parenchyma (chp).
708 Bars: A. 3 cm; B. 100 µm; C. 200 µm.

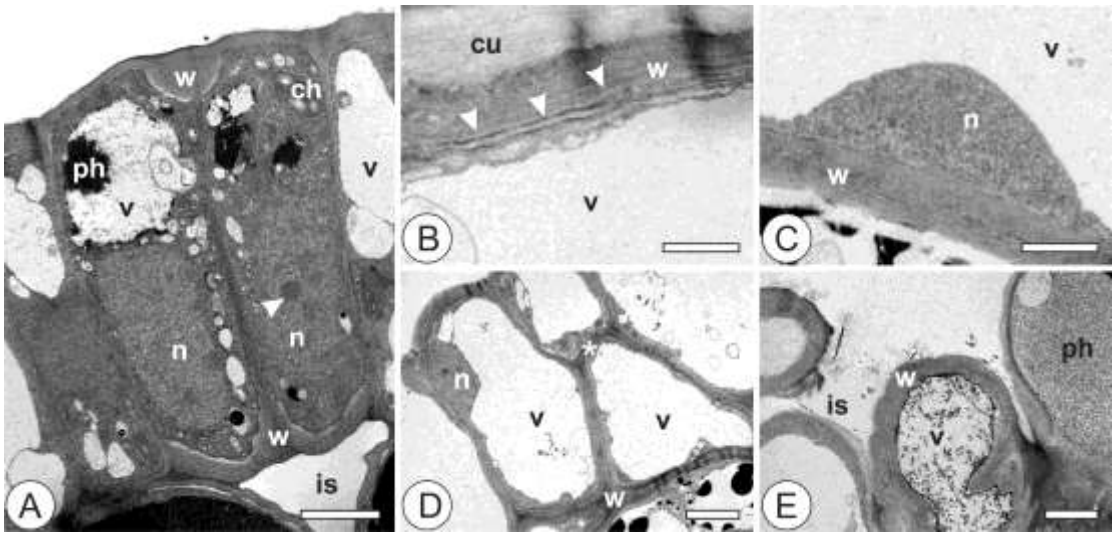


Figure 2. Cytology of epidermal cells in the leaves of *Psidium cattleianum* and galls of *Nothotrioza cattleiani*. A-C. Leaves. D-E. Galls. A. Cells with thin anticlinal primary walls (w), large nuclei (n) with conspicuous nucleoli (arrow head), few chloroplasts (ch) and small vacuoles (v), with phenolic inclusions (ph). B. Detail of a cell with polylamellated (arrows) cell wall (w), thick cuticle (cu), and hyaline vacuole (v). C. Detail of cell with homogenous secondary wall (w), small nucleus (n) and hyaline vacuole (v). D. Induction phase. Cells with homogenous thickened walls (w), peripheral nuclei (n), and hyaline vacuoles (v). Discrete sites of periclinal divisions are observed (asterisk). E. Maturation phase. Intermittent cell layer, with intercellular spaces (is), heterogeneous thickened and polylamellate walls, and inconspicuous cuticle. Vacuoles (v) may contain phenolic inclusions (ph). Bars: A-C. 2 µm; D, E. 5 µm.

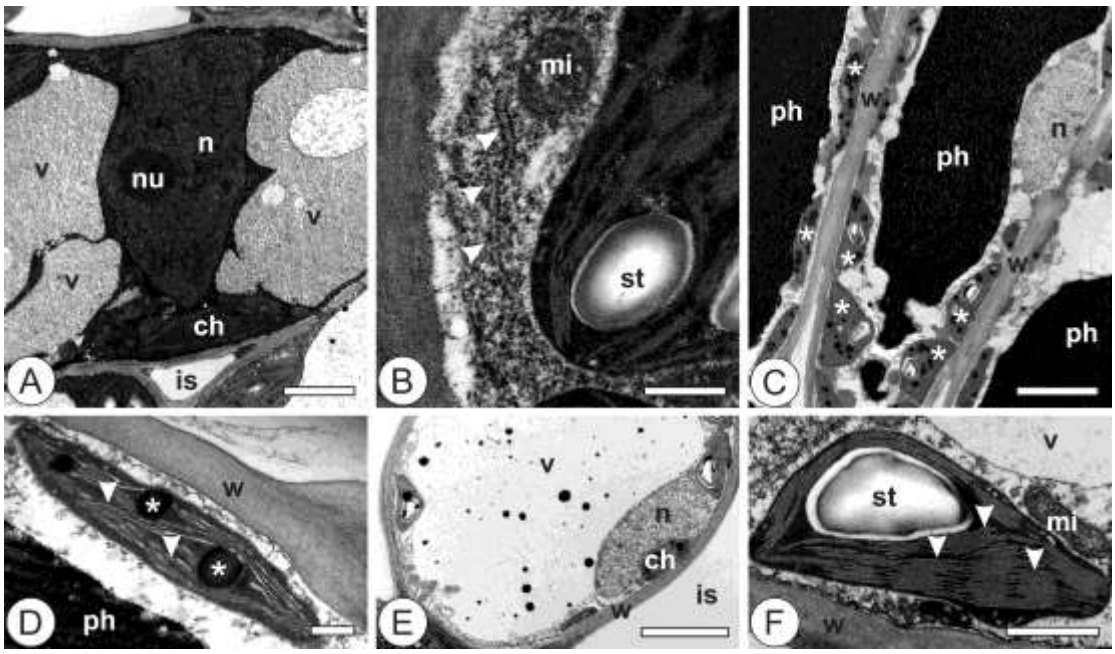


Figure 3. Cytology of chlorophyllous parenchyma cells in leaves of *Psidium cattleianum*. A, B. Young leaves. C-F. Mature leaves. A. Cell with primary thin walls, large central nucleus (n) with conspicuous nucleolus (nu), fragmented vacuole (v), and chloroplasts (ch) with well-developed grana. Intercellular spaces (is) are reduced. B. Detail of cell with dense cytoplasm, rough endoplasmic reticulum (arrow heads), and mithocondria (mi) associated to chloroplasts (ch) with well-developed grana and starch grain (st). C. Palisade parenchyma cells with homogenous walls (w), phenolic-rich vacuoles (ph), many chloroplasts with well-organized thylakoid lamellae and starch grains (asterisks), and peripheral nuclei (n) with inconspicuous nucleoli. D. Detail of chloroplast with low stack grana (arrow heads) and large plastoglobules (asterisks). E. Spongy parenchyma cell with homogenous primary wall (w), large vacuole (v), and peripheral nucleus (n) and chloroplasts (ch). Large intercellular space (is). F. Detail of chloroplast with well-developed thylakoid system, high stack grana (arrow heads), starch grain (st), and associated mitochondria (mi). Bars: A. 2 µm; B, D. 500 ηm; E, E. 5 µm; F. 1 µm.

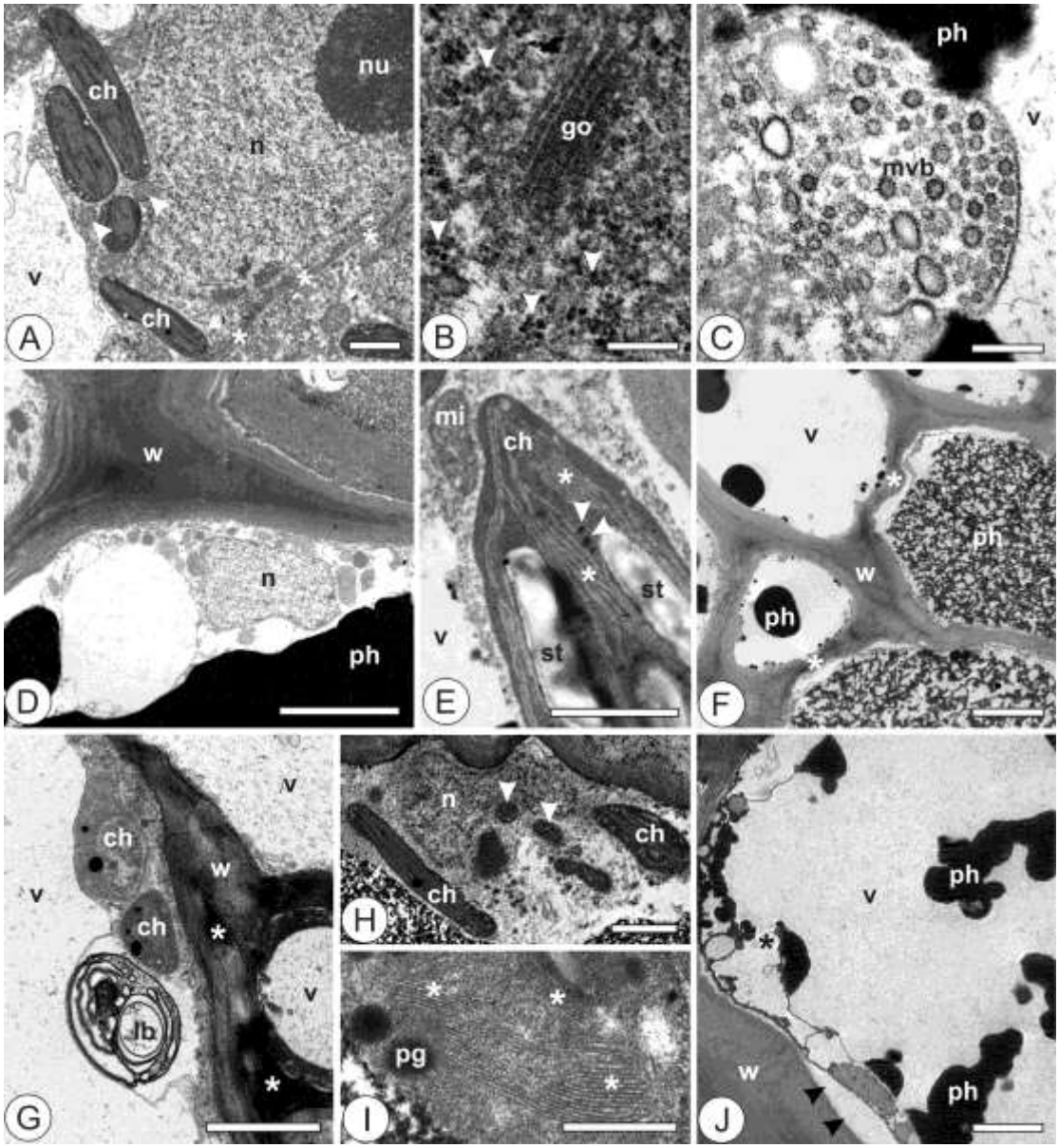


Figure 4. Cytology of chlorophyllous parenchyma cells in the galls of *Nothotrioza cattleiani*. A-C. Induction phase. D, E. Growth and development phase. F-H. Maturation phase. I, J. Senescent phase. A. Detail of cell with large nuclei (n) with conspicuous nucleoli (nu), small mitochondria (arrow heads) associated to chloroplasts (ch) with well-organized lamellae. B. Abundant polysomes (arrow heads) and Golgi apparatus (go). C. Multivesicular body (mvb) at cytoplasm periphery. D. Cells with secondary polylamellated walls (w), phenolic-rich vacuoles (ph), small nuclei (n) with inconspicuous nucleoli. E. Mitochondria (mi) associated to chloroplast (ch) with well-organized thylakoid lamellae (asterisks), starch grains (st) and small plastoglobules (arrow heads). F. Cells with irregularly thickened walls (w, asterisks), phenolic (ph) or hyaline vacuoles (v). G. Detail of lamellar body (lb), undeveloped chloroplasts (ch), and

cell walls (w) with sites of different electron-density (asterisks). H. Degraded nucleus (n), small mitochondria (arrow heads), and chloroplasts. I. Degraded chloroplast with vestigial grana (asterisks) and plastoglobules (pl). J. Cell with large periplasmic spaces (arrow heads), disrupted vacuole (v, asterisk) with phenolic inclusions (ph). Bars: A, E, H. 1 µm; B, C. 200 ηm; D, F. 5 µm; G, J. 2 µm; I. 500 ηm.

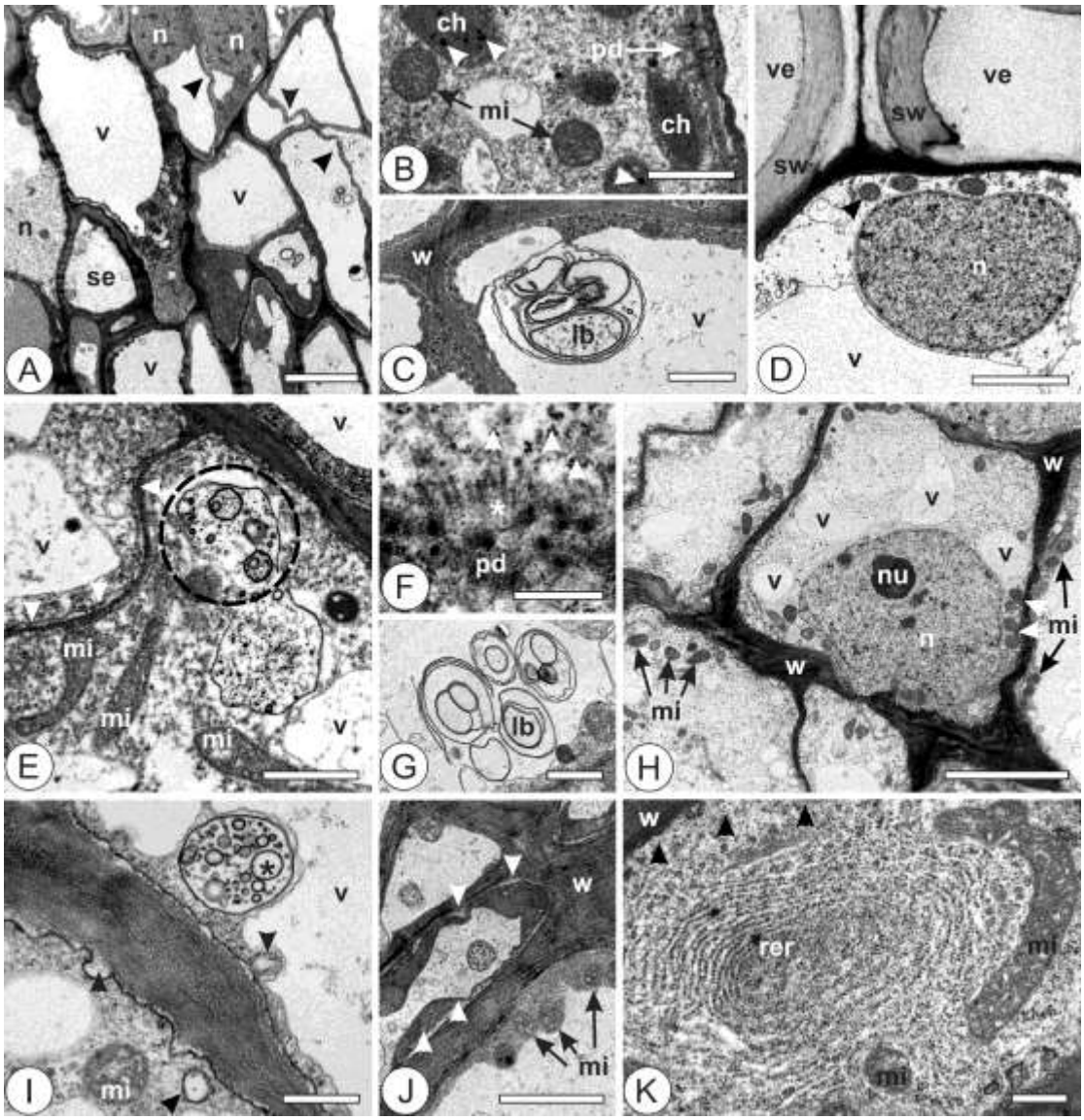


Figure 5. Cytology of vascular and perivascular parenchyma cells in the leaves of *Psidium cattleianum* and galls of *Nothotrioza cattleiani*. A-C. Young leaves. D. Mature leaves. E-K. Galls. A. Cells with thin and sinuous walls (arrow heads), large nuclei (n), hyaline vacuoles (v). Sieve elements (se) may be observed. B. Cell with dense cytoplasm, many plasmodesmata (pd), round mitochondria (mi), small underdeveloped chloroplasts (ch) with few small plastoglobules (arrow heads). C. Lamellar body (lb) near the cell wall (w). D. Cell with large nucleus (n) with dispersed heterochromatin, mitochondria (arrow

head), and hyaline vacuole (v). Vessel elements (ve) with secondary cell walls (sw) can be observed. E-G. Induction phase. E. Cells with thin sinuous walls (arrow heads), hyaline vacuoles (v), large mitochondria (mi), and multivesicular bodies (dashed circle). F. Detail of plasmodesmata (pd) with aligned microtubules (asterisks) and abundant polysomes (arrow heads). G. Detail of lamellar bodies (lb). H, J. Growth and development phase. H. Cells with irregularly thickened walls (w), fragmented hyaline vacuoles (v), large nuclei (n) with conspicuous nucleoli (nu), and abundant mitochondria (mi). I. Detail of cells with multivesicular bodies (mvb), and lomasomes (arrow heads). J. Cells during maturation phase, with thick walls (w), abundant mitochondria (mi) and periplasmic phases (arrow heads). K. Cell during senescent phase, with abundant rough endoplasmic reticulum (rer), polysomes (arrow heads), and large mitochondria (mi). Bars: A, H. 5 µm; B, C, E, G. 1 µm; D, J. 2 µm; F, I, K. 500 ηm.

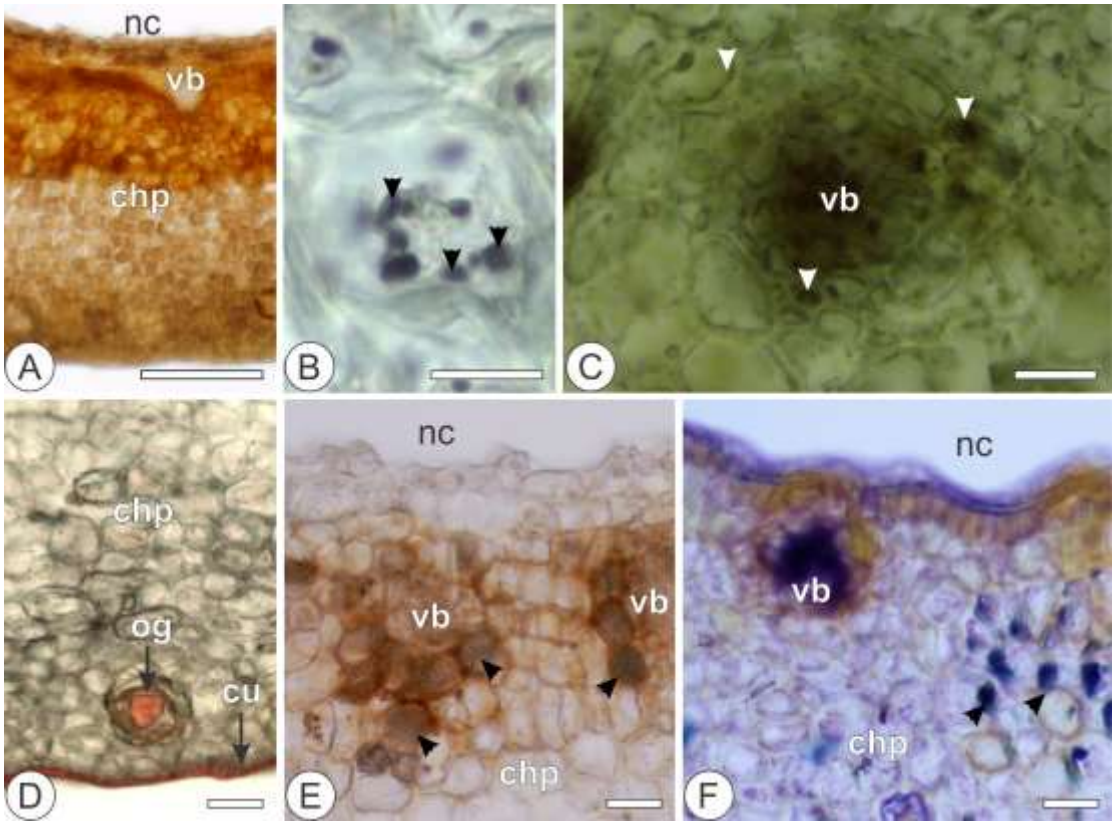
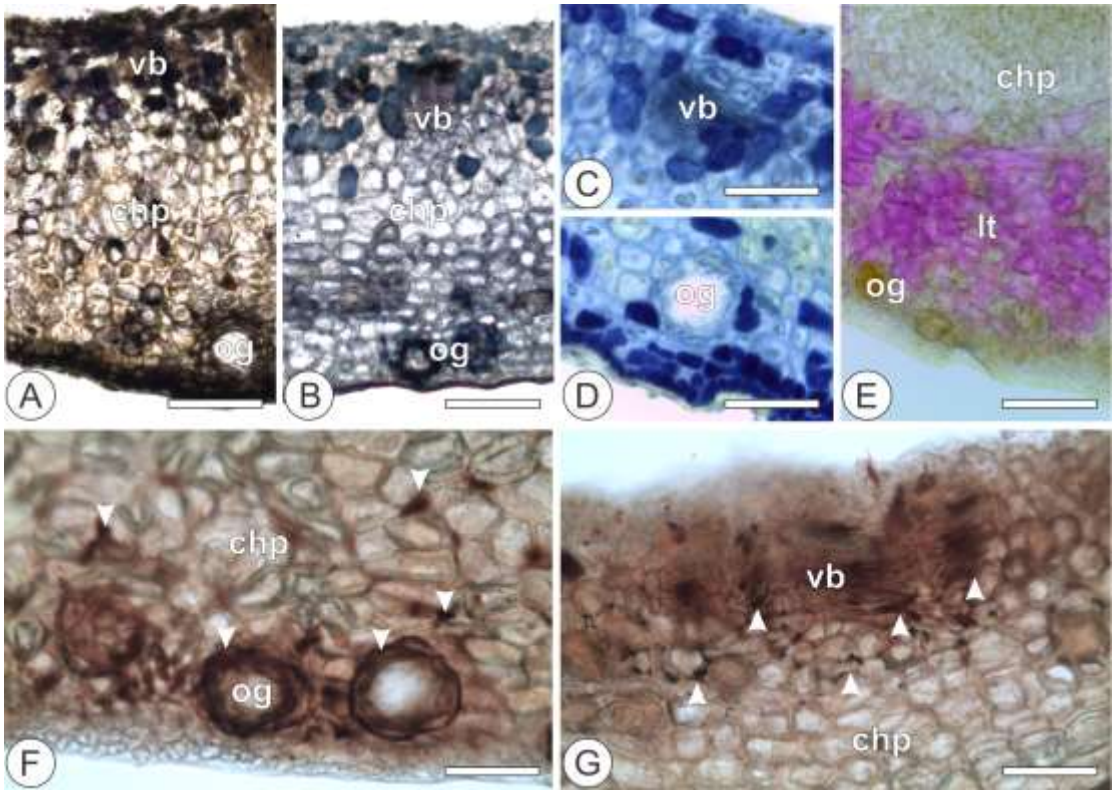


Figure 6. Histochemical detection of primary metabolites and carbohydrate-related enzyme activity in the galls of *Nothotrioza cattleiani*. A. Reaction of Fehling's reagent to reducing sugars in the chlorophyllous parenchyma (chp), with increased accumulation near the vascular bundles (vb) and tissues near the nymphal chamber (nc). B. Reaction of Lugol's reagent to starch (arrow heads) in outer cortical cells. C. Reaction of Coomassie blue to proteins (arrow heads) in a vascular bundle (vb) and perivascular parenchyma. D.

780 Reaction of Sudan red B to lipids in the oil glands (og) and cuticle (cu) of the outer
781 epidermis. E, F. Enzyme activity of glucose-6-phosphatase and invertases, respectively,
782 near vascular bundles (vb), and perivascular parenchyma (arrow heads), surrounding the
783 nymphal chamber (nc). Bars: A. 200 µm; B. 10 µm; C. 30 µm; D-F. 60 µm.



784

785 **Figure 7.** Histochemical detection of secondary metabolites and reactive oxygen species
786 (ROS) in the galls of *Nothotrioza cattleiani*. A-D. Reaction of ferric chloride solution,
787 NADI, and DMACA to total phenolics (A), terpenoids (B) and proanthocyanidins (C, D),
788 respectively. Most intense reactions are observed in the median and inner cortical cells
789 near the vascular bundles (vb), and in the outermost cortical cells, and in the epidermal
790 cells near the oil glands (og). E. Reaction of Wiesner's solution to lignins mainly in the
791 outer cortical cells of the chlorophyllous parenchyma (chp), near the oil glands (og). F, G.
792 Reaction of 3,3'- diaminobenzidine to ROS mainly in the median and inner cortical cells,
793 vascular bundles (vb), and in the outermost cortical cells and oil glands (og). Bars: A, B,
794 E. 150 µm; C, D, F, G. 75 µm.

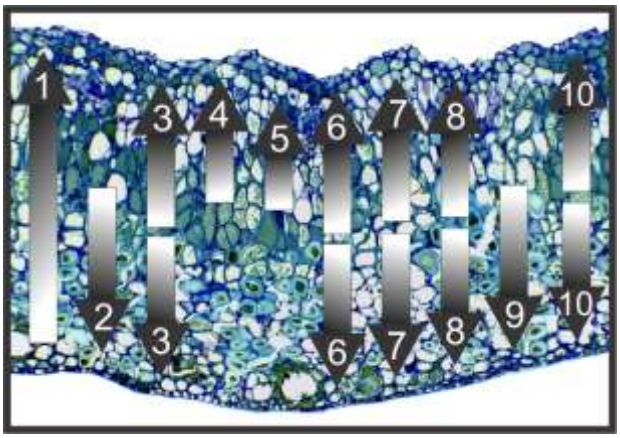
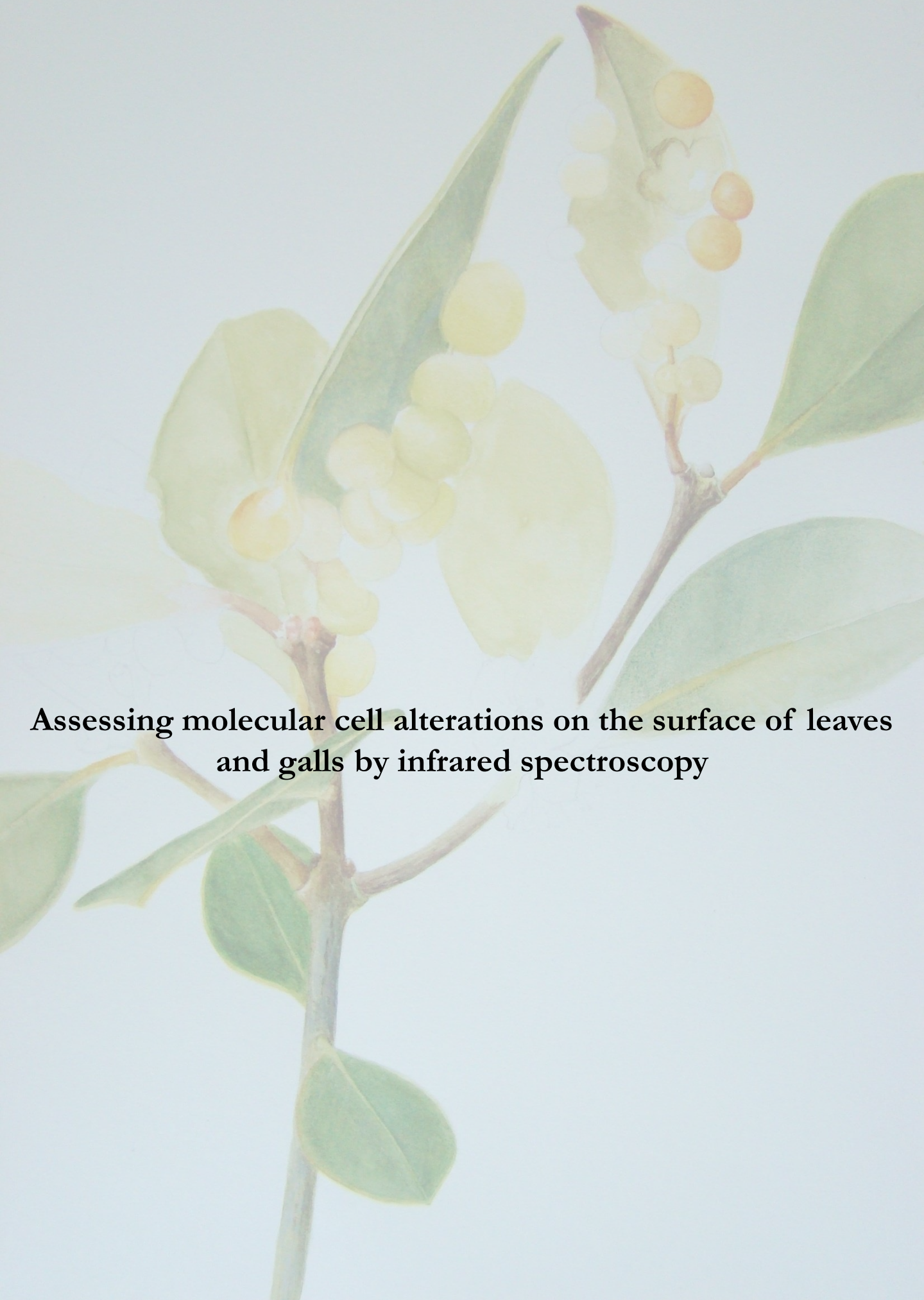


Figure 8. Histochemical gradients in the cortex of *Nothotrioza cattleiani* galls on *Psidium cattleianum*. Shading from white to black means increasing intensity of reaction. (1) Centripetal gradient of reducing sugars from the outermost cortical cells towards the innermost ones. (2, 9) Centrifugal gradients of starch and lignins, respectively, from the median cortical cells towards the outer cells. (3, 6, 7, 8, 10) Bidirectional gradients of proteins, phenolics, proanthocyanidins, terpenoids, and reactive oxygen species, respectively, from the median cortical cells towards both the inner and outer cell layers. (4, 5) Centripetal gradients of glucose-6-phosphatase and invertases activities, from the median cortical cells towards the inner cell layers, especially in the perivascular parenchyma.



Assessing molecular cell alterations on the surface of leaves and galls by infrared spectroscopy

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Assessing molecular cell alterations on the surface of leaves and galls by infrared spectroscopy

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Abstract

Plant tissues are altered by galling insects towards new structural and metabolical traits of plant cells, which should reflect into a different assembly of chemical functional groups. These functional groups may be recognized by Infrared Attenuated Total Reflectance Spectroscopy (IR-ATR) in complex mixtures, such as the plant cell walls. Molecular alterations were assessed in superficial cell walls in galls, and in comparison to non-galled tissues in the *Psidium myrtoides – Nothotrioza myrtoidis* system. The epidermal cells on the adaxial and abaxial surfaces were the focus of current analyses for they represent the two cytological limits, inner and outer cell layers of gall structure. The two epidermal layers undergo distinct degrees of alterations stimulated by gall induction and establishment, evidenced by anatomical, cytological and immunocytochemical analyses. Herein, we check the IR-ATR spectra of the inner and outer surfaces of galls in comparison to the adaxial and abaxial surfaces of leaves. We hypothesize that the IR-ATR spectrum of gall inner surface should be the most divergent due to its new structural and functional design. The spectrum of the outer surface of *N. myrtoidis* galls is similar to the spectrum of its equivalent non-galled tissue layer, the abaxial surface of leaves, but the spectrum of galls inner surface differs from the spectrum of the non-galled leaf adaxial surface, mainly in the regions between 3300-3000 cm^{-1} and 1700-1600 cm^{-1}. The differences are linked to the degree of pectin methylation in the cell walls of gall inner epidermis, and to the presence or absence of intramolecular hydrogen bonds. It is surprising that the structural and functional alterations do not seem to extend to the molecules at the level of pectin domains of the cell walls in both epidermal surfaces. Anyway, the cecidogenetic field is strongly corroborated at the molecular level.

Keywords: IR-ATR, epidermis, homogalacturonans, *Nothotrioza myrtoidis*, *Psidium myrtoides*.

Introduction

Structural properties of plant cells rely mostly on the composition of cell walls (Albersheim et al. 2011), which is altered under the influence of galling herbivores as evidenced by immunocytochemical analyses (Carneiro et al. 2014a; Formiga et al. 2013; Oliveira et al. 2014a). Such qualitative alterations may be followed by changings at the molecular level detected by the absorption of infrared light (IR – wavenumbers between 4000-400 cm^{-1}), whose energy excites molecular vibrations to high energy levels (Pasquini 2003). The systematic use of the infrared absorption spectra allows the identification of molecular structures by the fingerprint of chemical bonds and functional groups, which are analyzed by chemometric methods especially in the region of 1000-500 cm^{-1}. Technological and computational advances in chemometric analyses using IR absorbance spectra led to the development of the Attenuated Total Reflectance (IR-ATR) apparatus, which allows the construction of spectra and detailed structural analysis of any kind of sample (Andersson et al. 2007; Harrick 2000). Moreover, the fingerprints may be successfully assessed even in complex mixtures (Christy et al. 2004), which is the case of plant cell walls.

The influence of biotic and abiotic factors may alter the chemical composition of plant cells, which has been assessed by histochemistry and immunocytochemistry, as far as the influence of galling herbivores is concerned (Bedetti et al. 2013; Carneiro et al. 2014a; b; Ferreira and Isaias 2013; Ferreira and Isaias 2014; Formiga et al. 2013; Isaias et al. 2011; Oliveira and Isaias 2010; Oliveira et al. 2010; Oliveira et al. 2011; Oliveira et al. 2014a). Plant epidermal cells mediate the interactions between plant organs and the surrounding environment (Evert 2006, Fahn 1990). Their presence on the adaxial and abaxial leaf surfaces represents the two cytological limits, the innermost and the outermost cell layers within gall structure. In the *Psidium myrtoides* – *Nothotrioza myrtoidis* system, the two epidermal layers undergo distinct degrees of alterations stimulated by the gall inducer, as evidenced by anatomical, cytological and immunocytochemical analyses (Carneiro and Isaias 2014, Carneiro et al. 2014a). On Psylloidea galls, whose inducers feed by piercing cells and sucking their contents (Burckhardt 2005), the set of cell alterations are usually poor. Nevertheless, the set of structural alterations in the galls of *N. myrtoidis* indicate that the inner epidermis is functionally altered (Carneiro et al. 2014a; b).

The generation of a cecidogenetic field, i. e., the tissues directly influenced and changed by the gall inducer stimuli (*sensu* Mani 1964), implies in changes in the composition of plant cell walls, with those of the innermost tissue layers acquiring porosity and flexibility, while those of the outermost tissue layers acquire rigidity along gall development (Carneiro et al. 2014a; Formiga et al. 2013; Oliveira et al. 2014a). Even though the inner and outer gall surfaces have the same ontogenetic origin (Carneiro et al. 2014a), the structural alterations are distinct and so is the molecular composition of their cell walls. Based on this premise, current study proposes the investigation of the surfaces of non-galled leaves of *P. myrtoides* and leaf galls of *N. myrtoidis* using IR-ATR spectroscopy. The main objective is to check if gall establishment distinctly affects the molecular structure of the epidermal cell walls on both gall surfaces. Distinct fingerprint patterns in gall and leaf epidermises related to the neo-ontogenesis of the dermal system are expected, and greater cell alterations should occur in the inner epidermis when compared to the outer epidermis of the galls. Current data will be confronted with the pectin composition of cell walls and their influence on the cell biology.

Material and methods

Samples of non-galled leaves and galls induced by *Nothotrioza myrtoidis* (Psylloidea) on *Psidium myrtoides* (Myrtaceae) collected at Serra do Caraça, Minas Gerais state – Brazil (n = 3; from different individuals) were fixed in Karnovsky's solution in 0.1 M phosphate buffer (pH 7.2) (O'Brien and McCully 1981). Fixed samples were dehydrated

in ethanol series (Johansen 1940), embedded in glycolmethacrylate (Leica®), sectioned in rotary microtome (Jung biocut), and incubated with the monoclonal antibodies JIM5 and JIM7 (Centre for Plant Sciences, University of Leeds, UK). These antibodies specifically bind epitopes of low methylated homogalacturonans (HGAs), and high methylated HGAs, respectively (Knox et al. 1990; Willats et al. 2000).

Fresh samples were sectioned with a 1cm-diameter round sampler. The disks were put on a zinc selenium crystal ATR apparatus (Smart Orbit ATR, Thermo Fisher Scientific Inc., Madison, USA) coupled with the spectrophotometer FTIR Nicolet 380 (Thermo Fisher Scientific Inc., Madison, USA), and submitted to infrared radiation between 4000 - 525 cm^{-1} wavelengths to obtain the absorbance spectra. Series of 32 scannings were performed, with the resolution of 2 cm. All spectra were obtained in triplicate from two different samples of non-galled leaves and galls, and were transformed to transmittance spectra.

The optimized geometry of methylated and demethylated HGAs, and related IR absorption spectra were studied at the B3LYP/6-31G* level of theory, using MOLPRO (Werner et al. 2012).

Results

Immunolabeling of low methylated HGAs by JIM5 is observed in the adaxial epidermal cells of the non-galled leaves (Fig. 1a), and in the outer epidermal cells of the galls (not shown), while high methylated HGAs bound by JIM7 are evidenced in the abaxial epidermal cells of the non-galled leaves, and both in the inner and outer epidermal cell walls of the galls (Fig. 1b-d).

The IR-ATR spectra of the adaxial epidermal surface of leaves and of the gall inner epidermis evidence differences especially in the regions between 3300-3000 and 1700-1600 cm^{-1} (Fig. 2a). The IR-ATR spectra of the abaxial epidermal surface of the non-galled leaves and of the gall outer epidermis are similar (Fig. 2b).

The optimized geometry of low and high methylated HGAs indicates the establishment of intramolecular hydrogen bonds between the equatorial carbonyl and hydroxyl groups in low methylated HGAs, which does not occur in the high methylated HGAs (Fig. 3a, b).

Discussion

The differences on the degree of pectin methylation is confirmed by the IR-ATR spectra of non-galled leaves and galls of *P. myrtoides*, especially between 1700-1600 cm^{-1}, which is the fingerprint band region of carbonyl groups (R-C=O) in organic acids and esters (Barbosa 2007). In the galls, the carbonyl groups of HGAs may be either esterified (R-COO-R), with more intense absorption bands in the region of 1750-1735 cm^{-1}, or form carboxylates ($R\text{-}COO^{-}$), which have more intense absorption bands in the region of 1600 cm^{-1} (Barbosa 2007). Unexpectedly, the absorption peak of the spectrum of the leaf adaxial epidermis, whose cells have low methylated HGAs with carboxylates, shifted to a higher frequency region (around 1720 cm^{-1}). This shift is attributed to the intramolecular hydrogen bonds occurring between the equatorial hydroxyl groups and the equatorial carbonyl groups in the low methylated HGAs, which reduce the electronic density of the carbonyl carbons, and lead them to vibrate at higher frequencies. The occurrence of the hydrogen bonds should explain both the shifted carbonyl band and the retracted hydroxyl band in the leaf adaxial epidermis, as described for other molecules (Silverstein et al. 1991). In fact, in the gall inner epidermis, whose cells have high degree of carbonyl methylated groups in the HGAs, the absorption peak is shifted to a lower frequency region (1640 cm^{-1}), and the hydroxyl band is enlarged due to the absence of intramolecular hydrogen bonds.

The high and low methylated HGAs may have variable structures, which can be recognized by specific monoclonal antibodies (MAbs), namely JIM5 and JIM7, which bind to disperse or concentrated sites of methylation, respectively (Clausen et al. 2003). The HGAs are deposited in the cell walls in the high methylated form and undergo demethylation by the action of pectin methylesterases (PMEs) (Liu et al. 2013). Such dynamic control of HGA methylation occurs in the galls of *N. myrtoidis* on *P. myrtoides*, which confers different cell wall functionalities to galled tissues when compared to non-galled leaf tissues (Carneiro et al. 2014a). In fact, flexibility is assumed for cell walls with high methylated HGAs (Xu et al. 2011), which neither have intramolecular hydrogen bounds nor establish calcium-mediated bonds between adjacent carboxylate groups, as do the low methylated HGAs in rigid cell walls (Vincken et al. 2003; Willats et al. 2001). Likewise, the enhanced flexibility of the high methylated HGA-rich cell walls in the gall inner epidermis indicates divergent functionalities when compared to the low methylated HGA-rich cell walls in the leaf adaxial epidermis. Instead of being adaptive for *P. myrtoides* and mediate the plant-environment interface, the inner epidermis of the gall is adaptive for *N. myrtoidis* by facilitating the access to its feeding sites (*sensu* Price et al. 1987). The IR-ATR spectra together with the assessment of the optimized geometry of low and high methylated HGAs corroborate the immunocytochemistry of plant cell walls in the galls and leaves of *P. myrtoides* – *N. myrtoidis* system (Carneiro et al. 2014a).

In addition to the variations in the HGAs methylation, the cellular structure of the non-galled leaves and galls evidences well marked differences among the inner and outer epidermis of the galls, and the adaxial and abaxial surfaces of the non-galled leaf epidermis (Carneiro and Isaias 2014; Carneiro et al. 2014a). The greater alterations in the inner epidermis when compared to the outer epidermis of the galls corroborate the new functional designs proposed for the Neotropical galls (Oliveira et al. 2014b). Such functional designs are based on the establishment of gradients of tissue alterations from the innermost towards the outermost cell layers of the galls. Counterpoising to the anatomical alterations of the outer epidermis of the galls in relation to the abaxial epidermis of the leaves (Carneiro et al. 2014a), IR-ATR analyses demonstrated that such tissues are similar on the molecular-structural basis. Even though the walls of *N. myrtoidis* galls are relatively thin (~ 600 µm) (Carneiro et al. 2014a), the cecidogenetic field does not extend to the outermost cell layers, as far as molecular alterations in cell wall polymers are concerned. Herein, the chemometric analytical approach is employed for the first time to help understanding refined differences in the structure and functionalities of low and high methylated HGAs in galls. Further investigations on the intermolecular interactions between HGAs and other cell wall polymers should help clarifying the neo-established functionalities of plant cells under the influence of gall inducers at the molecular level.

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Figures

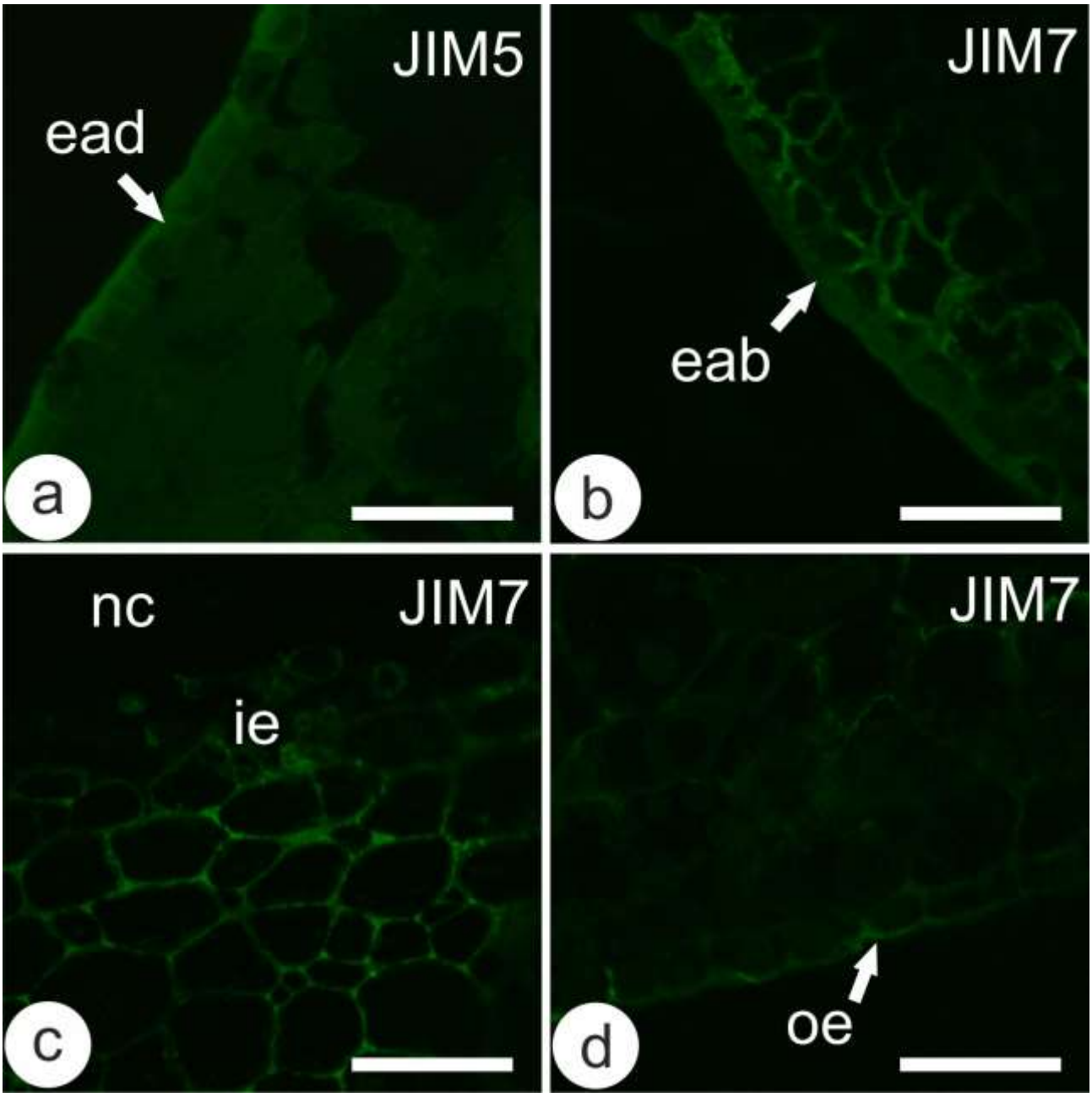

Figure 1 Immunolabeling of homogalacturonans (HGAs) in the epidermal cells of the non-galled leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. **a** Low methylated HGAs bound by the JIM5 antibody. **b-d** High methylated HGAs bound by the JIM7 antibody. **a, b** Non-galled leaves. **c, d** Galls. Bars: 50 µm. **ead** – adaxial surface of the epidermis; **eab** – abaxial surface of the epidermis; **ie** – inner epidermis; **nc** – nymphal chamber; **oe** – outer epidermis

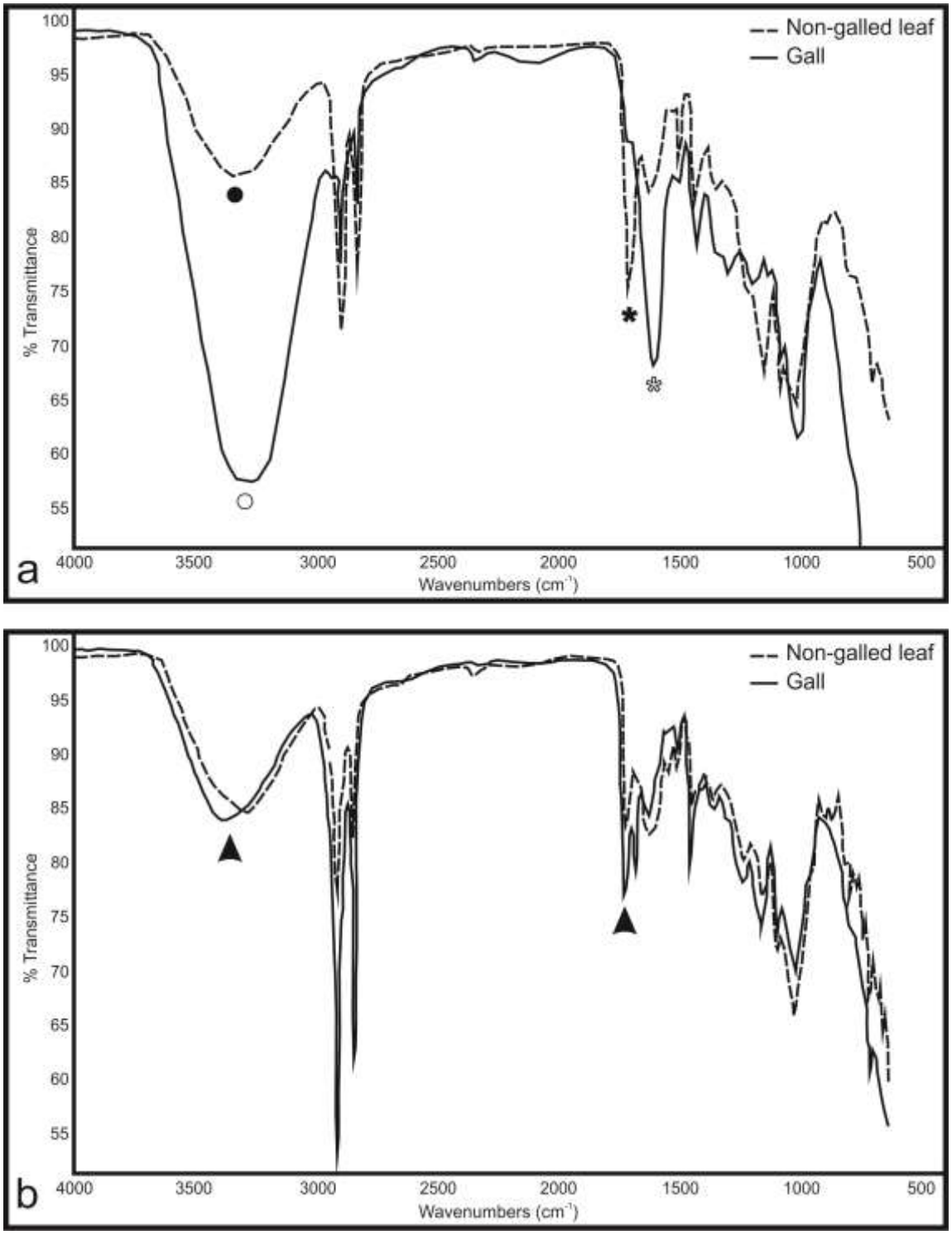

Figure 2 Infrared Attenuated Total Reflectance (IR-ATR) transmittance spectra of non-galled leaves of *Psidium myrtoides* and galls of *Nothotrioza myrtoidis*. **a** Comparative spectra of the adaxial epidermal surface of non-galled leaves and inner epidermis of galls. White and black circles point the difference of intensity on the bands between 3000 – 3300 cm^{-1}. Black asterisk points the band of 1720 cm^{-1} exclusive of the leaves, and white asterisk points the more intense band of 1640 cm^{-1} in the galls. **b** Comparative spectra of abaxial epidermal surface of non-galled leaves and outer epidermis of galls, without significant differences in band intensity and/or presence in the regions between 3300-3000 and 1700-1600 cm^{-1} (arrow heads)

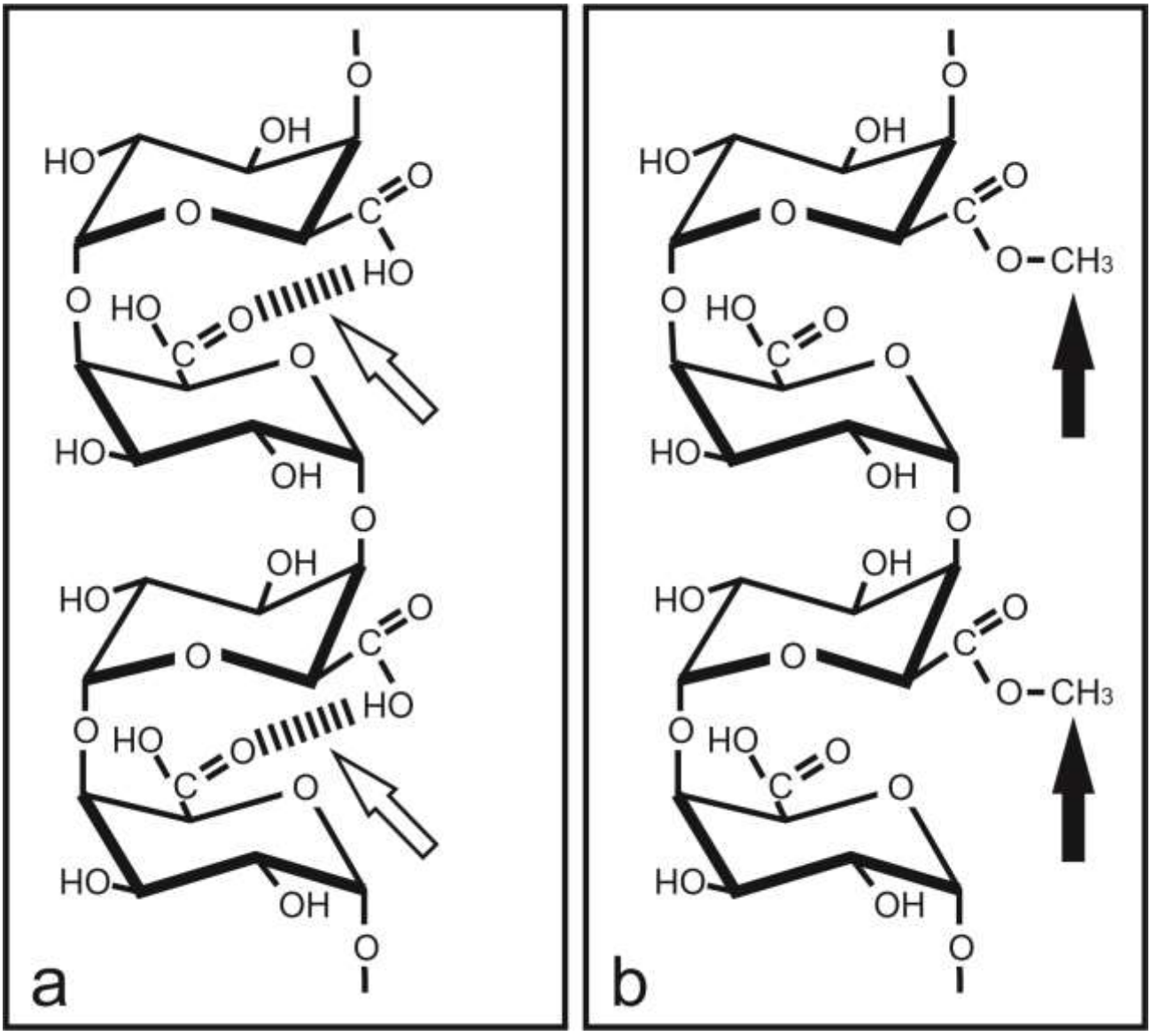

Figure 3 Optimized geometric structures of homogalacturonans (HGAs). **a** Low methylated HGAs with intramolecular hydrogen bonds (white arrows) between equatorial hydroxyl and carbonyl groups in adjacent sugar residues. **b** High methylated HGAs. The methyl esterification of the equatorial carbonyl groups (black arrows) prevents the formation of hydrogen bonds with the equatorial hydroxyl

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Galhas são excelentes modelos para testes de hipóteses relativas ao desenvolvimento vegetal devido aos padrões repetitivos de alterações morfológicas, metabólicas e funcionais dos tecidos que as constituem. O processo de rediferenciação celular no órgão hospedeiro durante a morfogênese de galhas requer um novo programa de desenvolvimento dos tecidos vegetais, que é aqui denominado neo-ontogênese.

A neo-ontogênese das folhas de *Psidium* spp. para a formação das galhas de *Nothotrioza* spp. ocorre por meio de alterações marcantes nos níveis químico, metabólico, histoquímico, citológico e tecidual. Embora o fenótipo macro-morfológico de ambas as galhas aqui estudadas seja o mesmo, isto é, galhas globoides, com câmara ninfal ampla e parede relativamente fina, aspectos da biologia celular funcional revelam características distintivas e únicas. O desenvolvimento citológico das galhas de *N. myrtoidis* ocorre através de um processo de rediferenciação em "*standby*", com poucas alterações por um longo período, diferentemente das alterações graduais e constantes observadas nas galhas de *N. cattleiani*. O parênquima cortical das duas galhas é constituído de células com aparato metabólico fotossintético e respiratório pouco desenvolvido e não apresenta gradientes citológicos. Entretanto, os gradientes funcionais determinados pela composição dinâmica das paredes celulares determinam: (1) porosidade para o trânsito de moléculas célula a célula no estabelecimento de gradientes histoquímicos distintos, e (2) balanço de rigidez e flexibilidade na regulação dos sítios de ocorrência de hipertrofia e hiperplasia. Células verdadeiramente nutritivas associadas aos feixes vasculares nas galhas de *N. cattleiani* e células semelhantes às nutritivas associadas aos feixes vasculares nas galhas de *N. myrtoidis* são reportadas aqui pela primeira vez. A atividade enzimática associada a esses sítios nutricionais parece ser crucial para o funcionamento das galhas como drenos de fotoassimilados, bem como para a biologia celular funcional das suas diferentes camadas.

Os resultados obtidos com a análise por espectrometria na faixa do infravermelho sinalizam a importância da interface biológica-química no entendimento da biologia celular funcional de galhas. Atualmente, o sistema *P. myrtoides* – *N. myrtoidis* é estudado em parceria com o Departamento de química – UFMG, utilizando-se o método analítico de espectrometria de massa por eletro-spray que possibilitará uma análise molecular mais refinada no que se refere ao acúmulo diferencial de metabólitos frente às alterações estruturais e funcionais ocorrentes nas galhas.

Os conhecimentos teóricos sobre o duplo sistema co-genérico *P. myrtoides – N. myrtoidis* e *P. cattleianum – N. cattleiani* constituem base sólida para que as investigações continuem avançando para níveis subcelulares e moleculares, com perspectivas de elucidar pontos-chave da resposta à pergunta ainda não completamente respondida, “Como as galhas são formadas?”.