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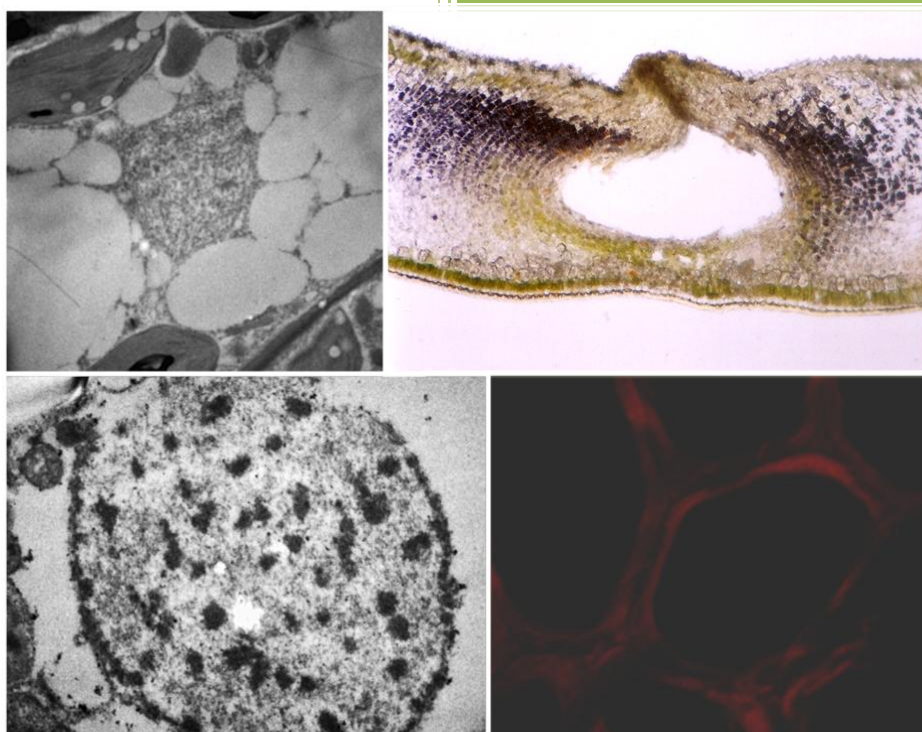
Tese de Doutorado



Gradientes citológicos e histoquímicos em galhas de insetos

Belo Horizonte

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Denis Coelho de Oliveira

Gradientes citológicos e histoquímicos em galhas de insetos

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Orientadora: Profa Dra Rosy Mary dos Santos Isaias

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PREFÁCIO

Antes mesmo de ingressar no curso de Doutorado em Biologia Vegetal do Programa de Pós Graduação em Biologia Vegetal da UFMG, já tinha em mente usar a citologia e a histoquímica como ferramenta no estudo da estrutura e função de galhas. Esta idéia apareceu ainda na iniciação científica, quando trabalhei com o sistema *Lonchocarpus muelhbergianus-Euphalerus ostreoides.* Neste sistema, a galha induzida pelo *E. ostreoides*, que é um sugador de floema, apresentava um zoneamento de tecidos muito semelhante às galhas induzidas por Cecidomyiidae descritas por Bronner, ou seja, um tecido que acumula nutrientes próximo à câmara ninfal, circundado por um tecido lignificado e por um tecido que acumula substâncias de reserva com células mais vacuoladas na porção externa da galha. Estes resultados encontrados em *Lonchocarpus*, definitivamente não demonstravam o padrão para galhas de insetos sugadores.

Nesta perspectiva, escrevi um projeto que tinha como objetivo propor um padrão de acúmulo de reservas nutricionais para galhas da região neotropical. A princípio parecia um projeto muito ousado, talvez ainda seja, mas foi com ele que recebemos o apoio da FAPEMIG e conseguimos entrar de vez no mundo da citologia e da histoquímica. A idéia inicial era trabalhar com duas galhas de *Copaifera langsdorffii* induzidas por Cecidomyiidae (a galha de nervura e a galha em chifre), uma galha induzida por *Pseudophacopteron* sp. (Hemiptera) em *Aspidosperma australe*, uma galha induzida por uma espécie não identificada de Cecidomyiidae em *A. spruceanum*, e como quinto tipo, as galhas induzidas por *Euphalerus ostreoides* (Hemiptera) em *Lonchocarpus mulhbergianus*. Com estes sistemas tínhamos galhas induzidas por galhadores com diferentes hábitos alimentares: sugadores de floema e raspadores. Protocolar os testes histoquímicos foi uma tarefa árdua, principalmente aqueles relacionados às atividades enzimáticas. Mas apesar disso, o desenvolvimento do projeto corria tranquilo.

Entretanto, a área de coleta de *Copaifera langsdorffii* pegou fogo por dois anos consecutivos, inviabilizando as coletas necessárias aos testes histoquímicos e às análises citológicas das galhas. O que poderia ser encarado com um desespero na verdade tornou-se uma oportunidade de explorar com mais tranqüilidade os sistemas *Aspidosperma* spp. – insetos galhadores. Quer dizer, esta tranqüilidade

toda não era minha, mas sim da Rosy, eu, na verdade, queria abraçar o mundo. E foi assim que surgiram os trabalhos publicados na Plant Science 178(2010): 350-358, apresentado na minha qualificação de doutorado, e na Protoplasma 242(2010): 81-93. Ambos fazem parte desta Tese de Doutorado, capítulos segundo e terceiro, respectivamente. Foi através destes trabalhos que surgiu a questão: qual seria a função dos plastoglóbulos nos cloroplastos das células do tecido clorofiliano das galhas? Discuti esta idéia com o Prof. Pires que se mostrou bastante interessado. A princípio nossa hipótese era de que os plastoglóbulos prejudicavam o sistema de membranas e por conseqüência a aparato fotossintético!!! É, mas o que mostramos foi exatamente o contrário. E assim surgiu o quarto capítulo desta Tese.

Por último, vem o primeiro capítulo, sugerido pela banca de qualificação composta pelos Professores: Dra. Jane E. Kraus, Dra. Maria Zabelê D. Moura e Dr. Geraldo W. Fernandes; uma revisão sobre o tema principal da tese, trabalho que, sem dúvida, foi fascinante!

RESUMO

Galha é uma estrutura vegetal que confere vantagem adaptativa aos galhadores em relação aos seus ancestrais de vida livre. Assim, compreender o desenho funcional desta estrutura é um passo importante para avaliar o valor adaptativo da galha para o galhador. A grande diversidade de formas presentes nas galhas reflete a grande diversidade de insetos, porém algumas questões importantes permanecem desconhecidas: Qual é a força moduladora desta diversidade de formas? Qual é a estrutura funcional dos tecidos da galha? E por fim, o que gera todo este gradiente funcional na galha? A presente tese discute estas questões e propõe um modelo para o desenvolvimento de galhas e formação dos gradientes citológicos e histoquímicos.

ABSTRACT

A gall is a plant structure that confers adaptive value to the galling herbivores in relation to their free-living ancestors. Thus, understanding the functional design of this structure is an important step to assess the adaptive value of the gall to the gall inducer. The great diversity in gall morphology reflects the great diversity of insects, but some important issues remain unknown: what is the force modulating the morphological diversity? What is the functional structure of the gall tissues? And finally, which are the forces that generate all these functional gradients in the galls? The current thesis discusses these issues and proposes a model for gall development, and the formation of the cytological and histochemical gradients.

Desenvolvimento de galhas de insetos: formação de gradientes citológicos e histoquímicos

Tecidos vegetais podem reagir prontamente a estímulos abióticos e bióticos alterando sua morfogênese. Dentre os fatores bióticos que alteram a morfogênese da plantas destacam-se os insetos galhadores, capazes de induzir estruturas compostas por tecidos vegetais, as galhas, dentro das quais eles se alimentam e buscam abrigo (Mani 1964, Shorthouse & Rohfritsch 1992). A formação da galha envolve ativa rediferenciação celular na planta hospedeira com a formação de células e tecidos com características e funções típicas de um novo órgão (Mani 1964, Lev-Yadun 2003, Oliveira & Isaias 2010). Os mecanismos moleculares que levam a formação de galhas ainda não foram elucidados. Entretanto, propostas datadas de mais de 10 anos atrás sugerem que a injeção de secreções durante a oviposição de afídeos e secreções larvais de cinipídios pode agir como gatilho para o desenvolvimento dos tecidos vegetais (Shorthouse & Rohfritsch 1992, Higton & Mabberly 1994). Contudo, a natureza da ação das secreções oriundas dos insetos ainda permanece desconhecida (Stone & Schönrogge 2003). Outras moléculas sinalizadoras, oriundas das plantas, foram propostas para a formação das galhas: auxinas, citocininas, aminoácidos e proteínas, entre outros (Shorthouse & Rohfritsch 1992). Em geral, galhadores podem usar sinais moleculares similares àqueles normalmente usados no metabolismo vegetal (Abrahamson & Weiss 1997, Shorthouse & Rohfritsch 1992), o que dificulta experimentos que elucidem os processos de formação da galha, não somente o controle quantitativo, mas também qualitativo das moléculas envolvidas. Em contrapartida, estudos comparativos entre a ontogenia dos órgãos hospedeiros, com mecanismos de estímulo e resposta documentados, e das galhas podem fornecer indícios relevantes dos mecanismos de ação dos insetos galhadores para a formação do novo órgão. O sentido das divisões e do alongamento celular é indicativo da direção do campo cecidogênico (Mani 1964, Oliveira & Isaias 2010), e consequentemente podem indicar os gradientes metabólico e funcional presentes nas galhas. Análises histoquímicas também podem se configurar como excelentes ferramentas para estudos de gradientes funcionais em galhas, principalmente na detecção de metabólicos primários e de espécies reativas de oxigênio (Bronner 1992, Nyman 2000, Oliveira & Isaias 2010, Oliveira et al. 2010)

O presente estudo discute os mecanismos de sinalização que podem levar a formação das galhas e dos gradientes citológico e histoquímico nos seus tecidos; propõe as espécies reativas de oxigênio (ERO) como principais moléculas sinalizadoras para a indução e formação das galhas e sua conseqüente relação com a formação do corpo final da estrutura, ou seja, que com a geração da diversidade de morfotipos de galhas.

Hipóteses para o valor adaptativo da galha para o galhador

Três principais hipóteses têm sido discutidas em torno do valor adaptativo da estrutura da galha para o galhador: a hipótese do microambiente, a hipótese do inimigo natural e a hipótese nutricional. A primeira, estabelece que os tecidos da galha protegem o galhador de condições desfavoráveis do ambiente, como a dessecação e o aumento de temperatura (Crespi et al. 1998; Price et al. 1987, Whitham 1992, Danks 2002, Fernandes & Price 1992, Stone & Schonrogge 2003), o que tem sido relacionado com várias características morfológicas e anatômicas das galhas. Em geral, a rediferenciação de tricomas no bordo da abertura das galhas e a deposição de cutícula são citadas como características que evitam a perda excessiva de umidade nas lojas e consequentemente ajudam a manter a temperatura interna (Kraus et al. 2002, Stone & Schonrogge 2003, Moura et al. 2009, Moura et al. 2008, Oliveira et al. 2006, Oliveira & Isaias 2010a). A presença de células epidérmicas papilosas recobertas por cutícula espessa e a formação de periderme também são características comumente relatadas em folhas como mecanismos de controle de umidade e temperatura (Fahn 1990, Woodman & Fernandes 1991, Gutschick 1999, Press 1999, Stone & Schonrogge 2003). Em galhas, estes caracteres podem exercer papel similar no controle da umidade e temperatura dentro da câmara. Outra característica anatômica importante é a homogenização do parênquima, que além de apresentar células grandes, vacuolizadas, com acúmulo de água, possui poucos espaços intercelulares (Kraus 2009), o que também é interpretado como um mecanismo para evitar o excesso de perda de água (Sack & Holbrook 2006, Kraus 2009). Em geral, tecidos que amenizam a perda de água nas galhas, e que mantém um microambiente favorável para o galhador estão localizados mais externamente na estrutura da galha, ou seja, no revestimento ou no córtex externo.

Segundo Stone & Schonrögge (2003), a pressão seletiva imposta pelos inimigos naturais é a principal força que modula a diversidade de galhas. Esta hipótese postula que os tecidos das galhas podem possuir características que protegem o galhador contra seus inimigos naturais, sendo estas, características vantajosas em relação a seus ancestrais de vida livre. Características anatômicas e morfológicas como aumento dos tecidos lignificados (Rohfritsch 1992, Kraus 2002, Oliveira et al. 2006, Oliveira et al. 2008, Oliveira & Isaias 2010a, Stone et al. 2002), aumento da espessura da galha pelo aumento do número de camadas de células e o desenvolvimento de tricomas tectores (Rohfritsch 1992, Kraus et al. 2000, Stone & Schonrögge 2003, Kraus 2009, Oliveira & Isaias 2009) são citadas como mecanismos de proteção contra à ação de inimigos naturais.

A hipótese nutricional, que sugere a manipulação dos tecidos vegetais pelos galhadores para a produção de recursos alimentares, se sustenta pela rediferenciação celular levando ao desenvolvimento de um tecido que acumula grande quantidade de nutrientes em relação aos tecidos não galhados, o tecido nutritivo (e.g. Bronner 1992). Segundo Bronner (1992), o tecido nutritivo, em geral, ocorre ao redor da câmara larval de galhas induzidas por cecidomiídios e cinipídios, contudo alguns autores relataram a presença de um tecido acumulador de nutrientes em galhas induzidas por insetos sugadores (Oliveira et al. 2006, Alvarez et al. 2009, Oliveira & Isaias 2010). Embora Stone & Schonrogge (2003) coloquem a hipótese do inimigo como principal fator de seleção para a diversidade de galhas, a hipótese nutricional como potencial para explicar esta diversidade não é descartada. Mesmo que insetos sugadores de floema não se alimentem diretamente do tecido ao redor da câmara ninfal, a presença de um organismo na galha causa injúria nas células. Assim, um gradiente citológico e fisiológico é estabelecido, e este, pode ser o responsável pelo campo cecidogênico na galha (Mani 1964, Shorthouse & Rohfritsch 1992, Oliveira & Isaias 2010, Oliveira et al. 2010) e conseqüente formação do corpo final da estrutura. Em galhas induzidas por cecidomiídios e cinipídios, o tecido nutritivo é o primeiro tecido especializado formado e, muito embora em galhas induzidas por sugadores um tecido nutritivo típico não seja diferenciado, as células ao redor da câmara ninfal podem exibir um gradiente funcional centrípeto. Por conseguinte, análises citológicas e histoquímicas de galhas podem se configurar como uma excelente ferramenta para a compreensão das relações entre o hábito alimentar do galhador e a forma final da galha. Rohfritsch (1992) relata que diversos autores propõem a existência de uma relação entre o modo de alimentação e a complexidade estrutural das galhas de insetos. Nesta proposta, insetos com peças bucais mandibuladas ou sugadoras induziriam galhas irregulares em forma e tamanho, enquanto cecidomiídeos e cinipídeos induziriam galhas estruturalmente complexas. Contudo esta proposta por si só não explica a grande diversidade de formas encontradas na literatura, além do mais, galhadores com hábito alimentar semelhante podem induzir formas completamente distintas.

Características citológicas e histoquímicas do tecido nutritivo

Em geral, o tecido nutritivo de galhas induzidas por cecidomiídios e cinipídios é caracterizado por abundância de citoplasma, vacuoma, núcleo e nucléolos hipertrofiados, numerosas organelas citoplasmáticas, alta atividade enzimática, riqueza em RNA e acúmulo de açúcares em geral, entre outras (Bronner 1992). Esta autora propõe que em galhas induzidas por cecidomiídios não há formação de gradientes citológicos, porém há

acúmulo de amido nas camadas mais externas das galhas durante sua formação, e de açúcares nas camadas mais internas. Em galhas induzidas por cinipídios, há formação de um gradiente citológico e ocorre acúmulo de lipídios.

Entretanto, alguns sistemas descritos na região neotropical fogem ao padrão proposto por Bronner (1992) para galhas da região temperada. Galhas induzidas por *Schismatodiplosis lantana* (Cecidomyiidae) em *Lantana camara* (Verbenaceae) apresentaram acúmulo de lipídios no tecido nutritivo (Moura et al. 2008). Em galhas induzidas por uma espécie não identificada de Diptera – Cecidomyiidae em *Aspidosperma spruceanum* (Apocynaceae), há acúmulo de amido nas camadas mais externas do tecido nutritivo propriamente dito. Ademais, nas células mais internas do tecido nutritivo nestas galhas, o protoplasto não foi observado desde as fases iniciais de formação da galha, denotando processo de morte celular, o que já foi relatado por Harris et al. (2006) para o tecido nutritivo de galhas induzidas por *Mayetiola destructor* (Diptera Cecidomyiidae) em *Triticum aestivum*.

Embora insetos sugadores não induzam a formação de um tecido nutritivo propriamente dito, o acúmulo de substâncias de reserva e a formação de um gradiente citológico têm sido relatados na literatura. Galhas induzidas por *Euphalerus ostreoides* (Psyllidae) em *Lonchocarpus muelhbergianus* (Fabaceae) não formam um tecido nutritivo propriamente dito, mesmo apresentando uma zonação de tecidos semelhante a galhas induzidas por Cecidomyiidae (Mani 1964, Shorthouse & Rohfritsch 1992, Oliveira & Isaias 2010). Esta galha acumula amido e lipídios nos tecidos próximos à câmara ninfal (Oliveira et al. 2006). Em galhas induzidas por *Pseudophacopteron* sp., um inseto sugador, em *Aspidosperma australe* há formação de um gradiente citológico e histoquímico nos tecidos da galha, inclusive com a armazenagem de amido. Alvarez et al. (2009) relataram a presença de grãos de amido em galhas induzidas por afídios em *Pistacia terebinthus*. Em geral, o papel deste polissacarídeo é relacionado à manutenção da estrutura da galha e não ao modo de alimentação do inseto.

Estudos sobre a estrutura das galhas utilizando como ferramentas a anatomia vegetal, a citologia e a histoquímica permitem verificar gradientes citológicos e histoquímicos nas galhas em geral. Estes gradientes podem indicar o gatilho inicial e os passos subseqüentes para os processos de indução e desenvolvimento das galhas. Independentemente do sistema em questão, dois grupos moleculares têm sido constantemente detectados via histoquímica nos tecidos das galhas, os açúcares e as espécies reativas de oxigênio (ERO). A partir desta detecção levanta-se uma questão fundamental: Quais as moléculas iniciam e mantém a funcionalidade da galha? Hipotetiza-

se que as respostas estejam na relação entre a produção de espécies reativas de oxigênio (ERO) e de açúcares para o metabolismo respiratório nas camadas mais internas das galhas.

Papel das ERO no desenvolvimento de galhas

- **Formação do gradiente citológico**

Devido à presença de grande quantidade de oxigênio molecular na atmosfera, todas as células vivas estão sujeitas a ação reativa e tóxica de uma forma reduzida de oxigênio. Estas espécies reativas de oxigênio (ERO), como o ânion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($HO^\blacksquare$) e o oxigênio singleto ($^1O_2$) são produzidos tanto durante o metabolismo fundamental da célula como resposta a estímulos abióticos e bióticos (Moller et al. 2007, Couée et al. 2006, Rossel et al. 2002, Pham & Desikan 2009). Os cloroplastos, peroxissomos e as mitocôndrias são as principais organelas produtoras de ERO. Os cloroplastos produzem $^1O_2$ e O_2^- nos fotossistemas, as mitocôndrias em geral produzem O_2^-, e os peroxissomos produzem H_2O_2 que pode ser convertido a $HO^\blacksquare$ (Moller et al. 2007).

A produção de ERO através do metabolismo fundamental das células pode gerar importantes sinalizadores durante o desenvolvimento vegetal, como por exemplo, o aumento da elasticidade da parede durante o crescimento celular (Bell et al. 2009). Contudo, as ERO também são capazes de oxidar todos os tipos de componentes celulares, podendo levar a célula a morte (Moller et al. 2007). A morte celular pode ser conseqüência da produção de uma cascata oxidativa como parte de uma resposta imune da planta durante o início da interação com outros organismos (Doke et al. 1996, Moller et al. 2007). Contudo, a produção de ERO não está somente relacionada com a defesa contra a ação de outro organismo, mas também a regulação gênica que pode conduzir a uma resposta de hipersensitividade, a morte celular programada localizada ou a sinalização para desenvolvimento celular local (Pham & Desikan 2009, Couée et al. 2006, Moller 2007, Del Rio et al. 2009, Doke et al. 1996). Nesta perspectiva, a ação de insetos galhadores provocam aumento na produção de ERO nos tecidos vegetais (Oliveira & Isaias 2010, Oliveira et al. 2010), sendo este aumento e sua localização coincidente com os gradientes citológico e histoquímico formados nas galhas.

Muito embora Bronner (1992) tenha citado a formação de um gradiente citológico apenas em galhas induzidas por Cynipidae, análises anatômicas e citológicas de galhas induzidas por Hemiptera e Diptera nos neotrópicos mostram diferenças significativas

entre as camadas celulares proximais e distais à câmara larval (Souza et al. 2000, Kraus et al. 2002, Arduin et al. 2005, Oliveira et al. 2006, Moura et al. 2008, Moura et al. 2009, Oliveira & Isaias 2009, Oliveira & Isaias 2010). Sendo assim, os tecidos do córtex interno, próximos a câmara larval, estão sujeitos a maior estresse oxidativo e como resultado da rediferenciação celular, as galhas, independentemente do taxa indutor, possuem um gradiente histológico e citológico. A diferença nos níveis de ERO do córtex interno para o externo seria a principal força geradora da forma final do corpo da galha a qual advém de mudanças nos padrões de alongamento celular (Oliveira & Isaias 2010, Oliveira et al. 2010). As ERO provocam mudanças na conformação da parede celular tornando-a mais elástica favorecendo a expansão e o alongamento celular (Del Rio et al. 2009, Barceló & Laura 2009) devido amudanças na organização de microtúbulos e microfibrilas de celulose (Barceló & Laura 2009). Assim, o sentido da divisão e a forma das células são alterados em função do gradiente formado pelo acúmulo de ERO no córtex da galha.

De fato, a direção do alongamento celular exerce um papel importante na formação e determinação morfológica de órgãos vegetais (Steeves & Sussex 1989, Obroucheva 2008). Nas galhas, a determinação de sítios distintos de hipertrofia celular e hiperplasia tecidual tem sido detectada (Meyer & Maresquelle 1983, Bronner 1992, Rohfritsch 1992, Souza et al. 2000, Arduin et al. 2005, Oliveira et al. 2006, Moura et al. 2008, Álvarez et al. 2009), mas raramente as causas de tais determinações têm sido propostas (Oliveira & Isaias 2010). Durante o desenvolvimento de galhas, a hipertrofia e o aumento de volume das células do córtex externo e a hiperplasia das camadas celulares do córtex interno são constantes para cada morfotipo e cada sistema e também parecem ser determinantes para a forma final do corpo da galha. Estas diferenças são geradas pelo campo cecidogênico imposto pelo galhador (Mani 1964), mas parece haver forte correlação com a produção de espécies reativas de oxigênio.

- **Formação do gradiente histoquímico**

O impacto causado pela alimentação do inseto galhador provoca alterações bioquímicas e histoquímicas nos tecidos vegetais, alterações estas condicionadas ao campo cecidogênico gerado pelo galhador (Bronner 1992, Mani 1964). Independente do hábito alimentar destes herbívoros, há formação de um gradiente histoquímico em uma ou em todas as fases de desenvolvimento da galha (Bronner 1992, Oliveira et al. 2010, Oliveira & Isaias 2010, Moura et al. 2008, Schönrogge 2000). Este gradiente histoquímico parece estar relacionado ao estresse oxidativo gerado pelo galhador nas camadas celulares mais internas da galha.

Segundo Bronner (1992), galhas induzidas por Cynipidae apresentam alta concentração de proteínas estruturais e enzimáticas, em especial as fosfatases ácidas, proteases, fosforilases e invertases. Além disso, uma alta concentração de amido é encontrada no córtex externo, decrescendo em direção a câmara larval. Em um gradiente contrário ao de amido, a glicose ocorre de forma abundante ao redor da câmara larval. Por outro lado, lipídios são considerados como a principal fonte de alimento para Cinipídios galhadores. Em galhas induzidas por Cynipidae em *Quercus robur*, uma alta concentração de proteínas foi encontrada nos tecidos próximos a câmara larval. Entre as principais proteínas identificadas no córtex interno destas galhas destaca-se a formato desidrogenase (FDH). A expressão da FDH é um importante indicador de estresse respiratório e conseqüente produção de espécies reativas de oxigênio nas mitocôndrias (Moller et al. 2007, Schönrogge et al. 2000). A alta atividade respiratória nas células ao redor da câmara larval de galhas induzidas por Cynipidae é corroborada pela alta concentração de proteínas, proteases, ácido fosfatases e principalmente glicose, que é um importante substrato para a respiração (Bronner 1992, Schönrogge et al. 2000).

Há algumas características metabólicas em comum entre o tecido nutritivo de galhas de Cynipidae e Cecidomyiidae, como alta atividade da amilase, invertase, glicose-6-fosfatase, fosforilase, fosfatases ácidas, e um gradiente decrescente de amido em direção a câmara larval. O acúmulo de glicose só ocorreria no tecido nutritivo de galhas em estágio de desenvolvimento mais avançado, sendo os carboidratos em geral a principal fonte de alimento para os Cecidomyiidae galhadores (Bronner 1992). Entretanto, Moura et al. (2008) detectaram a presença de lipídios no tecido nutritivo de galhas de *Schismatodiplosis lantanae* (Cecidomyiidae) em *Lantana camara*, conferindo este acúmulo ao metabolismo intrínseco da espécie hospedeira. A armazenagem de amido foi detectada no tecido nutritivo de galhas induzidas por Cecidomyiidae em *Aspidosperma spruceanum*, sendo este acúmulo relacionado a intensa vascularização neste tecido (Oliveira et al. 2010). Neste mesmo sistema, Oliveira et al. (2010) observaram um gradiente crescente de proteínas em direção a câmara larval, fato também observado anteriormente por Bronner (1990) e Harris et al. (2006) em outras galhas. A detecção de proteínas próximo a câmara larval coincide com a produção de espécies reativas de oxigênio (Oliveira et al. 2010, Oliveira & Isaias 2010, Schönrogge et al. 2000), o que pode levar a morte celular do tecido (Oliveira et al. 2010).

Insetos sugadores, em geral, se alimentam inserindo seu estilete em células do floema, não havendo acúmulo de substâncias de reserva nos tecidos das galhas (Bronner 1992). Entretanto, em alguns sistemas, principalmente na região neotropical, substâncias

de reserva têm sido detectadas no tecido das galhas induzidas por insetos sugadores. Galhas de *Lonchocarpus muelhbergianus* induzidas por *Euphalerus ostreoides* (Hemiptera) acumulam amido e lipídios nas células proximais à câmara ninfal (Oliveira et al. 2006). Álvarez et al. (2009) detectaram a presença de reserva de amido em diferentes galhas induzidas por Psyllidae em *Pistacia terebinthus*. Em outro sistema, *Aspidosperma australe* – *Pseudophacopteronidae*, a presença de amido ocorre nas células proximais à câmara larval, em um gradiente decrescente em direção ao córtex externo da galha (Oliveira & Isaias 2010). Desta forma, pressupõe-se a formação de um gradiente histoquímico e acúmulo de amido como padrão para galhas induzidas por insetos sugadores.

Independente do hábito alimentar do galhador, o metabolismo de carboidratos nas galhas pode exercer papel fundamental no desenvolvimento estrutural, na manutenção da maquinaria celular, e na sinalização, especialmente durante o estabelecimento do gradiente histoquímico. A atividade de duas enzimas, a sacarose sintase e as invertases, pode ajudar a compreender este metabolismo nas galhas. A sacarose sintase catalisa a quebra reversível da sacarose em frutose e UDP-glicose, participando principalmente de processos que envolvem a maturação de órgãos, acúmulo de amido e síntese de calose (Koch 2004, Asano et al 2002, Salnikov 2003, Subbaiah & Sachs 2001), além de diversos polissacarídeos de parede celular (Albrecht & Mustroph 2003, Doblin et al. 2002). Em *A. spruceanum*, a atividade da sacarose sintase foi detectada nos tecidos que apresentaram acúmulo de amido, principalmente nas galhas maduras, denotando sua importância na fase de maturação dos tecidos da galha (Oliveira & Isaias 2010, Oliveira et al. 2010). Em *A. spruceanum*, a sacarose sintase também pode estar relacionada à deposição de calose na parede do tecido nutritivo, facilitando a passagem de macromoléculas até as células proximais a câmara larval (Oliveira et al. 2010).

As invertases catalisam a reação irreversível da sacarose em glicose e frutose, sendo sua atividade associada ao estabelecimento de fortes drenos fisiológicos (Koch 1996, Koch & Zeng 2002, Rehill & Schultz 2003). A formação de sítios de divisão, expansão e respiração celular está associada à alta atividade das invertases (Koch 2004, Rehill & Schultz 2003). Os produtos da quebra da sacarose pela invertase participam da biossíntese e sinalização de hormônios, como o ABA,

AIA e citocininas (Koch 2004, Wachter et al. 2003). Bronner (1992) mostrou a importância da atividade desta enzima para o acúmulo de nutrientes no tecido nutritivo de galhas induzidas
por

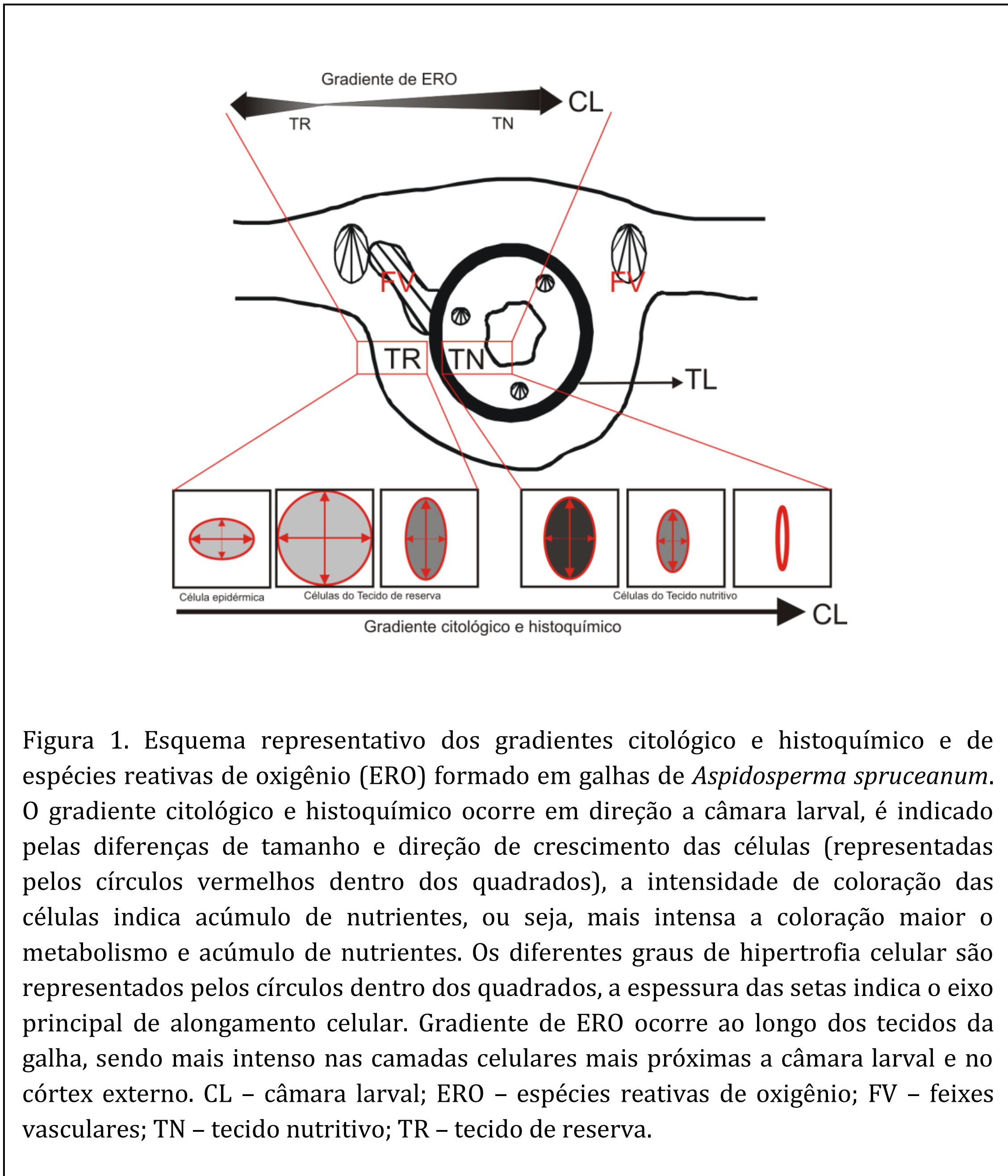

Figura 1. Esquema representativo dos gradientes citológico e histoquímico e de espécies reativas de oxigênio (ERO) formado em galhas de *Aspidosperma spruceanum*. O gradiente citológico e histoquímico ocorre em direção a câmara larval, é indicado pelas diferenças de tamanho e direção de crescimento das células (representadas pelos círculos vermelhos dentro dos quadrados), a intensidade de coloração das células indica acúmulo de nutrientes, ou seja, mais intensa a coloração maior o metabolismo e acúmulo de nutrientes. Os diferentes graus de hipertrofia celular são representados pelos círculos dentro dos quadrados, a espessura das setas indica o eixo principal de alongamento celular. Gradiente de ERO ocorre ao longo dos tecidos da galha, sendo mais intenso nas camadas celulares mais próximas a câmara larval e no córtex externo. CL – câmara larval; ERO – espécies reativas de oxigênio; FV – feixes vasculares; TN – tecido nutritivo; TR – tecido de reserva.

Cynipidae e Cecidomyiidae. Em galhas de *Aspidosperma spruceanum* induzidas por Cecidomyiidae, a atividade da invertase foi detectada nos tecidos próximos a câmara larval, sendo associada ao alto estresse oxidativo gerado pelo galhador (Oliveira et al. 2010). Em galhas de *A. australe* induzidas por *Pseudophacopteron*, a atividade da invertase foi relacionada a sítios de alto estresse respiratório e de divisão celular (Oliveira & Isaias 2010). Pela análise histoquímica comparativa das duas galhas, pode-se concluir que tanto a atividade da sacarose sintase como das invertases estão relacionadas ao estresse oxidativo gerado pelo galhador, podendo exercer função primordial no estabelecimento do gradiente histoquímico em galhas.

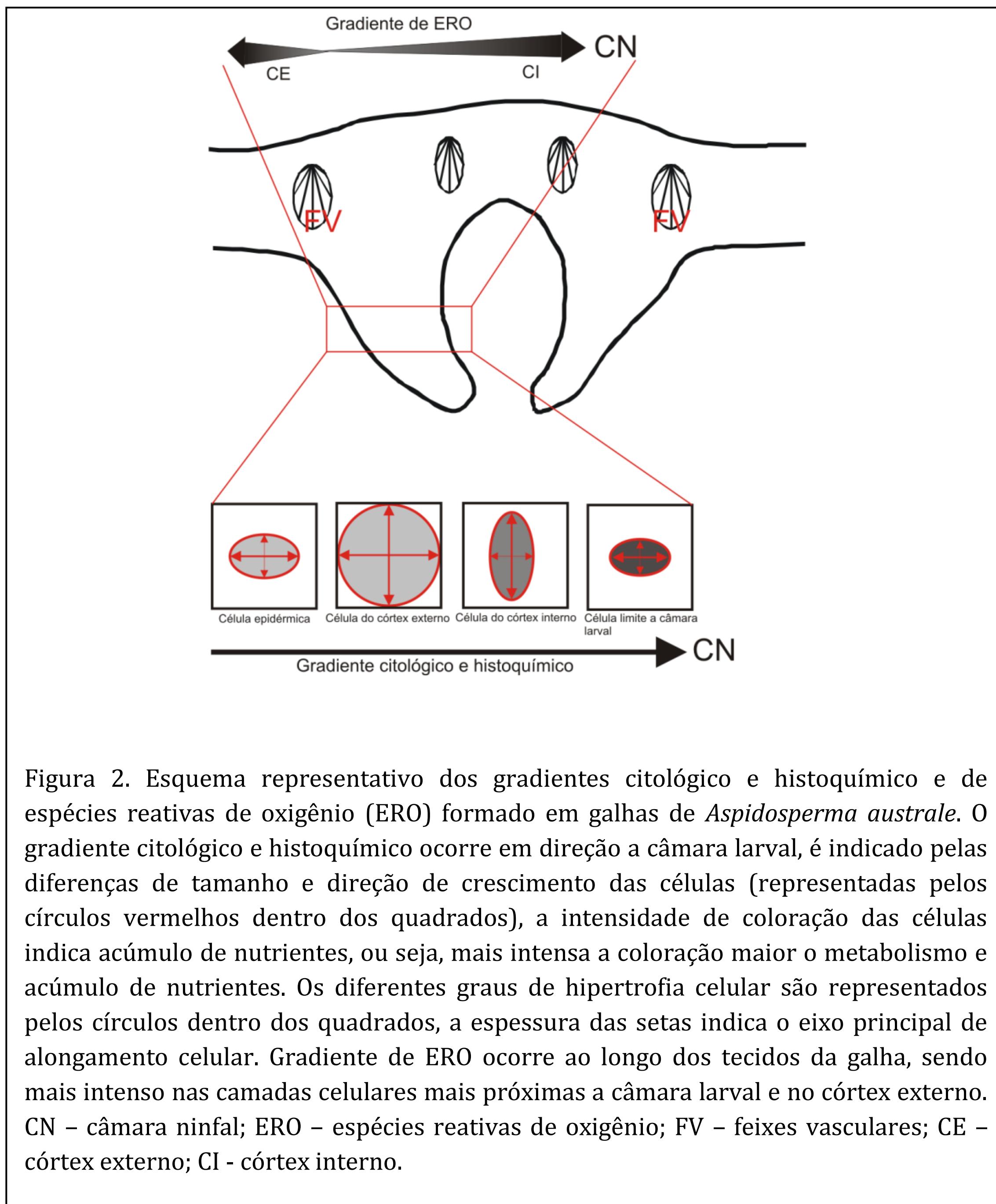

Figura 2. Esquema representativo dos gradientes citológico e histoquímico e de espécies reativas de oxigênio (ERO) formado em galhas de *Aspidosperma australe*. O gradiente citológico e histoquímico ocorre em direção a câmara larval, é indicado pelas diferenças de tamanho e direção de crescimento das células (representadas pelos círculos vermelhos dentro dos quadrados), a intensidade de coloração das células indica acúmulo de nutrientes, ou seja, mais intensa a coloração maior o metabolismo e acúmulo de nutrientes. Os diferentes graus de hipertrofia celular são representados pelos círculos dentro dos quadrados, a espessura das setas indica o eixo principal de alongamento celular. Gradiente de ERO ocorre ao longo dos tecidos da galha, sendo mais intenso nas camadas celulares mais próximas a câmara larval e no córtex externo. CN – câmara ninfal; ERO – espécies reativas de oxigênio; FV – feixes vasculares; CE – córtex externo; CI - córtex interno.

- **Formação e eliminação de espécies reativas de oxigênio (ERO) em galhas**

Os níveis de ERO em tecidos vegetais podem atuar como sinalizadores e agir diretamente no crescimento, desenvolvimento, respostas ao estresse e interações bióticas (Couée et al. 2006, Ryter & Tyrrel 1998, Mittler et al. 2004, Oliveira et al. 2010, Oliveira & Isaias 2010). Em galhas, a produção de ERO nos tecidos próximos a câmara larval ocorre

principalmente devido à alta atividade respiratória nas mitocôndrias. A grande quantidade de proteínas nestas camadas celulares e a alta atividade enzimática denotam o intenso metabolismo já detectado em diversos sistemas galhador-planta hospedeira (Bronner 1992, Schonrogge et al. 2000, Oliveira & Isaias 2010, Oliveira et al. 2010). Uma vez que a produção de ERO em excesso pode levar a morte celular (Moller et al. 2007), mecanismos que busquem a eliminação destes radicais são essenciais para os tecidos vegetais. Desta forma, a atividade de enzimas, tais como as invertases, pode desempenhar um papel importante na eliminação de ROS em galhas através da produção de açúcares solúveis nas camadas celulares mais internas, sítios de alto estresse oxidativo.

Os açúcares solúveis podem desempenhar um papel importante tanto nos mecanismos de eliminação de ROS quanto na sua produção (Couée et al. 2006). O metabolismo de açúcares e de esqueletos de carbono é essencial para a síntese de compostos que estão envolvidos na proteção anti-oxidante. Dentre os açúcares solúveis, a glicose atua como precursor inicial para síntese de carotenóides e de ascobarto entre outros aminoácidos, incluindo Cys, Glu e Gly, compostos envolvidos em mecanismos de defesa e eliminação de ROS (Couée et al. 2006, Pallet & Young 1993, Foyer 1993, Smirnoff et al. 2001). Assim, é plausível relacionar o acúmulo de açúcares solúveis em tecidos vegetais com mecanismos de defesa contra a ação prejudicial de ROS (Couée et al. 2006). Tendo em vista que o acúmulo de açúcares é um fato já descrito para diversas galhas (e.g. Bronner 1992) e devido ao envolvimento destes açúcares em mecanismos de eliminação de ERO, pode-se supor que, independente do modo de indução e de desenvolvimento, a armazenagem de açúcares nas células proximais à câmara larval seja uma resposta local a produção de ERO nas galhas. Ao contrário do córtex interno que é via de regra incolor, no córtex externo da galha, o estresse luminoso nos cloroplastos pode ser a principal fonte de ERO (Asada 1999, Moller et al. 2007, Oliveira et al. 2010, submetido).

Sob esta condição de estresse, os cloroplastos podem apresentar numerosos plastoglóbulos, corpúsculos tidos como geradores de um mecanismo para driblar os danos provocados pelas espécies reativas de oxigênio (Austin et al. 2007). Certas galhas, tais como as de *Aspidosperma australe* e *A. spruceanum* apresentam tecido clorofiliano com atividade fotossintética semelhante ao tecido não galhado. Nestas galhas, esta similaridade se deve, provavelmente, a presença de plastoglóbulos, um dos mecanismos de eliminação de ERO (Oliveira et al. 2010, submetido).

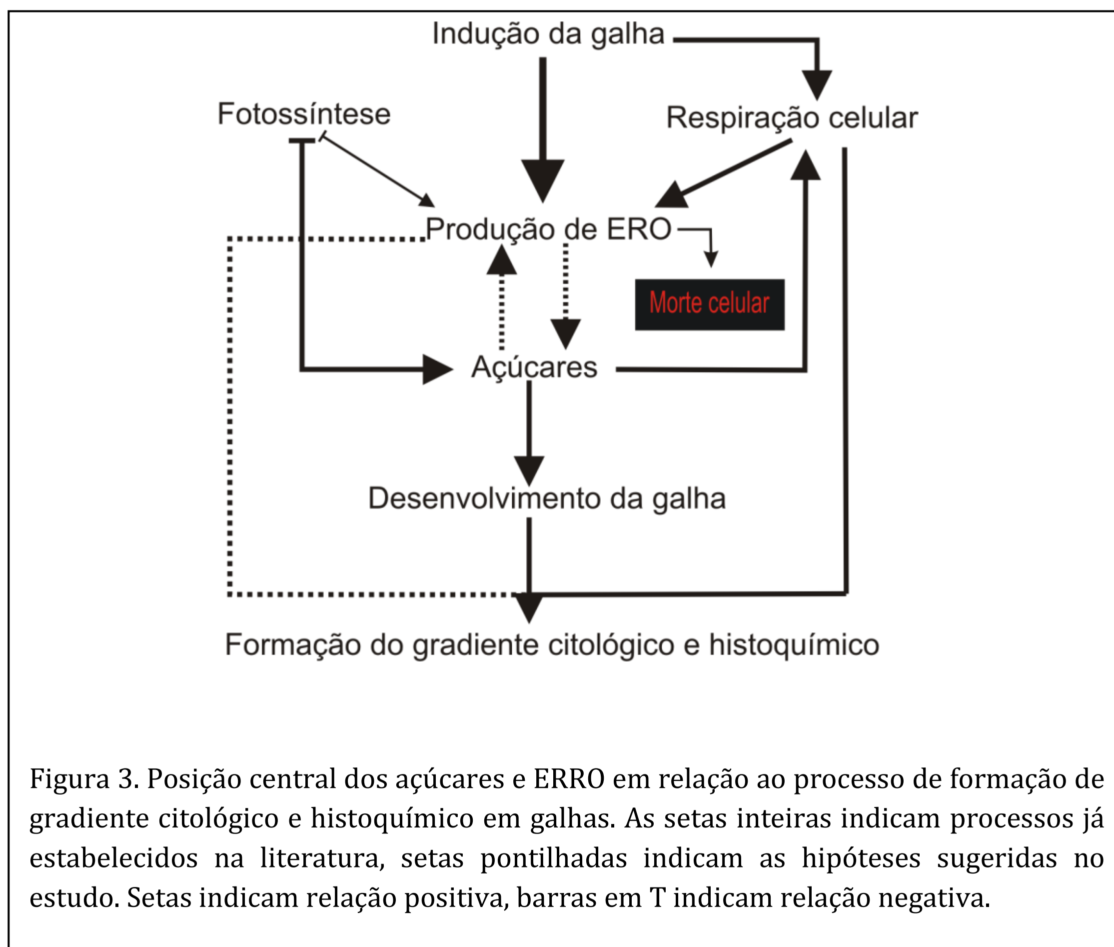

Figura 3. Posição central dos açúcares e ERRO em relação ao processo de formação de gradiente citológico e histoquímico em galhas. As setas inteiras indicam processos já estabelecidos na literatura, setas pontilhadas indicam as hipóteses sugeridas no estudo. Setas indicam relação positiva, barras em T indicam relação negativa.

Plastoglóbulos são corpúsculos de natureza mista produzidos por plastídios que podem conter tanto lipídios quanto proteínas e cuja função está associada à reserva de componentes moleculares e à recuperação do sistema de membranas dos tilacóides (Lichtenthaler 1968, Kessler et al. 1999, Vidi et al. 2006, Ytterberg et al. 2006, Hopkins et al. 2007). Seu envolvimento com a síntese e armazenamento de plastoglobulinas e moléculas como tocoferol tem sido relacionado a propriedades antioxidantes que protegem as membranas de foto-oxidação e o PSII de fotoinativação (Bréhelin et al. 2007, Havaux et al. 2005). De fato, a atividade fotossintética e o acúmulo de açúcares têm sido relacionados à expressão de genes envolvidos na remoção do excesso de fótons, síntese de flavonóides, ou em defesas contra ROS, principalmente na síntese da superóxido dismutase (Koch 1996, Rossel et al. 2002, Moller et al. 2007).

Conclusões e Perspectivas

A formação do corpo final da galha, e conseqüentemente a grande diversidade de formas encontradas na natureza, depende do impacto mecânico ou químico que o galhador causa nos tecidos vegetais, e como estes tecidos vão reagir a esta injúria. De forma geral, as galhas apresentam um gradiente histológico em direção a câmara larval ou ninfal, ou seja, as células do córtex externo da galha são maiores em relação às células mais internas. Este gradiente acompanha o gradiente histoquímico, as células proximais a câmara larval acumulam qualitativamente mais substâncias, são metabolicamente mais ativas e estão sujeitas à um maior estresse oxidativo. A produção de espécies reativas de oxigênio durante todo o processo de formação da galha pode ser o fator fundamental para o estabelecimento da interação e formação dos gradientes citológico e histoquímico. Uma vez que a forma das células reflete a forma e a função dos tecidos e órgãos, percebe-se um gradiente funcional estabelecido pela formação de ERO o qual não somente é responsável pela diferenciação de cada morfotipo de galhas, mas pode, além disso, conferir vantagens aos insetos galhadores.

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Cytological and histochemical gradients induced by a sucking insect in galls of *Aspidosperma australe* Arg. Muell (Apocynaceae)

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Cytological and histochemical gradients induced by a sucking insect in galls of *Aspidosperma australe* Arg. Muell (Apocynaceae)

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ABSTRACT

The storage of carbohydrates and lipids was previously investigated in nutritive tissues of galls of Cecidomyiidae and Cynipidae. Unexpectedly, starch accumulation has been detected in non-nutritive galls induced by Hemiptera, which feed directly from phloem bundles. Samples of non-galled leaves and galls induced by *Pseudophacopteron* sp. in *Aspidosperma australe* were processed for light and electron microscopy. Histochemical tests detected sites of ROS (reactive oxygen species), carbohydrates, and enzymes. PCD (programmed cell death) evidenced by plastoglobules and ROS formation also occurred. Phosphorylase and sucrose synthase activity indicated the steps of starch storage. The sites of glucose-6-phosphatase activity were related to the provision of sucrose for gall growth and nutrition of *Pseudophacopteron*. Acid phosphatase took part in the metabolism of starch and degradation of some organelles during the main trophic phase of the insect. The invertases were related to the sites of hyperplasia, and regulation of cell growth and intense respiration. The cytological and histochemical gradients validate the storage of starch as a pattern in galls induced by sucking insects. The detection of enzymes related to carbohydrate metabolism and sites of ROS production is described for the first time for galls induced by sucking insects in the Neotropical region.

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1. Introduction

Insect galls commonly store substances that provide nourishment to the gall inducer, and take part in regulating the morphogenesis of the gall itself. These substances are located in specialized cells [1]. Lipids accumulate in the galls induced by Cynipidae; whereas carbohydrate accumulation prevails in galls induced by Cecidomyiidae [1]. Variations in these patterns may occur, as demonstrated by the detection of lipid droplets in nutritive tissues of galls induced by *Schismatodiplosis lantanae* (Cecidomyiidae) in *Lantana camara*, a lipid-producing Verbenaceae [2]. This may be evidence that the storage of lipids and carbohydrates is potentially constrained by the host plant metabolism. Studies of cellular differentiation and histochemical detection of reserve substances in galls induced by insects with sucking feeding habits are few, especially in the Neotropical region, and so, to the best of our knowledge, no pattern of reserve metabolism has been proposed for these gall systems.

Bronner [1] used histochemical techniques to propose reserve metabolism patterns in Cynipid and Cecidomyiid galls. Histochemical techniques are precise methods of localizing metabolites, and consequently of assessing the metabolism patterns of plant cells related to the nutrition of galling herbivores. Moreover, histochemical and cytological techniques may diagnose some stresses generated by the presence of the gall inducer within plant tissues [3,4], as well as the impacts of their different feeding habits. Among the host plant's responses to oxidative and respiratory stresses is the generation of reactive oxygen species (ROS) with the breakdown of membrane systems in chloroplasts and mitochondria, as well as the formation of plastoglobules [5,6]. The generation of ROS in gall tissues can be easily assessed by cytological analysis and confirmed by histochemical techniques [7].

Sucking gall-inducing insects are common in the Neotropical fauna [8], and their feeding site may be restricted to phloem cells where they introduce their stylets. This behavior may cause the deposition of "wound callose", which forms a physical barrier at the plasma membrane, and consequently may be the first step in the plant's reaction to the presence of the herbivore [9]. In the *Pseudophacopteron* sp. (Hemiptera)–*Aspidosperma australe* (Apocynaceae) gall system, due to the use of phloem as the feeding site [10], a nutritive tissue should be absent, and the storage of nutritional reserves and the enzyme activity should be related to maintaining the gall structure, as proposed by Oliveira et al. [11]

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for the *Euphalerus ostreoides–Lonchocarpus muehlbergianus* gall system. Because of the amplitude of the cecidogenic field [12,13], the highest metabolic activity should be expected in the tissues near the nymphal chamber, where respiratory and oxidative stresses are greater. These assumptions should be confirmed by cytological and histochemical analyses.

By studying the cytological and histochemical features of the *Pseudophacopteron* sp.–*A. australe* gall system, this investigation aimed to answer the following questions: (i) does the feeding activity of *Pseudophacopteron* sp. alter the storage of metabolites in its host plant? (ii) Is there a gradient of storage substances and enzymes in *A. australe* gall tissues? (iii) Do gall cytological features indicate oxidative stress? (iv) Do galls induced by *Pseudophacopteron* sp. in *A. australe* follow the patterns described in the literature for sucking gall inducers?

2. Materials and methods

2.1. Plant material collection

Tissue samples of non-galled leaves ($n \geq 12$) and of galls in three developmental stages, sorted by size, (immature galls 1.0 ± 0.3 mm wide, mature galls, 5.0 ± 0.5 mm wide, and senescent galls, 5.0 ± 0.7 wide and open) ($n \geq 12$ per stage), were collected from *A. australe* individuals ($n = 10$) located on the Pampulha campus of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. The collections were made from January through December 2008, at intervals of 2 months, and the samples obtained were submitted to cytological and histochemical analyses.

2.2. Cytological analysis

The samples were fixed in 4% Karnovsky in 0.1 M phosphate buffer (pH 7.2) for 24 h [14], post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2), dehydrated in an ethanol series [15], and embedded in Araldite® [16]. The material was cross-sectioned in an Ultramicrotome Reichert-Jung – Ultracut, contrasted in uranyl acetate and lead citrate according to Reynolds [17], and examined in a Zeiss EM 109 transmission electron microscope.

2.3. Histochemical analysis

2.3.1. Nucleic acids

Samples of non-galled leaves and mature galls were fixed in Carnoy for 48 h, dehydrated in an ethanol series, embedded in Leica® historesin, and cross-sectioned (5–10 μm) in a Reichert Jung® rotating microtome [15]. The material was stained with 0.15% methyl green and 0.25% pyronin B in acetate buffer (pH 4.7) [18]. For the controls, RNA and DNA were extracted by incubating the slides in 0.1% ribonuclease (pH 6.8) for 1 h at 40 °C, and in 0.2 mg deoxyribonuclease ml^{-1} in 0.003 M magnesium sulphate (pH 6.5) for 1 h at 25 °C, respectively.

2.3.2. Primary metabolites

Handmade sections of fresh material were used for detection of proteins, lipids, starch, and reducing sugars. For proteins, the sections were stained in 0.1% bromophenol blue in a saturated solution of magnesium chloride in ethanol for 15 min, and then washed in acetic acid and water [19]. For starch detection, the sections were immersed in Lugol reagent (iodine potassium iodide) for 15 min [15]. For reducing sugars, sections were immersed in Fehling reagent [20]. For lipids, the sections were immersed in a saturated solution of Sudan III (CI 26100) in 70° GL ethanol [21]. For the controls, lipids were extracted with methanol:chloroform (1:1, v/v), and starch with salivary amylase. Blank sections were used for comparative analysis.

2.3.3. Callose

The samples were fixed in FAA for 48 h, washed in distilled water followed by 70% ethanol, and stained in 0.1% aniline blue (pH 9.5) for 10 min [15]. The material was mounted with the stain and observed in a scanning confocal laser microscope (Zeiss, LSM 510, Germany). The controls were treated in the same manner but without the stain.

2.3.4. Enzyme activity

Sections of fresh material were immersed in the appropriate detection solutions for acid phosphatase, phosphorylase, glucose-6-phosphatase, invertase, and sucrose synthase. For the detection of acid-phosphatase activity, the sections were incubated 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 (25 °C). Subsequently, sections were washed in distilled water and immersed in 1% ammonia for 5 min [22]. For the control, the samples were incubated in the same solution without potassium sodium glycerophosphate. For the detection of phosphorylase activity, the sections were incubated in 1% glucose-1-phosphate in 0.1 acetate buffer (pH 6.0) for 2 h at room temperature [23]. After the incubation, the sections were immersed in Lugol reagent [15]. For the control, samples were not incubated in glucose-1-phosphate. For the detection of glucose-6-phosphatase activity, the sections were incubated in a solution containing 20 mg of potassium glucose-6-phosphate in 125 ml of 0.2 M Tris–maleate buffer (pH 6.7), and 3 ml of 2% lead nitrate in 7 ml of distilled water, for 15 min to 2 h, at 37 °C. Following the incubation, the material was washed in distilled water, immersed in 1% ammonium sulphate for 5 min, and mounted in glycerin jelly [23]. For the control, the samples were not incubated in potassium glucose-6-phosphate. For the detection of invertase activity, the sections were incubated in a neutral reaction buffered medium containing 0.38 mM sodium phosphate (pH 7.5), 0.024% tetrazolium blue, 0.014% phenazin metasulphate, 30 U of glucose oxidase, and 30 mM of sucrose at room temperature for 3 h [24,25]. For the control, an incubation medium without sucrose was used. For detection of sucrose synthase (SuSy) activity, handmade sections of fresh samples and of samples fixed in 2% paraformaldehyde with 2% polyvinylpyrrolidone 40 and 0.005 M dithiothreitol were placed in incubation medium for 30 min, at 30 °C. The incubation medium contained 5 μl of 150 mM NADH, 5 μl (1 U) of phosphoglucomutase from rabbit muscle, 5 μl of 3 mM glucose-1,6-biphosphate, 5 μl (1 U) of glucose-6-phosphate dehydrogenase from *Leuconostoc*, 5 μl (1 U) of UDPG–pyrophosphorylase from beef liver, 280 μl of 0.07% aqueous nitro-blue tetrazolium (NBT), 350 μl of buffer, and 50 μl of substrate. The buffer consisted of 100 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 0.2% BSA, and 2 mM EGTA at pH 7.4. The substrate contained 0.75 M sucrose, 15 mM UDP, and 15 mM pyrophosphate. For one of the controls, glucose-1,6-biphosphate and pyrophosphate were not used. In a second control, sucrose was not used [26].

2.3.5. Reactive oxygen species (ROS)

For DAB (3,3′-diaminobenzidine) staining, handmade sections of fresh material were immersed in 0.5% DAB (Sigma®) solution for 20–60 min, in the dark [7]. The intensity of the reaction was examined every 15 min.

3. Results

3.1. General features

The leaf gall induced by *Pseudophacopteron* sp. in *A. australe* forms a slight projection of the adaxial surface and a more prominent projection of the abaxial surface of its host leaf (Fig. 1a

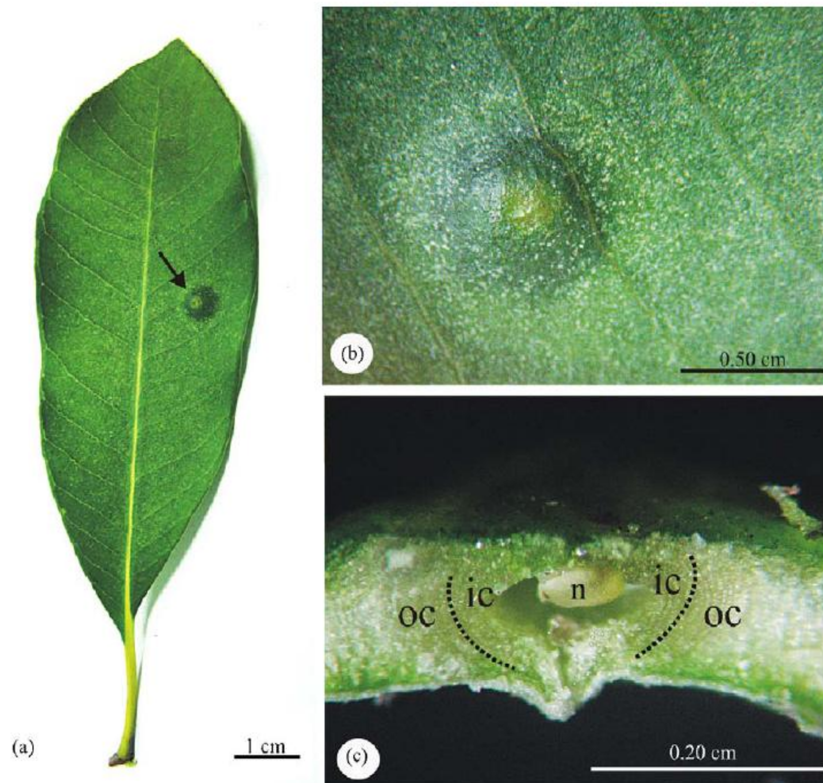

Fig. 1. (a) Leaf of *Aspidosperma australe* showing gall induced by *Pseudophacopteron* sp. (arrow). (b) Detail of gall projection. (c) Cross-section showing nymphal chamber, inner cortex (ic), outer cortex (ec), and nymph (n).

and b). The nymphal chamber is central, and shelters one individual of *Pseudophacopteron* sp. from its first instar until the adult phase (Fig. 1c). The gall is parenchymatic, with vascular tissues only at the top of the chamber, which is permanently opened to the abaxial leaf surface. This gall morphotype has two distinct tissue zones, an inner cortex, around the nymphal chamber, and an outer cortex with larger cells (Fig. 1c).

3.2. Cytological features

The cells of the non-galled mesophyll have small nuclei, thin cytoplasm, large vacuoles, chloroplasts with numerous primary starch grains, and plastids associated with mitochondria (Fig. 2a and b). The palisade parenchyma cells contain osmiophilic inclusions (Fig. 2c).

In immature galls, the cells near the nymphal chamber have lobed or large nuclei surrounded by numerous mitochondria associated with plastids and abundant starch grains (Fig. 2d and e). In the inner cortex, cells with transverse sinuous and thin cell walls indicate sites of hyperplasia (Fig. 2e). These cells have endoplasmic reticulum with electron-dense granules and oleosomes associated with their chloroplast thylakoids (Fig. 2f). Osmiophilic inclusions (Fig. 2d) can be observed throughout the cells over the entire gall cortex, but they concentrate in the cells around the nymphal chamber.

In mature galls, sites of hyperplasia are observed (Fig. 3a) in both the outer and inner cortex. The cells around the nymphal chamber have dense and hypertrophied nuclei, conspicuous nucleoli (Fig. 3b), and a developed vacuome (Fig. 3c). The chloroplasts of the outer cortex cells have abundant grouped and peripheral plastoglobules, and are associated with mitochondria (Fig. 3d). After the eclosion of the adult of *Pseudophacopteron* sp., in the senescent gall, the parenchyma cells of the outer and inner cortices enter cell death and degenerate. The nuclei and the organelles of the cells immediately surrounding the nymphal chamber are encapsulated and degenerate (Fig. 3e and f). These cytological events proceed towards the gall outer cortex.

3.3. Histochemical analyses

The non-galled and the galled tissues of *A. australe* in different stages of development are histochemically distinct in their qualitative aspects as well as in the sites of reaction (Table 1).

DNA was detected by red staining of the nuclei in both galled and non-galled tissues. In non-galled tissues, the DNA staining was detected in the nuclei of the cells of the palisade parenchyma. In mature galls, DNA staining was detected in the hypertrophied

Table 1
Histochemical tests in non-galled and galled tissues of the *Aspidosperma australe*–*Pseudophacopteron* sp. gall system.

Tests	Non-galled tissues	Galls		
		Young	Mature	Senescent
DNA	+	N.E.	+	N.E.
RNA	–	N.E.	+	N.E.
Proteins	+	+	+	+
Starch	+	+	+	+
Sugars	+	–	+	+
Lipids	+	+	+	–
Callose	–	–	–	–
Acid phosphatase	–	+	+	+
Phosphorylase	–	+	+	–
Glucose-6-phosphatase	–	+	+	–
Invertase	+	+	+	–
Sucrose synthase	+	+	+	–
ROS	+	+	+	+

+ = positive detection; – = negative detection; N.E. = not evaluated.

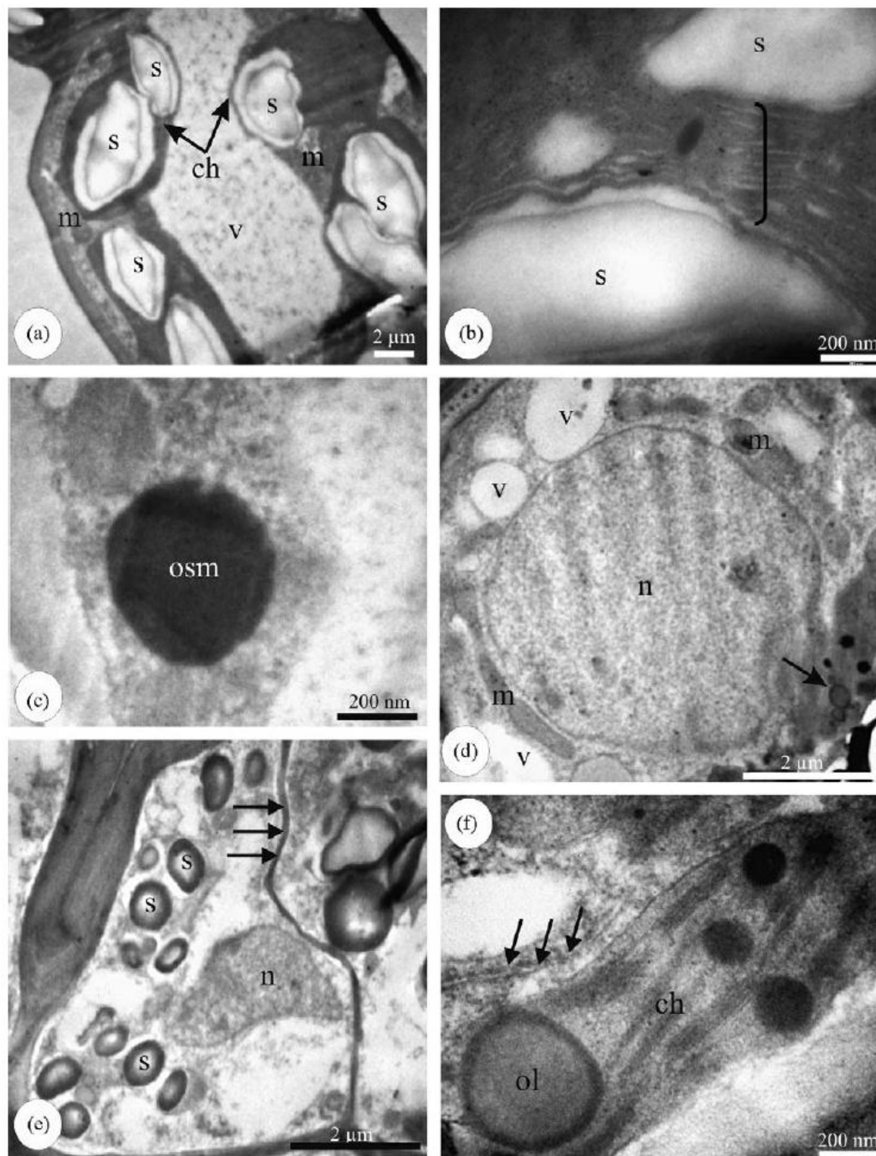

Fig. 2. Transmission electron micrographs of non-galled and galled tissues of *Aspidosperma australe*. (a–c) Non-galled leaf tissues. (a) Plastids of a mesophyll cell containing starch grains. (b) Detail of chloroplast starch grain and thylakoid membrane. (c) Detail of osmiophilic inclusions. (d–f) Immature galls. (d) Cell of the inner cortex tissue layer containing plastids with lipid droplets, numerous mitochondria, hypertrophied nucleus, and small vacuoles. (e) Detail of a cell of the inner cortex with a large lobed nucleus, and numerous plastids with starch grains. (f) Detail of a chloroplast with oleosome and associated plastoglobules. ch, chloroplast; osm, osmiophilic inclusion; m, mitochondria; n, nucleus; nu, nucleolus; ld, lipid droplet; s, starch; v, vacuole.

nuclei of the cells surrounding the nymphal chamber. RNA activity was also detected in the nucleoli of these cells (Fig. 4a).

In non-galled tissues, the histochemical reaction to proteins was more intense in the palisade parenchyma cells. In immature and mature galls, a centripetal gradient of proteins was established (Fig. 4b), but in senescent galls, proteins are equally distributed. Starch was detected in the palisade parenchyma cells of the non-galled mesophyll. In the cortices of the immature and mature galls, starch grains formed a centripetal gradient. In the mature galls, starch grains were sparse in the cells of the adaxial portion of the gall outer cortex (Fig. 4c), but during senescence, they dispersed throughout the gall cortex. Reducing sugars were detected in the cells next to the vascular bundles of the non-galled tissues, and of the mature (Fig. 4d) and senescent galls. Small lipid droplets were observed in the cells of the palisade parenchyma of the non-galled mesophyll. In both immature and mature galls, these droplets were only detected in the cells of the vascular parenchyma (Fig. 4e). The reaction for callose was negative for all of the samples.

Acid-phosphatase activity was not detected in the non-galled tissues, but was detected in the galls in all developmental stages. This enzyme activity was more intense in the cells surrounding the nymphal chamber of the mature galls (Fig. 4f). During senescence, the gradient of this enzyme activity extended to the cells of the gall outer cortex. Phosphorylase and glucose 6-phosphatase activity was not detected in the non-galled tissues or in the senescent galls, but in the immature and mature galls, the activity of these enzymes was detected in the cells of the inner cortex, next to the nymphal chamber (Fig. 5a). Slight invertase activity was detectable in the cells around the vascular bundles of the non-galled tissues. In the immature, mature, and senescent galls, the activity of this enzyme was detected by the formation of a salt of nitrous blue tetrazolium in the cytosol (Fig. 5b). The reaction was more intense

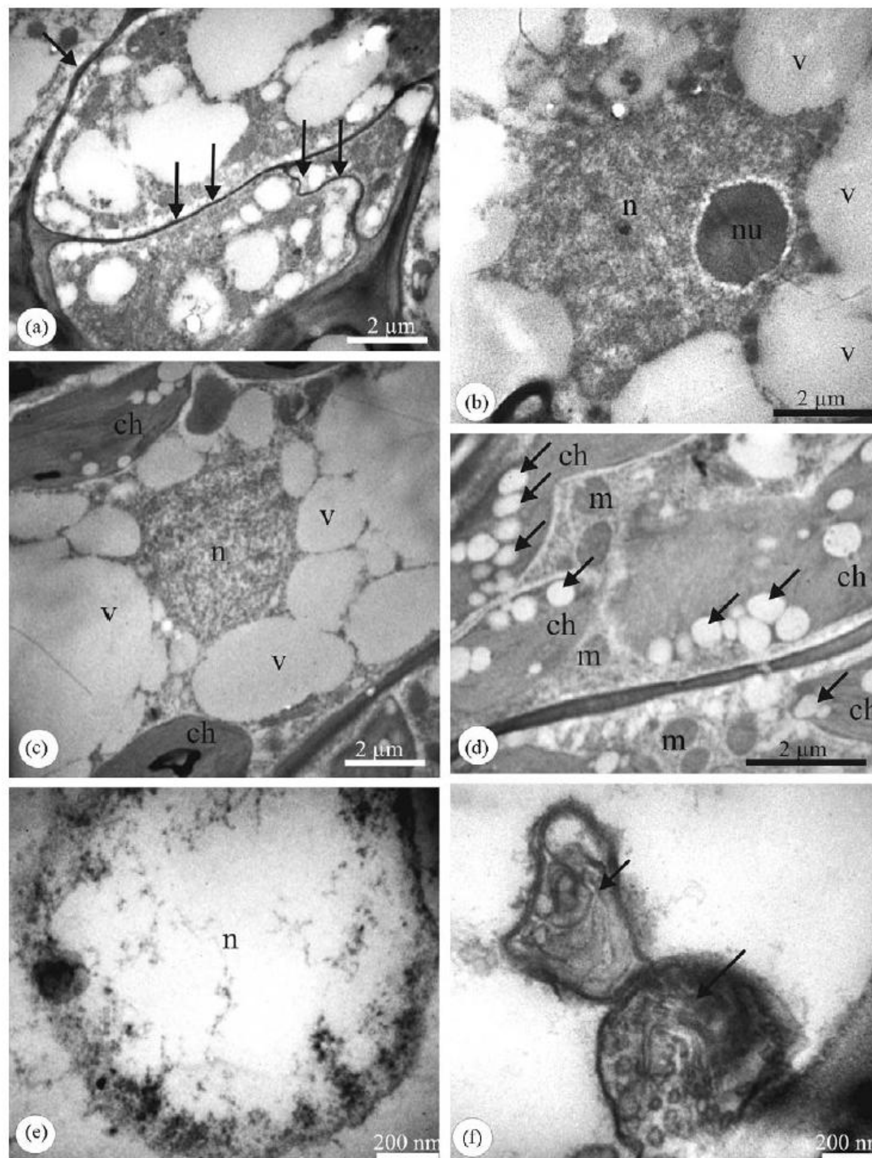

Fig. 3. Transmission electron micrographs of galled tissues of *Aspidosperma australe*. (a–d) Mature galls. (a) Cells of the outer cortex with thin sinuous cell walls (arrow), indicating a site of hyperplasia. (b and c) Cells near the nymphal chamber. (b) Hypertrophied nucleus containing evident nucleolus. (c) Intense vacuome, hypertrophied nucleus, and chloroplast with plastoglobules (arrow). (d) Plastoglobules (arrow) in cells of the outer cortex, with associated mitochondria. (e and f) Senescent galls. (e) Nucleus of a cell near the nymphal chamber, in the final stage of degradation. (f) Plastid encapsulation and degradation (arrow) in cells of the outer cortex. ch, chloroplast; m, mitochondria; n, nucleus; nu, nucleolus; s, starch; v, vacuole.

in the cells of the gall inner cortex, next to the nymphal chamber, and became less intense outwards, in a centripetal gradient. Marked sucrose synthase activity was detectable in the cells of the vascular bundles in non-galled tissues. In the immature and mature galls, this activity was localized in the cells of the vascular bundles next to the adaxial surface and in the cells surrounding the nymphal chamber (Fig. 5c and d). The gradients of sucrose synthase and invertase were similar to those of proteins and starch in the galled tissues.

The positive reaction to DAB appeared as a diffuse brownish color, and revealed the formation of hydrogen peroxide, among other free radicals. The reactive oxygen species (ROS) were detected in the cells of non-galled mesophyll. In the immature, mature, and senescent galls, the ROS were more concentrated in the cells of the inner cortex, next to the nymphal chamber (Fig. 5e and f), and became less intense outwards. Also, ROS was detected in chlorophyll parenchyma outwards from the galled tissues.

The gradients of the reserve substances and enzyme activities varied during the development of the galls of *A. australe* induced by *Pseudophacopteron* sp., and were established in the mature galls (Fig. 6).

4. Discussion

4.1. Cytological gradients and oxidative stress

Most hemipteran galls are relatively simple, both morphologically and anatomically [27]. Cytological, histochemical, and physiological analyses in gall tissues have revealed important differences from non-galled tissues [28,21,4,11], and have been commonly related to the feeding habits of the gall-inducing insects [27]. However, the storage of substances in the gall site may also be subject to constraints imposed by the metabolism of the host plant.

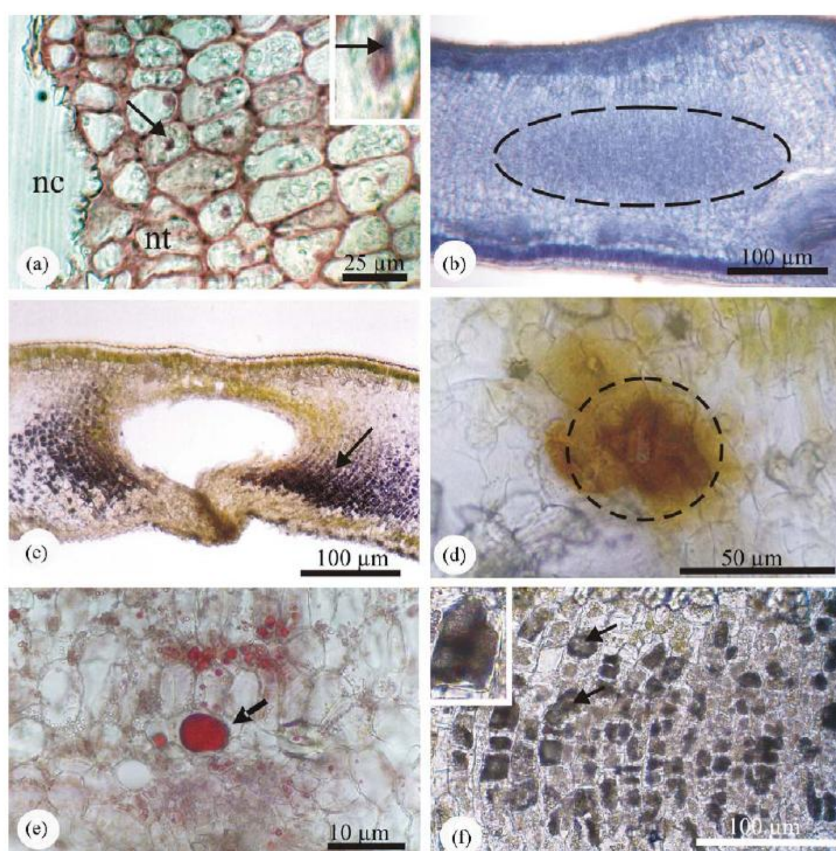

Fig. 4. Histochemistry of mature galls of *Aspidosperma australe*. (a) Test for the detection of DNA activity indicated by the red staining of nuclei, and RNA activity by the blue staining of nucleoli (arrow) near the nymphal chamber. (b) Protein gradient increasing towards nymphal chamber, indicating high metabolic activity. (c) Starch gradient (arrow) increasing towards the nymphal chamber on the abaxial surface. (d) Reducing sugars detected next to a vascular bundle located in the gall outer cortex. (e) Lipid droplets in cells of the vascular parenchyma in the gall outer cortex. (f) Phosphatase activity, detected mainly in the innermost layers of the gall tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In the immature galls induced by *Pseudophacopteron* sp. in *A. australe*, the cells surrounding the nymphal chamber are redifferentiated from chlorophyll parenchyma, which may confer physiological youth on the inner cortical cells of the gall. In the mature galls, the cytological features concord with Bronner's observation [1] for nutritive tissues of galls induced by Diptera: Cecidomyiidae, although *Pseudophacopteron* sp. is an hemipteran. This seems to be a novelty in studies on the development of galls induced by sucking insects, and has been detected for the first time in the Neotropical flora. Indeed, comparative studies on the histochemistry of immature and mature galls are not commonly performed. Moreover, during gall maturation, cellular redifferentiation is completed, and the synthesis of ROS seems to be increased. A cytological diagnostic feature of the ROS production and consequent oxidative stress in the galls of *A. australe* is the formation of plastoglobules in the chloroplasts. According to Rosseti and Bonatti [7] and Zentgraf [5], one of the early events activated by the hypersensitive response (HR) is the production of ROS, including hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-). The HR is a mechanism employed by plants to counteract pathogen growth by causing a localized cell death [29]. The positive reaction to DAB indicates the sites of ROS production, and is also a strong indication of the localized response to gall induction in *A. australe*. This response also leads to a disruption of membrane systems and consequently to cell death in the gall senescent phase. During the processes of senescence, thylakoid membranes are the first to degrade, followed by mitochondria membranes and the chloroplast envelope [30]. In the galls of *A. australe*, these symptoms are the latest cytological events under the influence of the cecidogenetic field, and occur just after the eclosion of the imago.

4.2. Histochemical gradients

The chemical impact caused by the feeding activity of *Pseudophacopteron* sp. results in the storage of primary metabolites, and is also under the direct influence of the cecidogenetic field. This storage of metabolites indicates the differentiation of a nutritive tissue, which is not typical of psyllid galls but has been previously described in two hemipteran galls in *Picea excelsa* [31]. Also, the redifferentiation of parenchyma cells may allow the establishment of the larvae without provoking hypersensitive reactions [27].

The high metabolic activity detected in the cells of the inner cortex is strongly influenced by the feeding activity of the insect, as stated by Bronner [1] and Schönrogge [4]. Distinct histochemical gradients were established in the galls of *A. australe* as products of metabolic changes induced by *Pseudophacopteron* sp., and must be more related to the growth and development of the gall structure than to the feeding requirements of the nymph. Moreover, the gradients of reserve substances and enzymes can be associated with the stresses generated by the presence of the parasitic life form within the host plant tissues.

In galls induced by Hymenoptera: Cynipidae in *Quercus* sp. and *Rosa* sp., large amounts of proteins were found in the innermost

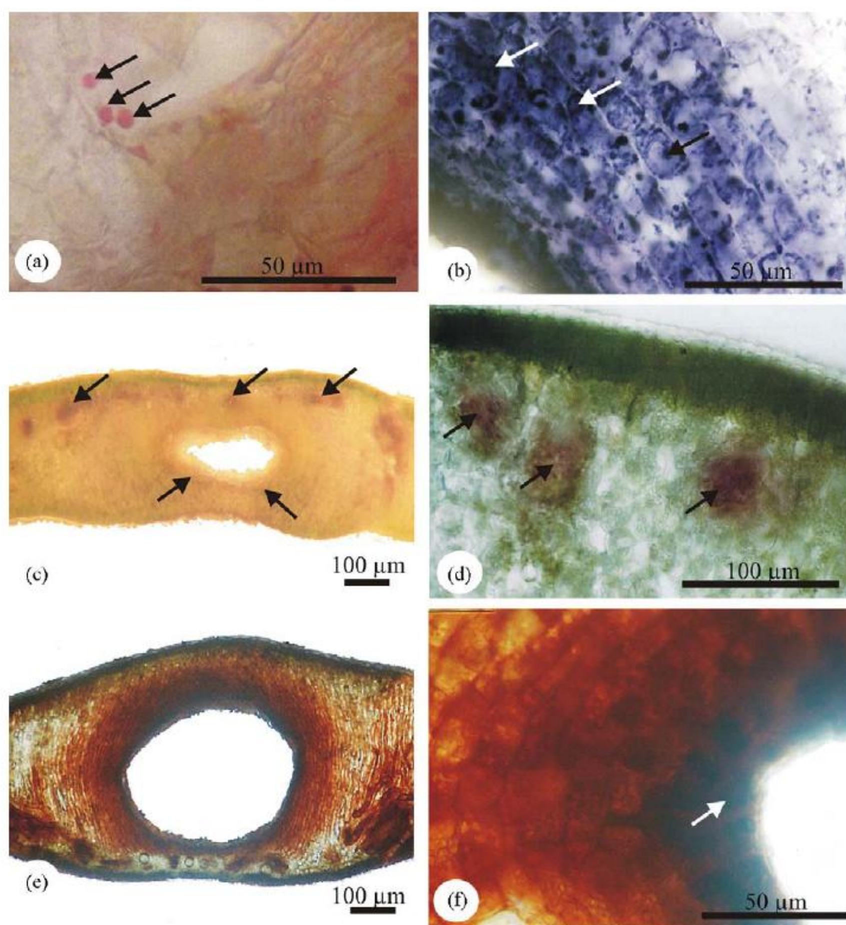

Fig. 5. Histochemistry of mature galls of *Aspidosperma australe*. (a) Phosphorylase activity detected in the cells of the outer cortex. (b) Invertase detected in an increasing gradient towards the nymphal chamber. (c and d) Sucrose synthase detected in vascular bundles (arrows) and at the abaxial cortex around the nymphal chamber. (e and f) ROS detected in cells surrounding the nymphal chamber (white arrow) in a decreasing gradient towards the gall outer cortex.

tissues, and in *Quercus* sp., a specific protein, format dehydrogenase (FDH), was expressed in correlation with the general protein gradient [4]. According to these authors, FDH is an indication of respiratory stress in the tissues surrounding the larval chamber. Similarly, the protein gradient found in the tissues of the mature galls of *A. australe* may indicate differential expression of proteins and must be related to high respiratory stress, mainly next to the nymphal chamber, and tends to disappear in the senescent phase. This observation is reinforced by the RNA activity, and the high production of ROS in the cells of the gall inner cortex. ROS production increased in the senescent phase.

The concentration of starch in the galls of *A. australe* is visually higher in the cells surrounding the nymphal chamber. In general, it is possible to identify a starch-free nutritive tissue in galls of Diptera: Cecidomyiidae, which accumulate in their outer cortex [1]. However, in galls of *Lonchocarpus muehlbergianus* induced by *E. ostreoides* (Hemiptera), starch reserves were also detected throughout the gall cortex, even in the cells surrounding the chamber [11]. Álvarez et al. [31] detected the storage of starch in three galls induced by different species of Psyllidae in *Pistacia terebinthus*. Therefore, it seems plausible to assume that, independently of the species of host plant, starch grains accumulate in non-nutritive galls induced by sucking insects.

The storage of lipids in the gall outer cortex may be inherent to the metabolism of *A. australe*, for in this host plant this metabolite is commonly detected in laticifers associated with the vascular bundles [32]. These substances have also been detected in other systems, such as *L. muelhbergianus–E. ostreoides* [11] and *L. camara–Aceria lantanae* [2]. In these systems, the presence of lipids were related to the maintenance of the gall structure, because lipids are molecules with high reserve capacity and energy, and are possible precursors of important components of plant metabolism [33].

4.3. Histochemistry of enzymes

Another intriguing and little-explored issue in galling insects–host plant systems is the demonstration of how storage substances become available for both the development of the gall structure and the feeding behavior of the galling insect. The histochemistry of some enzymes related to starch metabolism was first investigated by Bronner [1], and has never been assessed for galls in the Neotropical region.

Because starch is not directly used either for the feeding activity of *Pseudophacopteron* sp. or the development of gall structure, some enzyme activity related to carbohydrate metabolism is expected to occur in gall tissues. Phosphorylase is an important enzyme responsible for the polymerization and/or the breakdown of starch molecules. In general, there is a correlation between the activity of this enzyme and the amount of accumulated amyloplasts; therefore this correlation depends on the physiological status of the plant tissue [34]. This status may also differ within the same gall, making this model of study particularly intriguing. Phosphorylase activity was detected in the outer cortex of

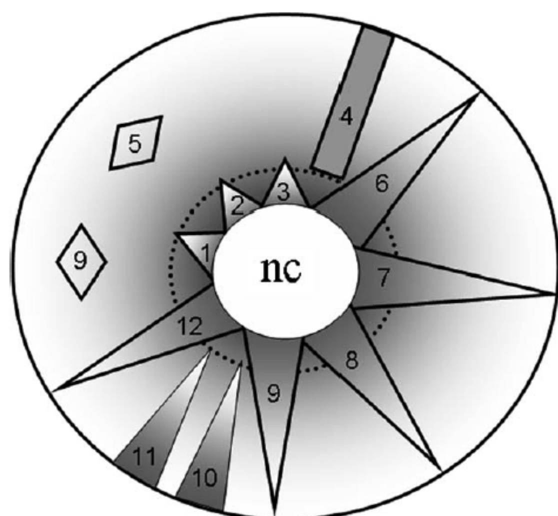

Fig. 6. Representative diagram of the histochemical gradients detected in mature galls of *Aspidosperma australe*. Arrow direction and intensity of gray color indicate histochemical and cytological gradients, respectively. DNA (1), RNA (2), and acid-phosphatase activities (3) are more intense and exclusively detected in the cells next to the nymphal chamber. Lipids (4) and reducing sugars (5) are detected exclusively in the gall outer cortex. Proteins (6), starch (7), and invertase (8) activity become more concentrated and more intense closer to the nymphal chamber. Sucrose synthase (9) activity is more intense near the nymphal chamber and vascular bundles. For glucose 6-phosphatase (10), and phosphorylase (11), the gradients are centripetal. The gradient of ROS-reactive species of oxygen (12) is more intense centrifugally.

immature and mature galls of *A. australe*, and was interpreted as an indication of the primary steps of starch storage in the gall tissues. The newly formed starch grains, stained red, could be distinguished from pre-existing ones, which stained blue.

Another enzyme investigated was glucose-6-phosphatase because of its involvement in the synthesis of intermediate compounds during the formation of sucrose [35]. This enzyme activity may be associated with the formation of sucrose after the breakdown of starch molecules, to provide resources for gall growth and the nutritional requirements of *Pseudophacopteron* sp.

The conversion of the products of starch metabolism into glucose and fructose, by the action of invertase, is quite important next to the nymphal chamber, a site of high stress and respiratory metabolism. In addition to catalyzing the irreversible breakdown of sucrose into glucose and fructose, the invertases take part in the biosynthesis of ABA, IAA, and cytokinins, and the perception of their levels in the formation of tumors [36,37], and consequently in cell hypertrophy. Moreover, both the cell wall and the vacuolar invertases maximize the production of hexoses, which promote cell division and expansion, and respiration [37], while the presence of sucrose favors the differentiation of storage organs and maturation of tissues [38,37]. Thus, the detection of active sites of invertases in the immature and mature gall tissues of *A. australe* might indicate the source of constant division and regulation of cell growth and intense respiration.

The detection of sucrose synthase may be important to determine the maturation of the gall tissues, once it is involved in organ storage and maturation [37]. The role of sucrose synthase in sucrose import may involve its dual capacity to direct carbon towards both polysaccharide biosynthesis and respiration. The UDPG product of sucrose synthase has been implicated in starch formation [39], so its storage in the immature and mature galls of *A. australe* may be a product of the activity of sucrose synthase in the cells of vascular bundles and around the nymphal chamber.

In addition to sucrose synthase, acid phosphatase may help break down starch molecules into smaller sugars for galling insect nutrition and/or the maintenance of the gall cellular machinery. In the *A. australe–Pseudophacopteron* sp. system, this enzyme may be responsible for the degradation of some organelles in the cells surrounding the nymphal chamber. In senescent galls, its activity spread throughout the gall tissues. Bronner [1] attributed to acid phosphatase, some changes in the cytoplasm in galls by the formation of autophagic structures, mainly in the nutritive tissue. Acid phosphatase hydrolyzes phosphomonoesters in many plant biochemical reactions, including the formation of sucrose during photosynthesis [40], and the release of Pi (inorganic phosphate), which is important for breaking down the starch molecule [41]. So, in the current system, besides the degradation of some organelles, acid phosphatase may take part in the metabolism of starch during the main trophic phase of *Pseudophacopteron* sp. This proposal is reinforced by the fact that the gradient of this enzyme activity homogenizes just after the imago emerges.

4.4. Conclusions

In conclusion, the effect of the feeding activity of *Pseudophacopteron* sp. alters the metabolism of *A. australe* during gall development, which was demonstrated by the histochemical and the cytological analyses. The detection of both reserve substances and enzyme activity revealed histochemical and cytological gradients, even though a nutritive tissue *per se* was not differentiated. The histochemical and cytological features detected in the *A. australe–Pseudophacopteron* sp. system validate the storage of starch as the pattern for galls induced by sucking insects. This is the first description of carbohydrate metabolic pathways and high oxidative stress for galls induced by sucking insects in the Neotropical region. The validation of this pattern will be checked by the analysis of other sucking feeding insects gall systems in comparison to those of other feeding modes herbivores.

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Do Cecidomyiidae galls of *Aspidosperma spruceanum* (Apocynaceae) fit the pre-established cytological and histochemical patterns?

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ORIGINAL ARTICLE

Do Cecidomyiidae galls of *Aspidosperma spruceanum* (Apocynaceae) fit the pre-established cytological and histochemical patterns?

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Abstract Cecidomyiidae galls commonly present a zonation of tissues with lignified cell layers externally limiting a reserve tissue and internally limiting a specialized nutritive tissue next to the larval chamber. The cytological aspects of this specialized tissue indicate high metabolic activity as well as carbohydrate accumulation. In *Aspidosperma spruceanum*–Cecidomyiidae gall system, ultrastructural and histochemical investigations corroborated this pattern and also revealed the storage of proteins in the nutritive cells. Reactive oxygen species (ROS), callose, and pectin accumulation were related to the feeding activity of the galling herbivore. Phosphorylase, glucose-6-phosphatase, acid phosphatases, invertases, and sucrose synthase activities were detected for the first time, in the Neotropical region, and discussed in relation to gall maintenance and the feeding activity of the Cecidomyiidae.

Keywords Invertase · Nutritive tissue · Storage tissue · ROS · Sucrose synthase

Introduction

According to the concept proposed by Raman (2007), galls are mostly symmetrical structures developed in response to the activity of specialist inducers and are distinct from the amorphous tumors induced by bacteria and fungi. The activity of these inducers, especially the insects, promotes cellular redifferentiation, leading to the formation of tissues with characteristics and functions typical of the neoformed organ, the gall (Oliveira and Isaias 2009). Several hypotheses have been postulated to explain the adaptive value of galls for the galling insects (Mani 1964; Price 1977; Stone and Cook 1998; Stone and Schönrogge 2003). Among them, the nutritional hypothesis has received major attention. This hypothesis suggests that the manipulation of plant tissues is optimized in order to produce and accumulate resources for the feeding of the galling organism. Thus, cytological and histochemical analyses can be used to visualize the cytological and enzymatic gradients established during the formation of the specialized nutritive tissue. These techniques may help assess the levels of cellular stress and the impact of the metabolic changes over the nutrition of the gall inducer.

Among the tissues formed as result of the feeding activity of the galling Cecidomyiidae, a nutritive tissue redifferentiates near the feeding sites of the larvae (Rohfritsch 1992). Bronner (1992) conducted a thorough and detailed study of this tissue in galls of *Liposthenes glechomae* (Cynipidae) in *Glechoma hederaceae*, *Biorhiza pallida* (Cynipidae) in *Quercus* sp., *Monarthropalpus buxi* (Cecidomyiidae) in *Buxus sempervirens*, and *Schizomyia galiorum* (Cecidomyiidae) in *Gallium mollugo*, and proposed patterns for the accumulation of substances related to the feeding behavior of the inducers in the temperate region. However, in the Neotropical region, few studies have been developed to characterize the histochemistry and cytology of neoformed tissues under the influence of galling insects (Oliveira et al. 2006; Moura et al. 2008).

The species focus of the current study, *Aspidosperma spruceanum* (Apocynaceae), is a host plant of galls induced

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by an undescribed species of Cecidomyiidae, which present several cycles over the course of a year (Formiga et al. 2009). The galling Diptera Cecidomyiidae is common in the Neotropical region (Espírito-Santo and Fernandes 2007), and their feeding habits (scraper and extra-cellular digestion) induce the formation of nutritive tissues. Due to the cecidogenetic field established around the larval chamber (Mani 1964, 1992), cytological and histochemical gradients are expected with higher metabolic activity in the nutritive tissue, where the respiratory and oxidative stresses should also be higher. In order to test this premise, this work aimed to answer the following questions: (1) are there cytological alterations in host plant tissues due to the feeding action of the Cecidomyiidae during gall development? (2) Do the gradients of reserve substances and enzymes in the gall tissues establish concomitantly to cellular redifferentiation? (3) Are the cytological patterns observed during gall development related to oxidative stress? And (4) do the galls of *A. spruceanum* follow the patterns described in the literature for Cecidomyiidae galls?

Material and methods

Material collection

Tissue samples of non-galled leaves ($n \geq 25$) and of galls in three developmental stages (immature galls, 0.9±0.5 mm wide, mature galls, 5.1±0.4 mm wide, and senescent galls, 5.0±0.5 wide and open) ($n \geq 25$ per stage), were collected from *A. spruceanum* individuals ($n=5$) located at the Pampulha campus of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. The collections were made from January through December 2008, at intervals of 2 months, and the samples obtained were submitted to cytological and histochemical analyses.

Cytological analysis

The samples were fixed in 4% Karnovsky in 0.1 mM phosphate buffer (pH 7.2) for 24 h (Karnovsky 1965, modified), post-fixed in 1% osmium tetroxide in 0.1 mM phosphate buffer (pH 7.2), dehydrated in an ethanol series (Johansen 1940), and embedded in Araldite® (Luft 1961). The material was cross-sectioned in an Ultramicrotome Reichert-Jung-Ultracut, contrasted in uranyl acetate and lead citrate according to Reynolds (1963), and examined in a Zeiss EM 109 transmission electron microscope (TEM). For scanning electron microscopy (SAM) samples of mature galls were fixed, gradually dehydrated through alcohol series, CO_2 critical point dried, and supported-coated with 35 nm of gold (O'Brien and MacCully 1981).

Histochemical analysis

Nucleic acids Samples of non-galled leaves and mature galls were fixed in Carnoy for 48 h, dehydrated in an ethanol series, embedded in Reichert Jung® historesin, and cross-sectioned (5–10 µm) in a Reichert Jung® rotating microtome (Johansen 1940). The material was stained with 0.15% methyl green and 0.25% pyronin B in acetate buffer (pH 4.7) (Brachet 1953). For the controls, RNA and DNA were extracted by incubating the slides in 0.1% ribonuclease (pH 6.8) for 1 h at 40°C and in $^{0.2\ mg\ ml^{-1}}$ deoxyribonuclease in 0.003 mM magnesium sulfate (pH 6.5) for 1 h at 25°C, respectively.

Primary metabolites Handmade sections of fresh material were used for detection of proteins, lipids, starch, and reducing sugars. For proteins, the sections were stained in 0.1% bromophenol blue in a saturated solution of magnesium chloride in ethanol for 15 min, and then washed in acetic acid and water (Baker 1958). For starch detection, the sections were immersed in Lugol reagent (iodine potassium iodide) for 15 min (Johansen 1940). For reducing sugars, sections were immersed in Fehling reagent (Sass 1951). For lipids, the sections were immersed in a saturated solution of Sudan III (CI 26100) in 70°GL ethanol (Brundett et al. 1991). For the controls, lipids were extracted with methanol:chloroform (1:1, *v/v*), and starch with salivary amylase. Blank sections were used for comparative analysis.

Callose The samples were fixed in FAA for 48 h, washed in distilled water followed by 70% ethanol, and stained in 0.1% aniline blue (pH 9.5) for 10 min (Johansen 1940). The material was mounted with the stain and observed in a scanning confocal laser microscope (Zeiss, LSM 510, Germany). The controls followed the same protocol but without the stain.

Pectins Handmade sections of fresh material were stained with ruthenium red for 15 min, washed in water, and observed immediately (Jensen 1962).

Enzyme activity Sections of fresh material were immersed in the appropriate detection solutions for acid phosphatase, phosphorylase, glucose-6-phosphatase, invertase, and sucrose synthase. For the detection of acid phosphatase activity, the sections were incubated in 0.012% lead nitrate and 0.1 mM potassium sodium glycerophosphate in 0.5 mM acetate buffer (pH 4.5) for 24 h, at room temperature (25°C). Subsequently, sections were washed in distilled water and immersed in 1% ammonia for 5 min (Gomori 1956). For the control, the samples were not incubated in potassium sodium glycerophosphate. For the detection of phosphorylase activ-

ity, the sections were incubated in 1% glucose-1-phosphate in 0.1 mM acetate buffer (pH 6.0) for 2 h at room temperature (Jensen 1962). After the incubation, the sections were immersed in Lugol reagent (Johansen 1940). For the control, the samples were not incubated in glucose-1-phosphate. For the detection of glucose-6-phosphatase activity, the sections were incubated in a solution containing 20 mg of potassium glucose-6-phosphate in 125 ml of 0.2 mM Tris–maleate buffer (pH 6.7), and 3 ml of 2% lead nitrate in 7 ml of distilled water, for 15 min to 2 h at 37°C. Following the incubation, the material was washed in distilled water, immersed in 1% ammonium sulfate for 5 min, and mounted in glycerin jelly (Jensen 1962). For the control, the samples were not incubated in potassium glucose-6-phosphate. For the detection of invertase activity, the sections were incubated in a neutral reaction buffered medium containing 0.38 mM sodium phosphate (pH 7.5), 0.024% tetrazolium blue, 0.014% phenazin metasulfate, 30 U of glucose oxidase, and 30 mM of sucrose at room temperature for 3 h (Zrenner et al. 1995; Doehlert and Felker 1987). For the control, an incubation medium without sucrose was used. For detection of sucrose synthase (SuSy) activity, handmade sections of fresh samples and of samples fixed in 2% paraformaldehyde with 2% polyvinylpyrrolidone 40 and 0.005 mM dithiothreitol were placed in incubation medium for 30 min at 30°C. The incubation medium contained 5 µl of 150 mM NADH, 5 µl (1 U) of phosphoglucomutase from rabbit muscle, 5 µl of 3 mM glucose-1,6-biphosphate, 5 µl (1 U) of glucose-6-phosphate dehydrogenase from *Leuconostoc*, 5 µl (1 U) of UDPG pyrophosphorylase from beef liver, 280 µl of 0.07% aqueous nitro-blue tetrazolium (NBT), 350 µl of buffer, and 50 µl of substrate. The buffer consisted of 100 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 0.2% BSA, and 2 mM EGTA (7.4). The substrate contained 0.75 mM sucrose, 15 mM UDP, and 15 mM pyrophosphate. For one of the controls, glucose-1,6-biphosphate and pyrophosphate were not used. In a second control, sucrose was not used (Wittich and Vreugdenhil 1998).

Reactive oxygen species (ROS)—For DAB (3,3'-diaminobenzidine) staining, handmade sections of fresh material were immersed in 0.5% DAB (Sigma®) solution for 20–60 min, in the dark (Rosseti and Bonnatti 2001). The intensity of the reaction was examined every 15 min.

Results

General features

The gall induced by Cecidomyiidae in *A. spruceanum* forms a slight projection of the adaxial surface and a prominent projection of the abaxial surface of its host leaf (Fig. 1a). The larval chamber is central and shelters one individual of the Cecidomyiidae. This morphotype has distinct tissue zonation, the nutritive tissue, around the larval chamber, and limited by lignified cells, and the reserve tissue with larger cells outside this mechanical zone (Fig. 1b, c).

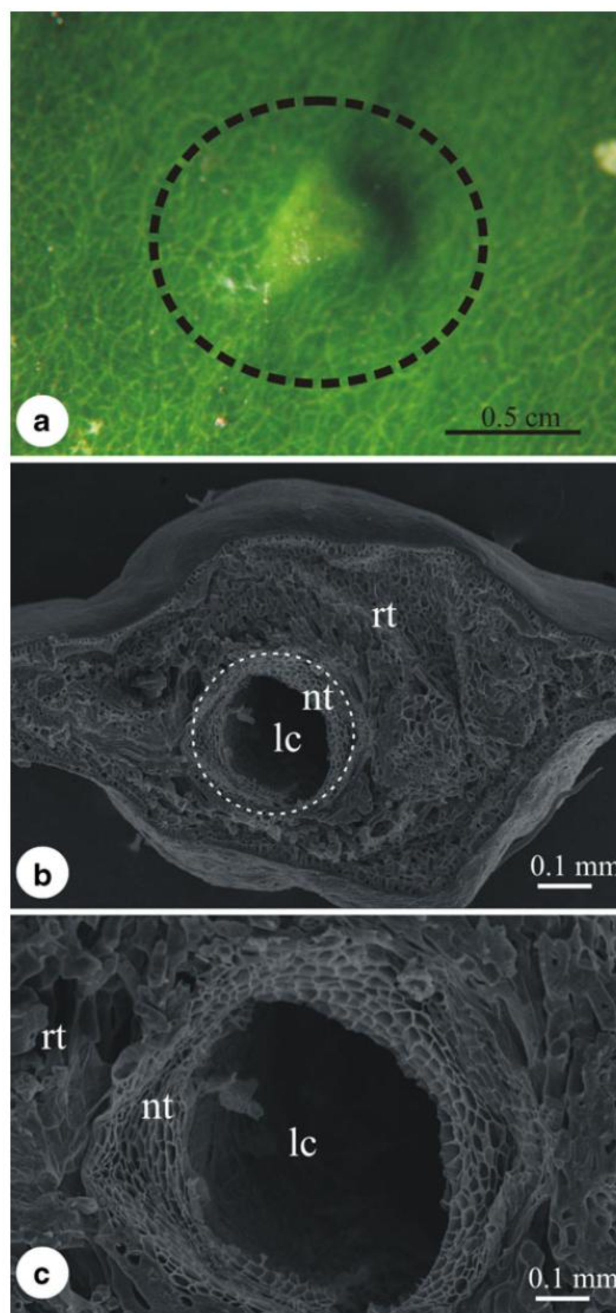

Fig. 1 **a** Gall of *Aspidosperma spruceanum*, detail of the projection to the adaxial surface. **b** Cross sections in scanning electron microscopy showing the larval chamber (*lc*), and tissue zonation with the reserve (*rt*), and nutritive tissues (*nt*). **c** Detail of the nutritive tissue around the larval chamber

Cytological analyses

In non-galled tissues, the palisade parenchyma cells have large vacuoles, numerous chloroplasts with primary starch associated with mitochondria (Fig. 2a). The plastids have intense lamellation in their grana (Fig. 2b). Lipidic droplets are distributed throughout the mesophyll (Fig. 2c), usually associated with plastids.

In the young galls, cellular degradation is more intense closer to the larval chamber (Fig. 2e). The nucleus of the cells surrounding the larval chamber enters cell death, evidenced by the disruption of the tonoplast, and highly condensed chromatin at its periphery (Fig. 2d). Besides the signs of degradation, some cells near the larval chamber have thickened primary cell wall (Fig. 2f), and some others have thin and sinuous walls indicating sites of hyperplasia, which extend to the reserve tissue. In this region, the chloroplasts have disaggregation of grana, reduction of the thylakoid system, and an increased number and size of plastoglobules (Fig. 3a). Starch grains are formed in chloroplasts of chlorenchyma tissue in the peripheral layers of the reserve tissue (Fig. 3b).

In the mature galls, the cells of the nutritive tissue have large vacuoles, protoplast with numerous mitochondria and large nucleus. The cell wall is thin and sinuous, with porous and striated appearance (Fig. 3c). The nucleus of these cells is dense with conspicuous nucleolus, and signs of chromatin condensation. These organelles are associated with mitochondria and chloroplasts, which have altered membrane systems (Fig. 3d). Cells containing numerous amyloplasts are still observed (Fig. 2e). The cells of the nutritive tissue surrounding the larval chamber have widely sinuous walls, and disorganized protoplast with signs of vacuole rupture (Fig. 3f). The cell wall shows electron dense portions, porous and striated appearance (Fig. 4a). Limiting the larval chamber, there are collapsed cells that

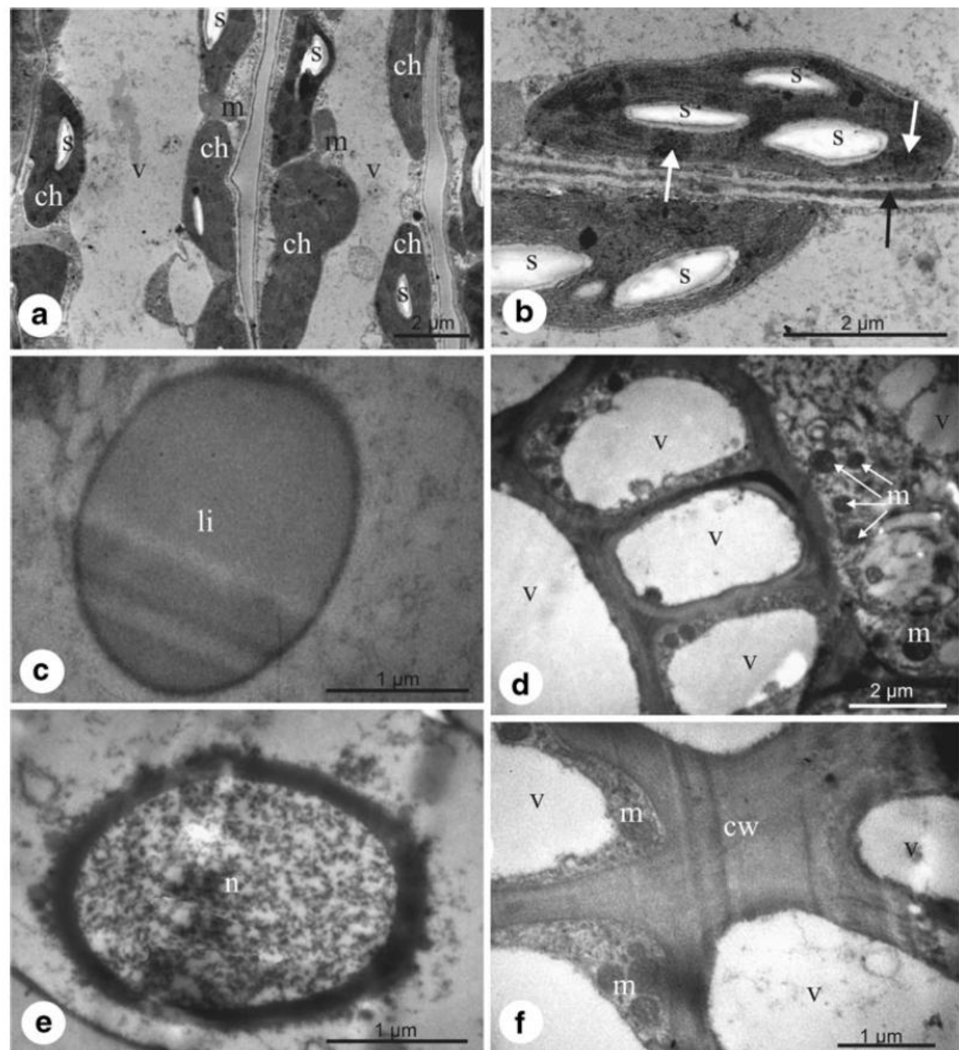

Fig. 2 Transmission electronmicrographs of galled and non-galled tissues of *Aspidosperma spruceanum*. **a–c** Non-galled tissues; **d–f** Nutritive tissue of a young gall; **a** Chloroplasts with primary starch, mitochondria and large vacuole associated with plastids in palisade parenchyma; **b** Detail of lamellation in grana; **c** Detail of a lipidic droplet; **d** Intense cellular degradation close to the larval chamber; **e** Detail of condensed chromatin in the nucleus; **f** Detail of thickened primary cell walls. *Ch* chloroplasts, *cw* cel wall, *l* lipid, *m* mitochondria, *n* nucleus, *s* starch, *v* vacuole

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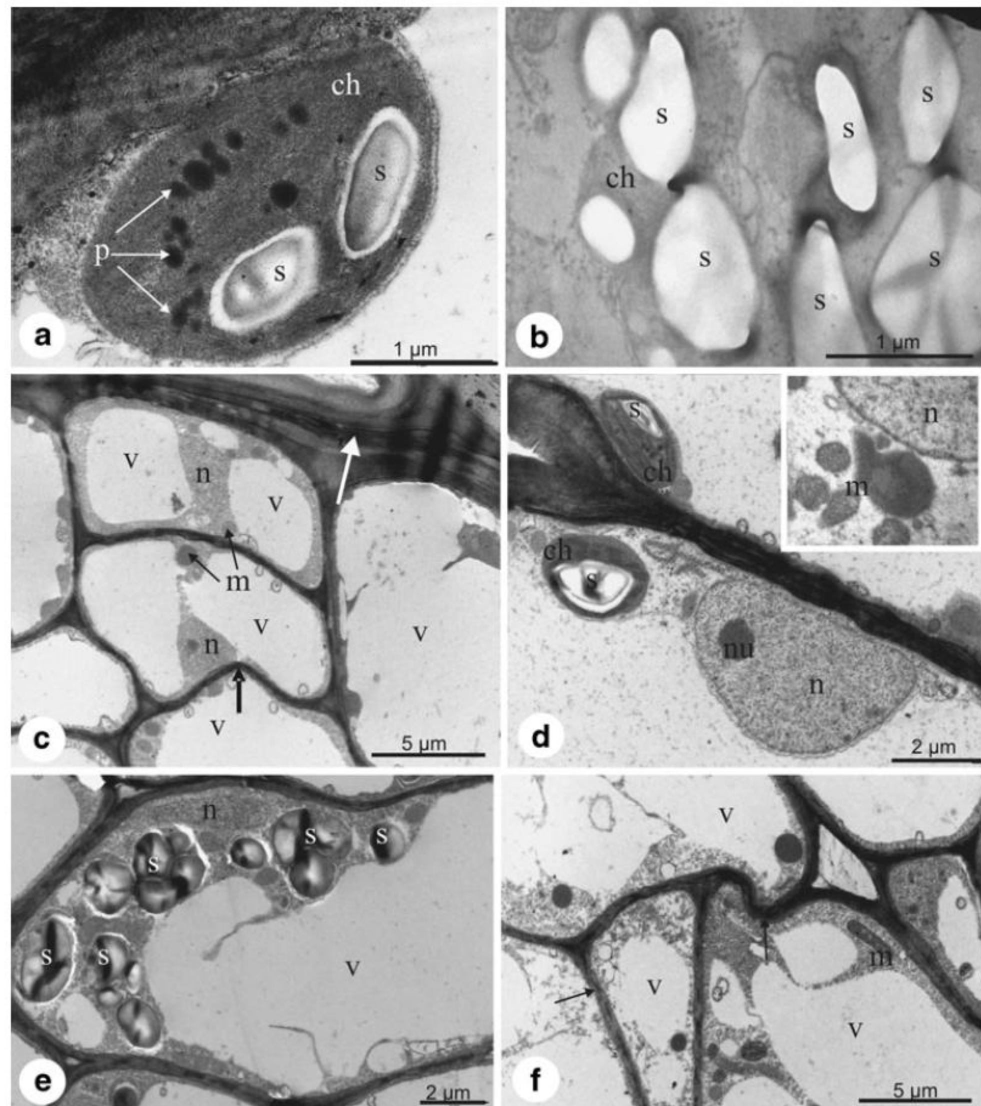

Fig. 3 Transmission electronmicrographs of galled tissues of *Aspidosperma spruceanum*. **a–b** Reserve tissue in a young gall; **c–f** Nutritive tissue of mature gall tissues; **a** Detail of starch and plastoglobules in chloroplast; **b** Detail of starch grains within chloroplasts; **c** Vacuoles, mitochondria, and thin walled cells; **d** Detail of nucleus and conspicuous nucleolus associated with plastids and mitochondria at the reserve tissue; **e** Detail of a cell containing numerous starch grains; **f** Cell with sinuous walls and signs of vacuole rupture. *ch* chloroplasts, *cw* cell wall, *l* lipid, *m* mitochondria, *n* nucleus, *p* plastoglobules, *s* starch, *v* vacuole

have lost their protoplasts (Fig. 4b). After the emergence of the gall maker, the cells have signs of degradation and PCD, characterized by pyknotic nuclei, loss of membrane integrity (Fig. 4c), and breakdown of the organelles membrane system (Fig. 4d). This event first occurs in the tissues of the inner cortex and then extends to the tissues of the outer cortex.

Histochemical analysis

Non-galled and galled tissues of *A. spruceanum* in different stages of development differed among performed histochemical tests (Table 1).

The activity of DNA was demonstrated by the reddish staining of the nucleus in both non-galled and galled tissues. In non-galled tissues, the reaction was observed in the nuclei of palisade parenchyma cells, and in the mature galls, in the hypertrophied nuclei of cells surrounding the larval chamber (Fig. 5a). The activity of RNA was detected neither in the nuclei of the cells of non-galled tissues nor in mature galls.

Total proteins were detected in all tissues of non-galled leaf, with stronger reactions in palisade parenchyma and in all the phases of gall development. In galls, the proteins were detected either in the tissues of the outer cortex or in the nutritive tissue surrounding the larval chamber (Fig. 5b). The gradient of proteins decreases towards the lignified tissue that divides the nutritive and the reserve tissues of the mature galls, and also decreases in the nutritive tissue towards the larval chamber (Fig. 5b). During senescence, the intensity of the reactions for proteins decreased from the outer to the inner gall cortex.

Starch grains were observed most notably in the palisade parenchyma tissue of non-galled tissues and in young and mature stages of the galls. In these stages of gall development, a gradient of starch was established in the

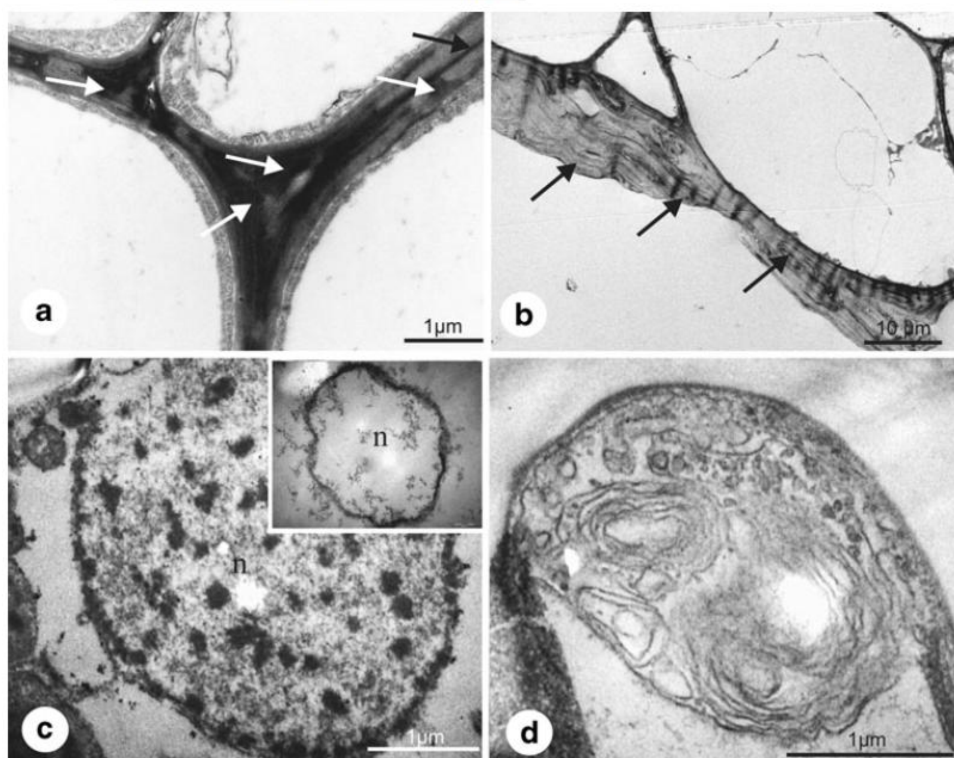

Fig. 4 Transmission electronmicrographs of the nutritive tissue of the gall of *Aspidosperma spruceanum*. **a–c** Mature galls. **a** Detail of electron dense portions of the cell wall (*white arrows*); **b** Cell wall collapse (*black arrows*); **c–d** Senescent galls. **c** Pyknotic nucleus and loss of membrane integrity; **d** Breakdown of the organelles membrane system. *n* nucleus, *v* vacuole

reserve tissue with higher concentrations in cells distal to the lignified tissue (Fig. 5c). Starch was also observed in the nutritive tissue, with the highest concentration in the layers of cells adjacent to the lignified cells (Fig. 5d).

Table 1 Histochemical tests in non-galled leaves and galls induced by a Cecidomyiidae in *Aspidosperma sprucenaum*

Test/tissue	Non-galled tissue	Galls		
		Young	Mature	Senescent
DNA	+	N	+	N
RNA	−	N	−	N
Proteins	+	+	+	+
Lipids	+	+	+	−
Starch	+	+	+	−
Reducing sugar	+	−	+	−
Callose	−	−	+	−
Pectin	+	+	+	+
Phosphorilase	−	+	−	−
Glucose-6-phosphatase	−	+	+	−
Invertase	+	+	+	+
Sucrose synthase	+	+	+	−
Acid phosphatase	−	+	+	+

+ = positive detection

− = negative detection

N.E. = not evaluated

During senescence, starch was not observed in any of the tissue layers of the gall. Reducing sugars were detected in the cells surrounding the vascular bundles in non-galled tissues and in galls. In mature galls, reducing sugars were detected in the outermost cells of the nutritive tissue (Fig. 5e), forming a gradient similar to that of starch. Lipids were detected in the palisade parenchyma of non-galled leaves and in the reserve tissue of young and mature galls (Fig. 5f) in a decreasing gradient towards the lignified tissue. In the nutritive tissue, lipid droplets were not detected. Callose was only detected in the cell walls of the nutritive tissue at the phase of maturation (Fig. 5g). Also, the walls of the nutritive tissue cells were highly pectic in all gall developmental phases (Fig. 5h).

The activities of phosphorylase and glucose-6-phosphatase were not detected in non-galled tissues and in senescent galls. In young galls, the phosphorylase was detected in the cells of the reserve tissue, whereas glucose-6-phosphatase (Fig. 6a) was detected in the most external cell layers of the nutritive tissue, either in young or mature galls.

The activity of acid phosphatase was not observed in non-galled tissues. In galls, this enzyme was detected in all developmental phases, with greater intensity at the maturation phase in cells surrounding the larval chamber (Fig. 6b). During senescence, the gradient of this enzyme activity extends up to the most external cell layers.

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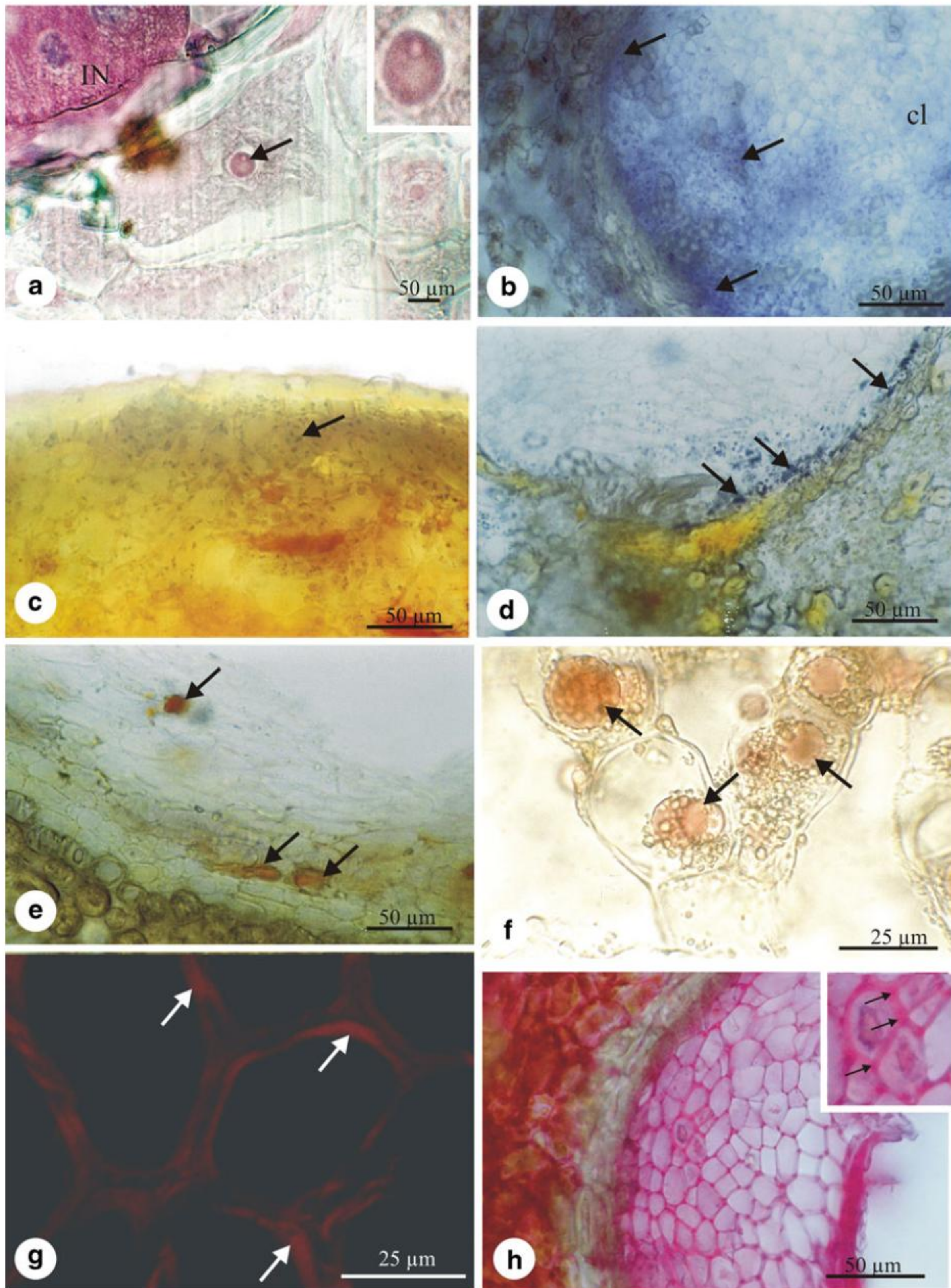

Fig. 5 Histochemical tests in mature galls of *Aspidosperma spruceanum*. **a** Detail of DNA staining in the nuclei of nutritive tissue cells; **b** Protein gradient decreasing towards the larval chamber; **c** Centrifugal gradient of starch in the reserve tissue; **d** Presence of starch on outermost cells of the nutritive tissue; **e** Reducing sugars in the outermost cell layers of nutritive tissue; **f** Detail of lipids in the reserve tissue of the gall; **g** Callose in the cell walls of nutritive tissues (*arrows*); **h** Pectic compounds in nutritive cell walls

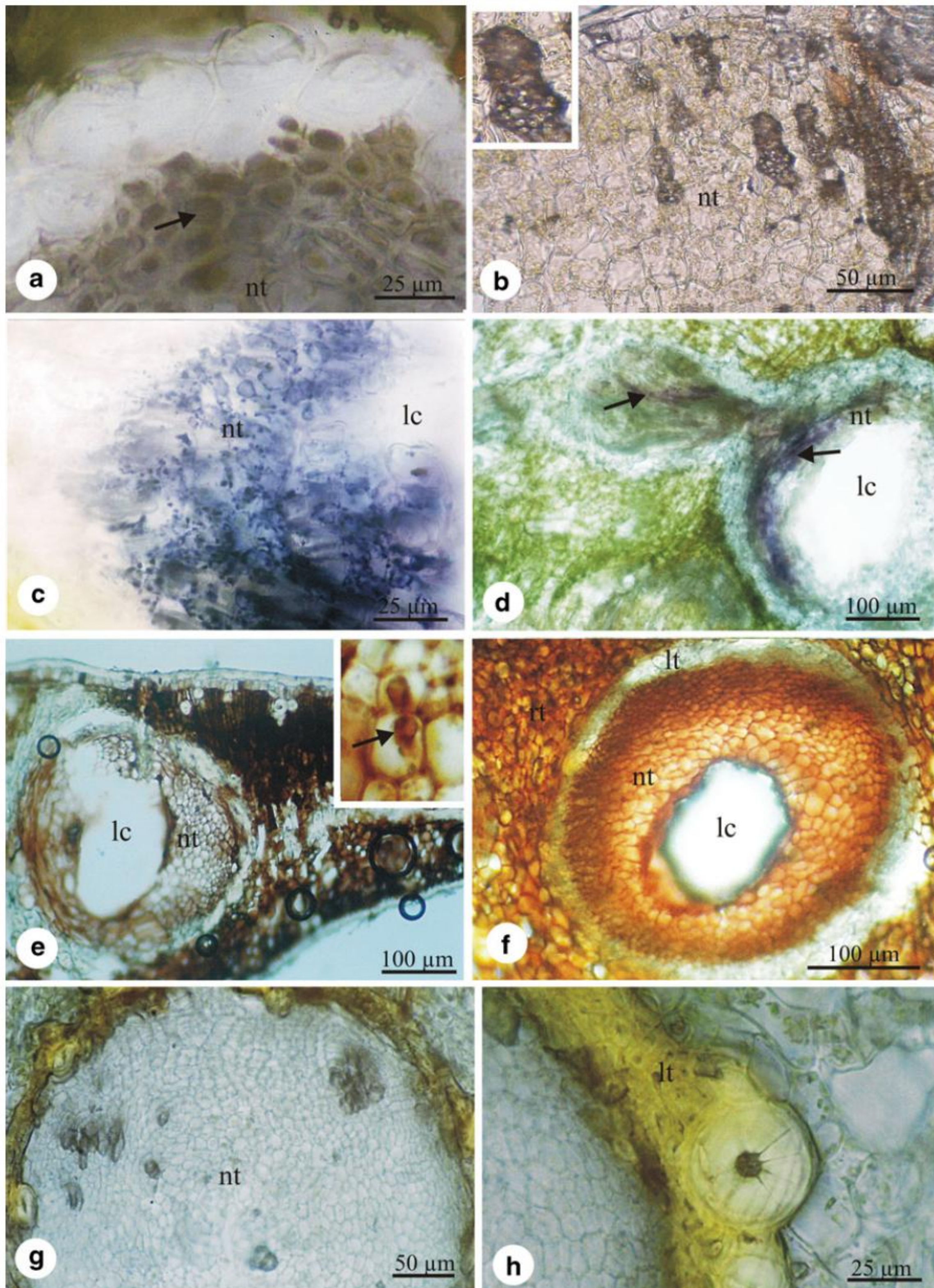

Fig. 6 Histochemical tests in galls of *Aspidosperma spruceanum*. **a**–**d**, **f**–**h** Mature galls. **e** Young gall. **a** Glucose-6-phosphate in the outermost layers of the nutritive tissue (*arrow*); **b** Acid phosphatase activity in the nutritive tissue; **c** Invertase activity in the cells of the nutritive tissue; **d** Activity of sucrose synthase in the vascular bundles and in the outermost cell layers of the nutritive tissue; **e** ROS in the outermost layers of chlorophylous parenchyma and in the cells of the nutritive tissue (higher magnification); **f** ROS detection throughout the reserve and nutritive tissues; **g** Neoformed vascular bundles within nutritive tissue; **h** Detail of the numerous pits in the cells of the lignified tissue. *lc* larval chamber, *lt* lignified tissue, *tn* nutritive tissue

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The activity of invertase was detected in non-galled tissues surrounding the vascular bundles and in some cells of the palisade parenchyma. In young and mature galls, invertase activity was limited to the cells of the nutritive tissue (Fig. 6c). In senescent galls, the activity of invertase was more discrete, extending to the most external cell layers of the reserve tissue. In galls, the activity of sucrose synthase was limited to vascular bundles surrounding the gall and at the most distal cell layers of the nutritive tissue (Fig. 6d). The activity of this enzyme was not detected in senescent galls.

ROS were detected either in ungalled tissues or in galls. In non-galled tissues, the ROS were limited to the cells of spongy parenchyma and were scarcer in the cells of the palisade parenchyma. In the young galls, the ROS were strongly detected in the outermost layers of chlorophylous parenchyma and in the cells of the nutritive tissue (Fig. 6e). In mature galls, the ROS were detected throughout the most external cell layers, and in the cells of the nutritive tissue, just adjacent to the lignified zone (Fig. 6f). In cell layers close to the nutritive tissue, DAB reaction occurred with lower intensity, and together with the cytological observations indicated that these cells have lost part of their protoplast.

Interspersed to the cells of the nutritive tissue in young and mature galls, neoformed vascular bundles (Fig. 6g) connected nutritive and reserve tissues. The mechanical barrier formed by the lignified zone was transposed by numerous pits (Fig. 6h).

During the development of galls of Cecidomyiidae in *A. spruceanum,* cytological and histochemical gradients were established. The compounds detected were related to primary metabolism of plants and were important for the development and maintenance of the gall structure and the nutrition of the gall maker. These gradients varied during the development of the gall (Fig. 7).

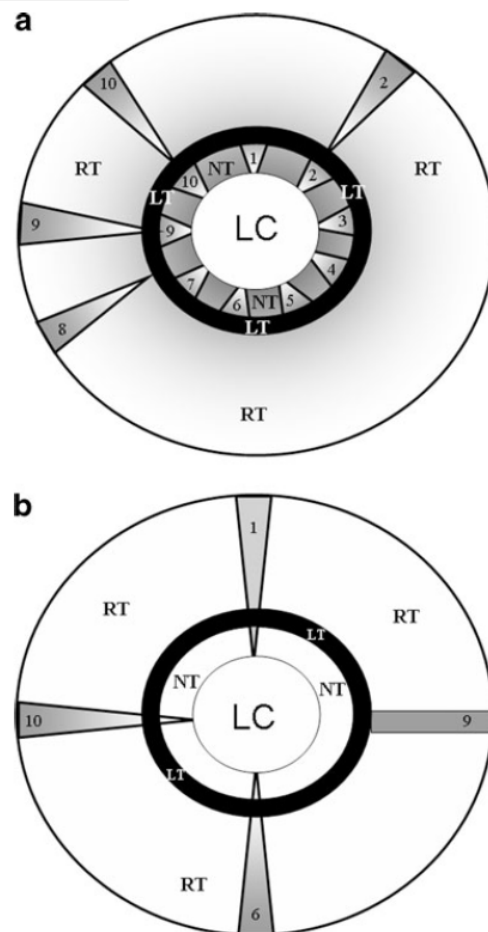

Fig. 7 Representative diagram of the histochemical gradients detected in mature (**a**) and senescent (**b**) galls of *Aspidosperma spruceanum*. *Arrow* direction and intensity of *gray color* indicate histochemical and cytological gradients, respectively. Invertase (*1*), starch (*2*), sucrose synthase (*3*), glucose-6-phosphatase (*4*), reducing sugars (*5*), acid phosphatase activities (*6*), callose (*7*), lipids (*8*), ROS-Reactive Species of Oxygen (*9*), proteins (*10*). *LT* lignified tissue, *NT* nutritive tissue, *RT* reserve tissue

Discussion

Although new substances have not been detected in gall tissues when compared to non-galled ones, galled samples have cytological and physiological characteristics different from those of non-galled tissues (Bayer 1992; Bronner 1992). The galls of *A. spruceanum* induced by the unidentified species of Diptera: Cecidomyiidae accumulated reserve substances and presented differences in enzyme activities when compared to non-galled tissues. Among the specialized tissues of the gall, the nutritive tissue accumulates nutrients in a sufficient amount so as to guarantee the complete development of the gall maker (Bronner 1992; Rohfritsch 1992; Harris et al. 2006).

The main cytological features observed in the galls of *A. spruceanum* were indicatives of the production of ROS and consequent cell death. These features had not been previously associated to gall development, and occurred since its young stages. The cytological analysis of the young galls showed that the cells surrounding the larval chamber had degradation of the membrane systems and condensation of chromatin in the nuclei, which were related to advanced PCD during some plant organ development (Beers 1997). These signals were previously related to the action of the larvae by Harris et al. (2006) in the nutritive tissue of galls of Diptera: Cecidomyiidae in wheat plants. The degradation of the cell membrane system observed in the nutritive tissue of young galls of *A. spruceanum* might be related to the production of ROS evidenced by the positive reaction to DAB. Also, these cells entered a form of programmed cell death (PCD), a kind of reaction reported by other plant pathogen interactions, as a genet-

ically controlled process induced by the ROS (Overmyer et al. 2009). In fact, PCD is engaged in active plant mechanisms against the spread of viruses (Lam et al. 2001) and bacteria (Hutcheson 1998), but it should be regulated by the pathogen to ensure that it stays in the host tissues (Wang et al. 1996), and by the host through autophagy to prevent systemic death (Patel et al. 2006). As a natural event in plant body, orchestrated PCD might have prevented hypersensitive responses of the plant against the gall maker, and should have helped in shaping the structure and in the establishment of the galling herbivore.

During the phases of maturation and senescence, cell degradation and oxidative stress continue. In *A. spruceanum*, this process started in the cells surrounding the larval chamber and extended to the outermost layers of the reserve tissue. In the innermost layers of the nutritive tissue, closest to the larval chamber, the cells have practically lost all their protoplast, and some of them collapsed. The collapse of the cell wall is considered the final stage of PCD (Beers 1997; Gunawardena et al. 2004), and were indicatives that the continuity of this process was necessary for the establishment of the shape and function of the galls induced by Cecidomyiidae.

Galls induced by Cecidomyiidae may have the same cytological characteristics of Cynipidae galls (Rohfritsch 1992; Mani 1964). However, the cytological alterations may be more discrete, and the nutritive tissue can accumulate specific nutrients (Bronner 1992). In fact, the nutritive cells are subject to high oxidative stress due to the proximity of the larval chamber (Bronner 1992; Schönrogge et al. 2000), which should be the primary stimulus for the histochemical gradients observed in *A. spruceanum* galls.

The gradients of reserve substances and enzymes in the gall tissues established concomitantly to cellular redifferentiation, and were directly associated to the high metabolic activity and oxidative stress to which cells are subjected to in the vicinity of the larval chamber, as proposed by Schönrogge et al. (2000). In addition, the nutritive tissue in *A. spruceanum* was protein rich, and functioned as the nutritional source for the galling Cecidomyiidae, similarly to the observations of Bronner (1992) and Harris et al. (2006) for other gall systems. In the absence of food stimulation by the galling agent, the gradient of proteins tended to homogenize and then disappeared. This was particularly characteristic of the gall senescence.

The gradients of substances in the nutritive tissue were directly driven to the feeding supply to the galling herbivore, while the gradients in the reserve tissue were related either to the maintenance of the gall structure or to the translocation of substances to the nutritive tissue. The presence of starch in the nutritive tissue around the larval chamber of mature galls of *A. spruceanum* is a new record for this group of galling herbivores, and the accumulation of this substance and its location seems to be related to the intense vascularization. In general, the gradient of starch increases towards the most external cell layers of Cecidomyiidae galls, and is absent in the nutritive tissue, around the larval chamber (Bronner 1992). During senescence, the gradient of starch disappeared together with the end of the feeding stimulus of the gall maker. This proved that the feeding activity of the gall maker was the primary stress for the maintenance of the gradients.

In *A. spruceanum*, sugars were found in the outermost layers of nutritive tissue in mature galls, and followed a gradient similar to that of starch. However, in cells that boundary the larval chamber these substances were not detected. Since the proteins were detected in the most internal layers of the nutritive tissue, it was suggested that these were the main components of the gall maker's diet, whereas starch and sugars were mainly related to the maintenance of cellular machinery.

Lipids were detected in the most external cell layers in young and mature galls, but the storage of this substance might be inherent to the metabolism of the host plant as previously proposed by Oliveira et al. (2006) for *Lonchocarpus muelhbergianus–Euphalerus ostreoides* (Psyllidae) system, and by Moura et al. (2008) for *Lantana camara–Aceria lantanae* (Acari) system. The authors suggested that these substances could be used for the maintenance of gall structure, as these molecules are highly energetic and may donate precursors of important components of plant metabolism as stated by Buchanan et al. (2000).

Cell wall lignification in galls of Cecidomyiidae is generally interpreted as a mechanical protection against the attack of natural enemies (Stone and Schönrogge 2003; Price et al. 1986). Nevertheless, the presence of cell wall components, other than lignin, has not been explored in studies of gall development. The cell walls of the nutritive tissue of mature galls of *A. spruceanum* were thickened due to the accumulation of callose and pectin. The presence of callose is another indicative of the stress provoked by the activity of the Cecidomyiidae, once the deposition of this substance can occur on physiological conditions in which the plasma membrane is disrupted, including chemical treatment, physiological stress or attack by pathogens (Doblin et al. 2007; Mellersh and Heath 2001). The species of Cecidomyiidae are known for their reduced and simplified digestive system, making extraintestinal digestion. Their habitual scraping ruptures the plant cell walls thus allowing access to nutrients (Roskan 1992; Bronner 1992; Harris et al. 2006). This injury might have induced the deposition of callose in the cell walls of nutritive tissue especially in mature galls of *A. spruceanum*. Moreover, the callose found in the cell walls of nutritive tissue in galls of *A. spruceanum* can add permeability and thus facilitate the

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flow of nutrients to the feeding site, as proposed by Bronner (1992). This proposal is supported by Yim and Bradford (1998) statement that the deposition of callose surrounding the endosperm of seeds of melon serves as a molecular filter and facilitates water movement. Also, Harris et al. (2006) showed changes in the structure of the cell wall in galls induced by Cecidomyiidae which feed on a liquid that moves through this wall. Besides the callose, a large amount of pectin in cell walls may increase tissue porosity, which would also facilitate the passage of macromolecules (Willats et al. 2000, O'Neill and William 2003). Relatively immobile microfibrils and matrix polymers make the primary cell wall behave like a sieve, which blocks the passage of large molecules and microorganisms. However, small molecules and ions pass through the aqueous channels of the matrix, and so do some small proteins and polysaccharides. Also, some large proteins and proteoglycans seem to penetrate the cell wall too (Brett and Waldron 1996).

Besides the accumulation of nutrients, high enzymatic activity is necessary for the dynamic functioning of the gall. The breakdown of starch molecules in the most external cell layers of the nutritive tissue of young and mature galls of *A. spruceanum* should be done by the activity of acid phosphatase. Among the functions of this enzyme, its participation in the route of sucrose synthesis (Tanksley 1983), and the release of Pi (inorganic phosphate) are key roles in the breakdown of starch (Lytovchenco et al. 2007). Another role of the acid phosphatase in galls is the formation of autophagic structures mainly in the nutritive tissue, causing changes in the cytoplasm (Bronner 1992). The inverse reaction, namely, the polymerization of starch was at least partly performed by the activity of phosphorylase, slightly detected in tissues of young galls of *A. spruceanum*. This enzyme seems to be correlated to the amount of amyloplasts in plant organs (Gerbrandy and Verleur 1971), and in galls, as well.

Once the starch cannot be directly used either for the feeding of the gall maker or the gall maintenance, some conversion is necessary. The glucose-6-phosphatase, an enzyme involved in the synthesis of intermediate compounds during the formation of sucrose (Baroja-Fernandez et al. 2003), was detected in young and mature phases of the gall, especially in the outermost cells of the nutritive tissue next to the lignified zone. Moreover, the gradient of invertases in mature galls coincided with the gradient of starch and sugars, so the activity of this enzyme might have provided resources to the nutrition of the gall maker, as well as to the cell expansion and metabolism of the nutritive tissue. The invertases are enzymes that catalyze the irreversible breakdown of sucrose into glucose and fructose, and their activities are associated with plant tissues that act as physiological sinks (Koch 1996; Koch and Zeng 2002; Rehill and Schultz 2003). The sinking of these carbohydrates to gall site is crucial to cell division and expansion, and cellular respiration (Koch 2004). Rehill and Schultz (2003) showed that the development and expansion of tissues are correlated with the activity of invertases. Bronner (1992) resumed the importance of this enzyme to the accumulation of nutrients in the cells of the nutritive tissue of Cynipidae and Cecidomyiidae galls. The study with *A. spruceanum* galls checked this pattern for the first time for a Cecidomyiidae gall in the Neotropical region.

From the metabolical and structural points of view, an important enzyme is sucrose synthase, which participates in several processes that involve maturation and storage of nutrients (Koch 2004). The UDPG, a product of the breakdown of sucrose by sucrose synthase, has been linked with the formation of starch (Asano et al. 2002; Koch 2004) and the synthesis of callose (Salnikov et al. 2003; Subbaiah and Sachs 2001; Koch 2004). The detection of this enzyme in vascular bundles and the nutritive tissue of mature galls of *A. spruceanum* might indicate its involvement in the synthesis of starch and callose, and the maturation of gall tissues.

The current cytological and histochemical analyses showed that the feeding activity of the galling Cecidomyiidae changed the metabolism of *A. spruceanum* host organs during gall development. The primary trigger for these changes seemed to be ROS production and resulted in gradients of reserve substances and enzymatic activities important for gall metabolism and for the availability of resources to the diet of the galling herbivore. In the galls of *A. spruceanum*, a true nutritive tissue differentiated, and the innermost cell layers entered cell death processes at the beginning of gall development, and at the maturation phase, these cells ended up losing their protoplasts. The events here related to ROS formation and PCD progressed from gall maturation till its senescence. Moreover, some lights were set upon the enzymatic activities responsible for gall development, and a viable approach to study gall metabolism was revisited, this time in Neotropical region.

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Is the oxidative stress caused by *Aspidosperma* spp. galls capable of altering leaf photosynthesis?

Is the oxidative stress caused by *Aspidosperma* spp. galls capable of altering leaf photosynthesis?

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ABSTRACT

The generation of ROS (reactive oxygen species) in plant galls may induce the degradation of the membrane systems of a plant cell and increase the number of plastoglobules. This numerical increase has been related to the prevention of damage to the thylakoid systems, and to the maintenance of photosynthesis rates. To investigate this hypothesis in gall systems, a comparative study of the ultrastructure of chloroplasts in non-galled leaves and in leaf galls of *A. australe* and *A. spruceanum* was conducted. Also, the pigment composition and the photosynthetic performance as estimated by chlorophyll fluorescence measurements were evaluated. The ultrastructural analyses revealed an increase in the number and size of plastoglobules in galls of both species studied. The levels of total chlorophylls and carotenoids were lower in galls than in non-galled tissues. The chlorophyll a/b ratio did not differ between the non-galled tissues and both kinds of galls. The values of maximum electron transport rate (ETR_{MAX}) were similar for all the samples. The occurrence of numerous large plastoglobules in the galled tissues seemed to be related to oxidative stress and to the recovery of the thylakoid membrane systems. The maintenance of the ETR_{MAX} values indicated the existence of an efficient strategy to maintain similar photosynthetic rates in galled and non-galled tissues.

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1. Introduction

Plant galls induced by insects have typical tissues [1–3] that are formed by cell redifferentiation in their host organ. Consequently, the neoformed tissues alter the host organ and the functions of the gall structure itself. The attack of gall inducers may cause either negative [4–7] or positive effects on the photosynthesis of the host organs [8,9]. In the case of galls caused by aphids, these effects may be neutral [6]. In most previous studies, the levels of photosynthetic rates in galls were estimated by measuring gas exchange. However, more recent studies have found significant alterations in photosynthetic quantum yield in galls, with damage to the PSII in addition to alterations in gas exchange and reduction in pigment contents [7–13].

The induction and development of galls expose plant tissues to high oxidative stress [14–17]. From the cytological point of view, the oxidative stress can stimulate the degradation of the thylakoid system and the formation of plastoglobules [18–20]. The plastoglobules are subcompartments of the chloroplasts, and can contain several kinds of lipids and proteins. Their functions are associated with the storage of molecular components, with the recovery of the thylakoid membrane systems [21–25], and with the scavenging of reactive oxygen species in chloroplasts [26,27]. The involvement of oxidative stress with the synthesis and storage of plastoglobulines and other molecules, such as tocopherol, has been correlated with the protection of the membranes from photooxidation and the PSII from photoinactivation [28,29]. Thus, because of the high oxidative stress, it is probable that the differentiation of plastoglobules occurs in gall tissues as a strategy to maintain photosynthesis in this neoformed organ. This differentiation has not been investigated previously. The presence of ROS (reactive oxygen species) in galls of *Aspidosperma australe* and *A. spruceanum* was detected in previous studies [16,17], and inspired an approach to check if there were more plastoglobules in the cells where the ROS were detected. This may indicate a strategy to prevent damage to the thylakoid systems. In addition, the feeding activity of *Pseudophacopteron* sp. in *A. australe* differs from that of the Cecidomyiidae in *A. spruceanum*. *Pseudophacopteron* is a phloem-sucking insect while the Cecidomyiidae possesses scraper feeding habit, which provokes greater damage to plant cells. Thus, the feeding habit of the gall inducing herbivore can influence the photosynthetic activity of the gall. To investigate these hypotheses, a comparative study of the ultrastructure of the chloroplasts in non-galled leaves and in leaf galls of *A. australe* and *A. spruceanum*

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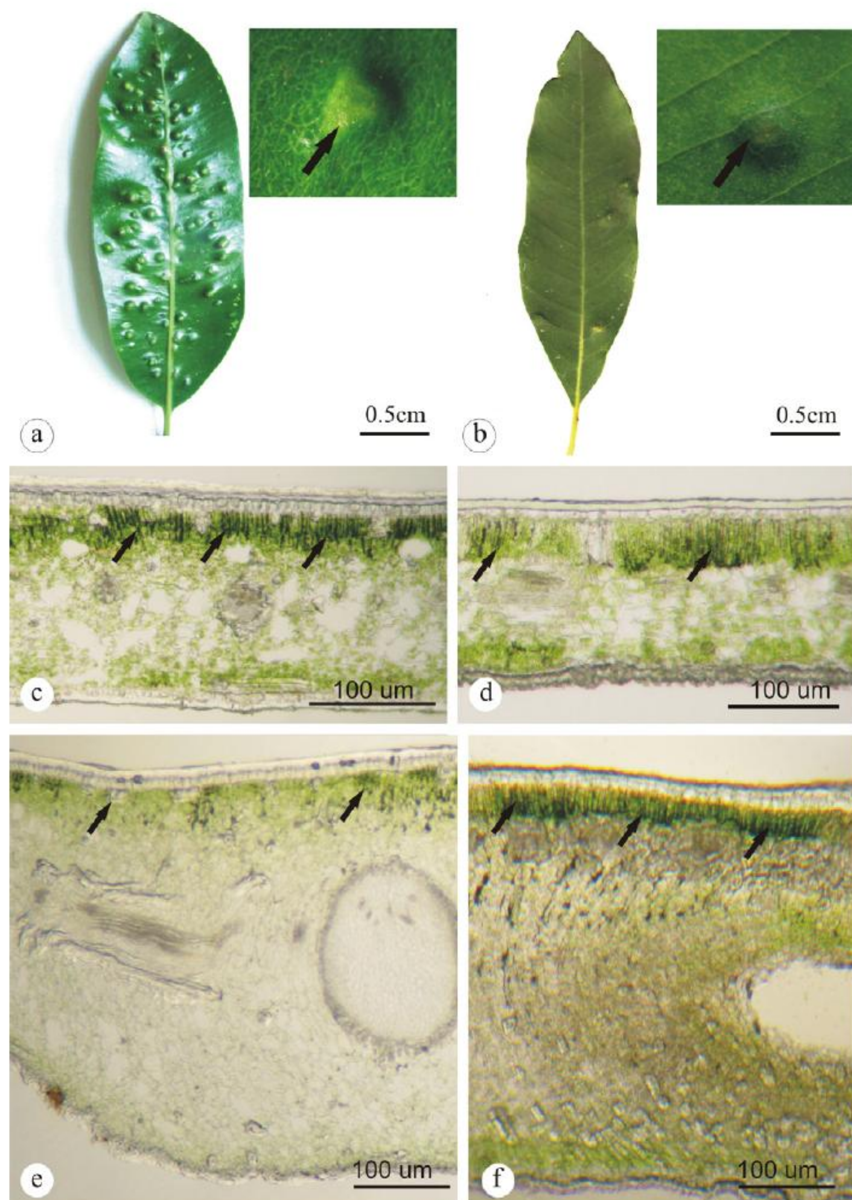

Fig. 1. Morphological and anatomical aspects of the galls of *Aspidosperma*. (a) Gall of *A. spruceanum*, detail of gall projection on the adaxial leaf surface (arrow). (b) Gall of *A. australe*, detail of gall projection on the adaxial leaf surface (arrow). (c) Cross sections of non-galled tissues (leaf) of *A. spruceanum* with chlorophyll tissue on the adaxial surface (arrows). (d) Cross sections of non-galled tissues (leaf) of *A. australe* with chlorophyll tissue on the adaxial surface (arrows). (e) Cross sections of gall tissues of *A. spruceanum* with chlorophyll tissue on the adaxial surface (arrows). (f) Cross sections of gall tissues of *A. australe* with chlorophyll tissue on the adaxial surface (arrows). oc = outer cortex, ic = inner cortex.

was conducted. The results were related to their pigment composition, and to the photosynthetic performance as estimated by measurements of chlorophyll fluorescence.

2. Materials and methods

2.1. Plant material collection

Trees of *A. australe* Müell. Arg. (n = 10) and *A. spruceanum* Benth. ex Müell. Arg. (n = 10), remnants of a semi-deciduous forest, were marked on the campus of the Universidade Federal de Minas Gerais (43°57′51″W and 19°52′11″S). Non-galled leaves and mature galls of *A. australe* induced by *Pseudophacopteron* sp. (Hemiptera), and of *Aspidosperma spruceanum* induced by an unidentified species of Diptera: Cecidomyiidae were collected for cytological and pigment studies, and analyzed *in situ* for chlorophyll fluorescence measurements.

2.2. Histometric analysis of chlorophyll parenchyma

The areas of chlorophyll parenchyma were measured in cross-sections of non-galled leaves (n = 40) and galls (n = 40) with the Axio-Vision 4.7® software. The data were expressed as the percentage of chlorophyll tissue within the total tissue, per unit surface area. Numerical data were submitted to an ANOVA, followed by Tukey's test ($P \leq 0.05$) using Graphpad Prism® software.

2.3. Cytological analyses

The samples were fixed in 4% Karnovsky for 24 h [30], modified by the addition of 0.1 M phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2), dehydrated in an ethanol series [31], and embedded in Araldite® [32]. The material was cross-sectioned in a Reichert-Jung Ultracut ultramicrotome,

contrasted in uranyl acetate and lead citrate [33], and analyzed in a ZEISS EM 109 Transmission Electron Microscope.

2.4. Pigment content and chlorophyll fluorescence

Pigments of non-galled leaves non-galled tissues of galled leaves (disks of 1 cm^2), and mature galls (n = 10) were extracted in 80% acetone (v/v) after maceration and centrifugation All samples were weighted, the galls were scanned, and the area of their tissues were calculated by the Axio-vision software. The contents of chlorophylls a and b, and carotenoids were obtained using the equations proposed by Lichtenthaler and Wellburn [34], and the pigment contents were calculated on area and fresh weight basis.

The photosynthetic performances of non-galled leaves, non-galled tissues of galled leaves, and galls (n = 5) were evaluated by fluorescence measurements using a modulated chlorophyll fluorometer (MINI-PAM, Walz, Effeltrich, Germany). The values of the potential quantum yield of PSII (F_V/F_M) were determined in the leaves of four individuals of each species at pre-dawn and at midday, after adaptation in the dark for 30 min. The photosynthetic performance under increasing levels of light was determined using the light curve program of the instrument, with increasing levels of photosynthetically active radiation (PAR) for 4 min, in 8 steps of 30 s each. At the end of each light level, a saturation pulse was applied and the chlorophyll fluorescence parameters were recorded. The effective quantum yield of PSII ($\Delta F/F_m'$) was determined by the formula $\Delta F/F_m' = (F_m' - F)/F_m'$, where F is the steady-state chlorophyll fluorescence yield of light-adapted samples and F_m' is the maximum chlorophyll fluorescence caused by the saturation pulse [35]. The apparent electron transport rate was estimated considering the ETR = 0.5 ($\Delta F/F_m'$) × PPF × 0.84, where 0.5 is the fraction of excitation energy distributed to PII, PPF is the incident photosynthetic photon flux, and 0.84 is the fractional light absorbance [36,37]. By the instantaneous light curves, the maximum apparent electron transport (ETR_{MAX}), saturating photosynthetically active radiation (PAR_{SAT}), and PAR in 1/2 of ETR_{MAX} was obtained [38].

3. Results

3.1. General features of galls

The leaf galls induced by Cecidomyiidae in *A. spruceanum*, and those induced by *Pseudophacopteron* sp. in *A. australe* form a slight projection of the adaxial surface (Fig. 1a and b), and a prominent projection of the abaxial surface of their host leaves. The larval chamber is central, and shelters one individual insect in both cases.

3.2. Histometric analysis of chlorophyll tissue

The chlorophyll tissue in the leaves of both *Aspidosperma* spp. is differentiated into palisade and spongy parenchymas (Fig. 1c and d). This tissue is restricted to the outer cortex of the galls of *Aspidosperma* spp. located at the adaxial side of the leaf lamina (Fig. 1e and f). The percentage of chlorophyll tissue decreases during the development of the two gall morphotypes (Fig. 2a and b).

3.3. Cytological analyses

The cells of the mesophyll, mainly those of the palisade parenchyma, of the non-galled tissues of *A. australe* have many chloroplasts with primary starch grains (Fig. 3a). The membrane systems of these chloroplasts have normal grana and thylakoid (Fig. 3b). In the mature galls of this species, the chloroplasts are concentrated in the outer cortex. These plastids have conspicuous lamellation, even though disorganization of the thylakoid system is apparent, and therefore the stacking of the membranes is less evident, altering the structure of the grana (Fig. 3c and d). Plastoglobules (Fig. 3c) associated with the thylakoid systems (Fig. 3d and e), and a few starch grains are observed at the periphery of the chloroplasts.

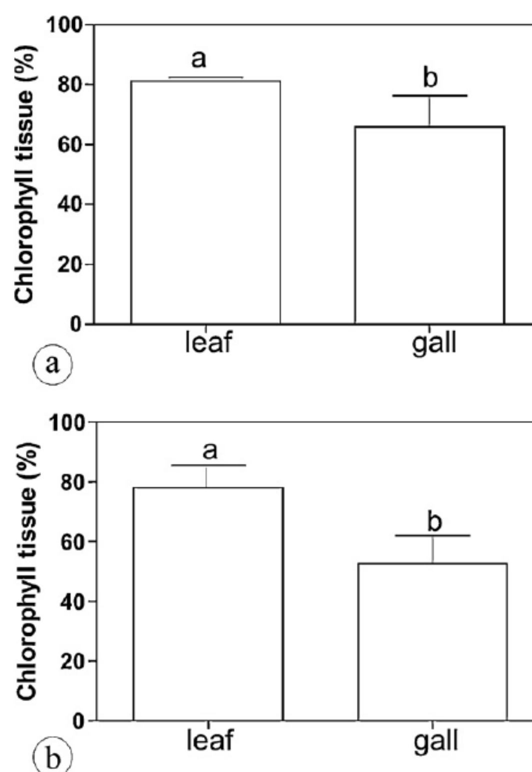

Fig. 2. Histometric analysis of the chlorophyll tissue of *Aspidosperma*. (a) Percentage of non-galled and galled chlorophyll tissues of *Aspidosperma australe*. (b) Percentage of non-galled and galled chlorophyll tissue of *Aspidosperma spruceanum*. Bars followed by the same letter do not differ significantly at $P < 0.05$ level by the Tukey's test.

The cytological features of *A. spruceanum* are similar to those of *A. australe*. The non-galled tissues have chloroplasts with strong lamellation associated with primary starch grains (Fig. 4a and b). The galls also have starch grains, plastoglobules mainly at the periphery of the chloroplasts (Fig. 4c and d). The disorganization of the thylakoid membrane system, and the loss of the integrity of the grana are also apparent (Fig. 4c and d).

3.4. Photosynthetic pigments and chlorophyll a fluorescence

The photosynthetic pigment contents (total chlorophylls and carotenoids), and the ratios of chlorophyll a/b and total carotenoids/chlorophyll were similar in both the non-galled leaves and in the non-galled portion of the galled leaves of both species, either on area or weight basis (Table 1). However, the total contents of chlorophylls and carotenoid in the galls were lower than those in the non-galled leaves of *A. spruceanum*, and *A. australe*. The carotenoid/chlorophyll ratio was lower in the galls of *A. spruceanum*. The chlorophyll a/b ratio did not differ among the samples of both species.

The values of F_V/F_M at midday were higher than 0.8 (data not presented), and photoinhibition was not detected either in non-galled or in galled leaves (in non-galled portions and in the galls) of both species. The values of ETR were similar for all the samples of both host plant species (Table 1). The galls of *A. australe* had values of ETR_{MAX} of 64.0 ± 11.9 μmol m^{-2} s^{-1}, while the non-galled leaves, and the

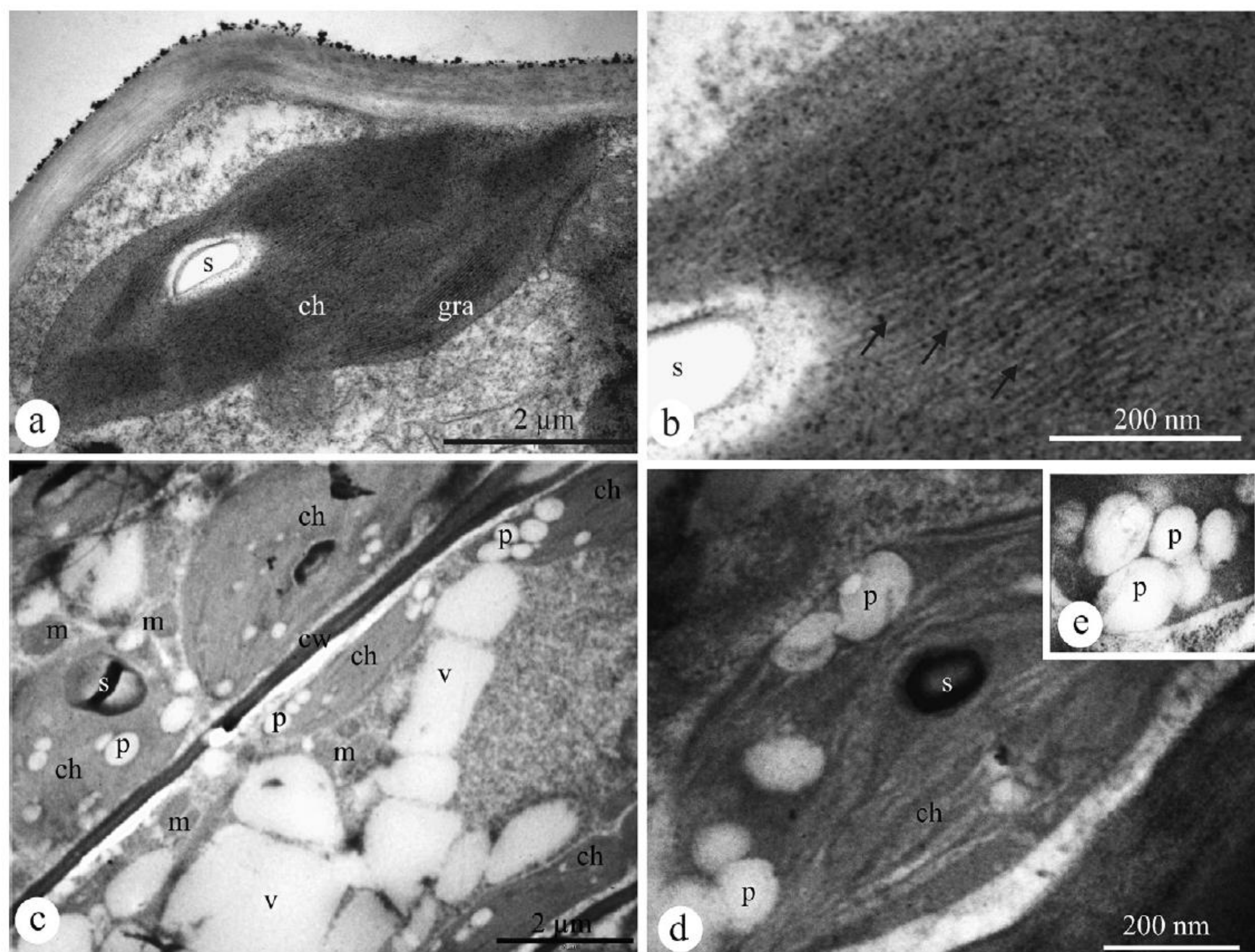

Fig. 3. Transmission electron micrographs of the cells of the mesophyll in non-galled leaves and galls of *Aspidosperma australe*. (a and b) Detail of a chloroplast in non-galled leaf. Intense lamellation and associated starch grains. (c) Cells of the outer gall cortex with plastids and plastoglobules. (d) A chloroplast with disaggregation of the membrane system and plastoglobules grouped at its periphery. (e) Detail of a plastoglobule in a chloroplast. ch – chloroplast, cw – cell wall, gra – grana, m – mitochondria, p – plastoglobule, s – starch, v – vacuole.

non-galled tissues of galled leaves had values of 74.6 ± 29.7 and $73.6 \pm 30.6\ \mu mol\ m^{-2}\ s^{-1}$, respectively. The galls of *A. spruceanum* showed values of ETR_{MAX} of $62.1 \pm 30.1\ \mu mol\ m^{-2}\ s^{-1}$, while in non-galled leaves and in non-galled tissues of galled leaves, these values were about 50.3 ± 16.3 and $42.7 \pm 16.6\ \mu mol\ m^{-2}\ s^{-1}$, respectively. The light curves showed no statistical differences between PAR_{SAT} and PAR in $1/2ETR_{MAX}$. *A. australe* showed a saturating PAR between 606.3 ± 22.1 and $676.8 \pm 181.8\ \mu mol\ m^{-2}\ s^{-1}$, while *A. spruceanum* PAR ranged between 452.7 ± 128.7 and $524.2 \pm 128.7\ \mu mol\ m^{-2}\ s^{-1}$.

4. Discussion

4.1. Histometric analysis of chlorophyll tissue and pigment contents

The galls of *Aspidosperma* spp. contained smaller amounts of photosynthetic pigments, as previously observed by Yang et al. [10] in galls of *Marchilus thunbergii* induced by a cecidomyiid. Also, the chlorophyll tissues were limited to the outer cortex, in both *A. australe* and in *A. spruceanum*. This position is several cell layers away from the feeding site of the galling larvae, which is the area of the gall with the highest level of oxidative stress [16,17]. Thus, it is plausible that the alterations in the pigment contents were not due to a reduction in the synthesis or degradation of pigments, but to an increase of non-chlorophyll tissues, as observed in the galls of *A. australe* and *A. spruceanum*. Also, there is no indication that the galls alter the phenology of their host plants [39], which proves that the reduction in pigment contents is not associated to leaf senescence.

4.2. Chlorophyll a fluorescence and chloroplast ultrastructure

The increase in number and size of plastoglobules at the periphery of the chloroplasts is cytological evidence of the increase of oxidative stress in gall tissues. The plastoglobules contain, among other proteins, tocopherol cyclase, an enzyme whose activity increases with oxidative stress. Tocopherol cyclase protects the thylakoid membranes and the proteins related to photosynthesis from damage caused by the ROS [40,41]. Also, this enzyme is proposed as a molecule that protects the membrane lipids from photooxidation, and photosystem II from photoinactivation [28]. The plastoglobules are functionally coupled, and structurally linked to each other and to the membranes of the thylakoids. This coupling allows the free exchange of lipophilic molecules such as plastoquinones, carotenoids, and tocopherol (VTE1) between the thylakoids and the plastoglobules, which is the site of synthesis

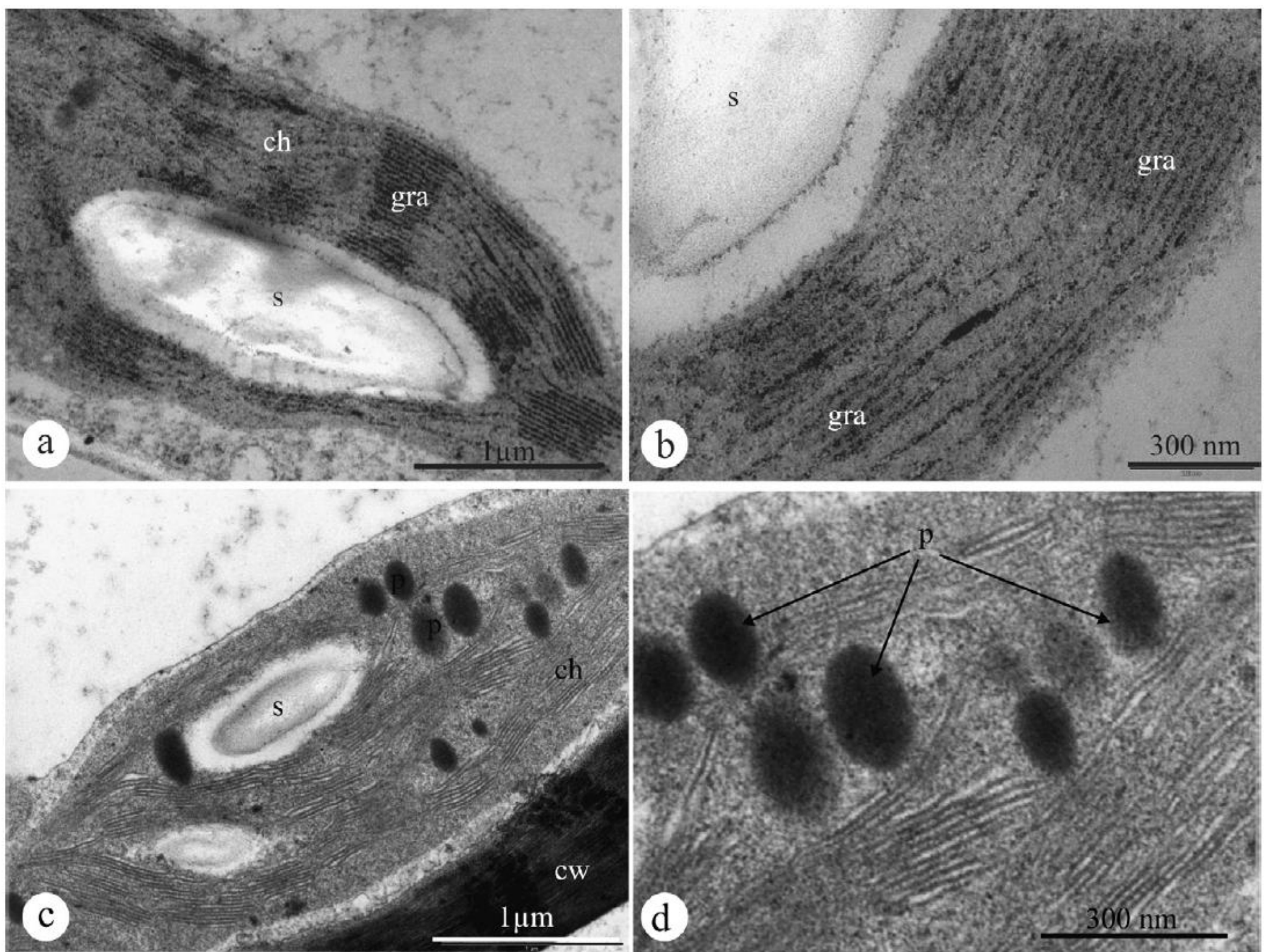

Fig. 4. Transmission electron micrographs of mesophyll cells in non-galled leaves and galls of *Aspidosperma spruceanum*. (a and b) Detail of a chloroplast in non-galled leaf. Intense lamellation and associated starch grains. (c) Cells of the outer cortex of the gall with plastoglobules in a chloroplast. (d) Detail of a chloroplast with disaggregation of the membrane system and grouped plastoglobules at its periphery. ch – chloroplast, cw – cell wall, gra – grana, m – mitochondria, p – plastoglobule, v – vacuole.

and storage of these substances. These molecules serve as electron corridors, protecting the photosynthetic apparatus from damage caused by the free radicals [19]. Also, tocopherol cyclase catalyzes the penultimate stage in the process of tocopherol synthesis (vitamin E) [19,41]. The production of carotenes and tocopherol is the mechanism of scavenging of the ROS in chloroplasts [26,27]. In the galls of *A. australe* and *A. spruceanum*, there was no variation in the ratio of carotenoids/total chlorophyll, which may suggest that the increase in number and size of plastoglobules did not result in changes in the pool of carotenoids. Nevertheless, the functioning of the PSII was not affected by gall induction.

By the larger number and size of the plastoglobules in the galls of *Aspidosperma* spp, there should be an increase in tocopherol and plastoglobulins synthesis [19,29]. In *Arabidopsis* and tobacco leaves, the increase in plastoglobulines increased the tolerance to light stress, while plants with reduced levels of this protein were

Table 1
Pigment contents (chlorophylls a and b, and carotenoids) in non-galled leaves, non-galled tissues of galled leaves and galls of *Aspidosperma australe* and *A. spruceanum*. Values followed by the same letters in the same line do not differ significantly at $P \leq 0.05$ level by the Tukey's test ($n = 10 \pm$ SD for pigment contents, and $n = 5 \pm$ SD for ETR dates).

	Total chlorophylls		Carotenoids		Chl a/b	Carot/chl	ETR_{MAX} (μmol m^{-2} s^{-1})	PAR sat (μmol m^{-2} s^{-1})	PAR $1/2ETR_{MAX}$ (μmol m^{-2} s^{-1})
	(μg cm^{-2} FM)	(mg g^{-1} FM)	(μg cm^{-2} FM)	(mg g^{-1} FM)					
A. australe									
Non-galled leaves	76.8 ± 24.9^{a}	3.7 ± 1.2^{a}	16.6 ± 4.1^{a}	0.8 ± 0.2^{a}	3.0 ± 0.1^{a}	0.22 ± 0.02^{a}	74.6 ± 29.7^{a}	621.1 ± 229.6^{a}	186.9 ± 69.3^{a}
Non-galled tissues	$51,6 \pm 17.1^{ab}$	3.0 ± 1.0^{a}	12.0 ± 3.4^{ab}	0.7 ± 0.2^{a}	3.7 ± 0.8^{a}	0.23 ± 0.1^{a}	73.7 ± 30.6^{a}	606.3 ± 222.1^{a}	182.7 ± 67.0^{a}
Galls	35.5 ± 19.7^{b}	0.9 ± 0.5^{b}	7.9 ± 3.9^{b}	0.2 ± 0.1^{b}	3.4 ± 1.4^{a}	0.22 ± 0.07^{a}	64.0 ± 12.0^{a}	676.8 ± 181.8^{a}	203.9 ± 54.7^{a}
P	0.0008	0.0006	0.042	0.030	0.536	0.969	0.722	0.808	0.807
A. spruceanum									
Non-galled leaves	51.5 ± 13.6^{a}	1.9 ± 0.5^{a}	13.6 ± 2.7^{a}	0.5 ± 0.1^{a}	4.04 ± 1.7^{a}	0.23 ± 0.03^{a}	50.4 ± 16.3^{a}	473.6 ± 117.6^{a}	142.7 ± 53.4^{a}
Non-galled tissues	48.8 ± 10.8^{a}	1.8 ± 0.4^{a}	8.4 ± 2.7^{a}	0.31 ± 0.1^{a}	3.92 ± 1.0^{a}	0.18 ± 0.07^{a}	42.7 ± 16.6^{a}	452.7 ± 128.7^{a}	136.4 ± 38.7^{a}
Galls	10.5 ± 3.5^{b}	0.6 ± 0.2^{b}	1.7 ± 0.8^{b}	0.1 ± 0.05^{b}	3.55 ± 0.7^{a}	0.15 ± 0.05^{b}	62.1 ± 30.1^{a}	524.2 ± 128.7^{a}	159.5 ± 57.6^{a}
P	<0.0001	<0.0001	0.0001	0.0001	0.774	0.050	0.300	0.557	0.618

strongly photoinhibited [29,42,43]. This would constitute an alternative strategy to protect the photosynthetic apparatus against the damage caused by oxidative stress during gall formation. This supposition was corroborated by the similarity between ETR values in the non-galled and galled tissues.

4.3. Feeding habit × photosynthesis

In *A. australe*, the galls induced by *Pseudophacopteron* sp, a sucking insect, did not present a nutritive tissue [16]. However, its feeding activity. could increase the sinking of photoassimilates towards the gall site. In *A. spruceanum*, a typical nutritive tissue, spatially separated from the chlorophyllian tissue by layers of lignified cells, was differentiated [17]. The development of this specific site for the feeding of the galling herbivore should explain the low damage caused to the photosynthetic cells and, consequently, the maintenance of the photosynthetic rates. According to Crawley [44], the photosynthetic rates may be increased in the host plants in function of additional sinks induced by sucking insects. Similarly, insect galls that do not cause mechanical damages to the photosynthetic cells can increase the photosynthetic rates in their host plant [45,46], or simply do not to alter these rates as was observed in *A. australe*. Although the symptoms detected during the development of the galls of *A. australe* and *A. spruceanum* indicated an increase in the oxidative stress, and damages to the membrane system of the chloroplasts, the photosynthetic performance assessed by chlorophyll *a* fluorescence remained unaffected. Also, plant cells may respond to galling stimuli by differentiating plastoglobules, which additionally can explain the maintenance of the photosynthetic performance in its host plant tissues.

4.4. Conclusions

Even though structural changes and sites of oxidative stress were detected during the development of the galls of *A. australe* and *A. spruceanum* [16,17], the damage to the chloroplast membrane system was not accompanied by a reduction in photosynthetic performance. Our results indicate that the two systems have distinct plant anatomical responses to the feeding activity of their associated galling herbivores. Nevertheless, the plastoglobules as ROS scavengers may generate an equilibrium in the physiology of the galls converging to the maintenance of the photosynthetic performance. This is the first evidence of a similar cytological strategy in response to two distinct galling herbivores attack in galls of Neotropical plants.

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Considerações finais e perspectivas

De forma geral, galhas são estruturas vegetais complexas que possuem tecidos típicos com características e funções semelhantes a um órgão vegetal. A funcionalidade destes diferentes tecidos rediferenciados a partir dos tecidos da planta hospedeira proporciona uma série de estudos ontogenéticos e fisiológicos. Assim, galhas podem ser consideradas verdadeiros microlaboratórios para estudos estruturais e funcionais.

Independente do taxa do indutor, as galhas apresentam um gradiente citológico associado. Em geral, as camadas de células mais próximas a câmara larval, apresentam células com metabolismo maior, muitas mitocôndrias, núcleo volumoso e nucléolo conspícuo entre outras características. Entretanto, estas células também podem se apresentar mortas na maturidade. O gradiente citológico é acompanhado por um gradiente histoquímico, principalmente em relação a substâncias de reserva para o galhador e para a manutenção da maquinaria celular da galha. Também, a detecção de atividade enzimática nos tecido configura-se uma ferramenta importante para estudos de gradiente histoquímico. Nesta Tese, nós hipotetizamos que o principal gatilho para a formação destes gradientes citológicos e histoquímicos seja a formação de espécies reativas de oxigênio (ERO). As ERO atuariam como sinalizadores para várias etapas do desenvolvimento das galhas, incluindo o acúmulo de reservas nos tecidos.

Este estudo representa mais um passo importante para a compreensão das interações inseto-planta que levam a formação de galhas. Várias questões surgiram no final desta Tese e uma nova linha de pesquisa, imunocitoquímica de parede celular em galhas, já está em eminência. Dentre elas, destaca-se a análise de gradiente funcionais. Neste caso, a imunocitoquímica possibilita avaliar o impacto da interação nas paredes celulares, e conseqüentemente na funcionalidade da galha. As possibilidades que a imunocitoquímica de parede celular permitem discutir são tão relevantes para o conhecimento do desenvolvimento celular e histológico que uma nova tese de doutorado já encontra-se em andamento. Investir em análises de ERO e sua importância no desenvolvimento vegetal também é um caminho a ser seguido, e que pode, sem dúvida, levantar várias outras questões.

De fato, o melhor produto de uma Tese ou de um sistema biológico são as perguntas que elas geram.