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ANETE TEIXEIRA FORMIGA

DISTRIBUIÇÃO TEMPORAL E RESPOSTAS CELULARES DE *BACCHARIS RETICULARIA* DC. (ASTERACEAE) A INTERAÇÕES BIÓTICAS

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

Área de Concentração Anatomia Vegeral

Orientadora: Profa. Dra. Rosy Mary dos Santos Isaias
Universidade Federal de Minas Gerais

Coorientador: Prof. Dr. Geraldo Wilson Fernandes
Universidade Federal de Minas Gerais

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Banca Examinadora

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_____ Prof. Dr. Hildeberto Caldas de Souza (UFOP)

_____ Prof. Dr. Dênis Coelho Oliveira (UFU)

_____ Prof. Dr. Fernando Vale (UFMG)

_____ Profa Dra Rosy Mary dos Santos Isaias (Orientadora)

_____ Prof. Dr. Geraldo Wilson A. Fernandes (UFMG) (Coorientador)

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Resumo

Galhas são modelos de estudo elegantes de desenvolvimento celular vegetal sob influência de um organismo externo, o galhador. Apesar de comumente incluírem dois organismos, a planta hospedeira e o indutor, as galhas podem incluir sistemas tri- ou multitróficos. Os padrões de desenvolvimento observados nestes sistemas podem ser avaliados por meio do estudo das modificações celulares, anatômicas e histoquímicas que resultam nos diferentes morfotipos encontrados, além da relação desses morfotipos com variações ambientais. As super-hospedeiras, plantas nas quais podemos encontrar diversos morfotipos de galhas, são interessantes para estudos de desenvolvimento vegetal, pois em um mesmo *pool* gênico, diferentes estruturas se desenvolvem, resultado de um misto de influências dos estímulos dos insetos indutores e das restrições impostas pela planta hospedeira. O presente estudo apresenta as similaridades estruturais e histoquímicas entre os morfotipos de galhas desenvolvidos em *B. reticularia* e discute a similaridade das galhas foliares e caulinares quanto aos níveis de complexidade estrutural e a maior ou menor proximidade ao padrão morfogenético dos órgãos hospedeiros. No nível microscópico, as alterações pécticas da parede celular das galhas foram comparados utilizando marcação específica para seis epitopos pécticos: extensinas, HGA de alta e baixa metil esterificação, AGP glicanos, galactanos e arabinanos. Os resultados indicam a manutenção do potencial de alongamento e flexibilidade ao longo do desenvolvimento das galhas, comprovando a existência de uma dinâmica péctica crucial ao estabelecimento das galhas. O fato de extensinas terem sido detectadas somente nos tecidos da galha em bolso nos leva a crer que o desenvolvimento deste morfotipo resulta em modificações para o maior alongamento e flexibilidade celular necessários para atingir sua forma final. No nível macroscópico, pode ser verificado o estabelecimento de duas síndromes sazonais reflexo das flutuações dos diversos morfotipos ao longo do tempo. Finalizando, a interação tritrófica da planta hospedeira, organismos galhadores e fungos endofíticos influenciou positivamente o valor nutricional das plantas com maiores valores de nitrogênio e fósforo encontrados nas plantas com presença da galha reniforme.

Palavras chave: galhas, morfotipos, epitopos pécticos, sazonalidade, interações tritróficas.

Abstract

Galls are elegant models for the study of plant cell development under the influence of an external organism, the gall inducer. Although commonly occurring due to the relationship between two organisms, the host plant and the inducer, the galls may also include tritrophic or multitrophic systems. Developmental patterns observed in these systems can be evaluated through the study of structural and histochemical modifications, as well as the influence of environmental variations that result in different gall morphotypes. The plants which host different gall morphotypes are known as superhosts and are interesting for studying plant development. In these cases, different structures are developed as a result of the manipulation of the same pool of genes, under the influence of the insects' stimuli *vs.* host plant developmental constraints. This study analyzes the structure and histochemistry of the gall morphotypes developed in *Baccharis reticularia*, focusing on the similarities between leaf and stem galls. The structural complexity of each gall is discussed under the perspective of the greater or lesser proximity to the morphogenetic pattern of their host organs. At the microscopic level, changes on the composition of the cell wall in the galls were compared using six specifically labeled epitopes: Extensins, HGA's with high and low methyl esterification, AGP glycans, galactans and arabinans. The results indicate that the stretching and flexibility potential of the plant cell wall is maintained throughout the development of the galls, and the dynamic constitution of the cell wall was, thus, proved to be crucial to their establishment. The fact that extensins were only detected in the tissues of the pocket gall morphotype is believed to be due to the greater cell elongation and flexibility required on the achievement of its final form. At the macroscopic level, two seasonal syndromes were found to be determinant for the fluctuations of the different gall morphotypes over time. Finally, the tritrophic interaction between the host plant, endophytic fungi and galling organisms positively influenced the nutritional value of plants, ensuring higher nitrogen and phosphorus content in plants bearing the kidney-shaped gall morphotype.

Keywords: galls, morphotypes, pectic epitopes, seasonality, tritrophic interactions.

Introdução geral

Dentre as relações ecológicas entre plantas e animais, aquelas estabelecidas entre insetos galhadores e plantas hospedeiras são notavelmente especializadas, determinando a morfogênese de estruturas anômalas ao crescimento vegetal padrão. Os insetos galhadores induzem alterações morfológicas específicas em suas plantas hospedeiras que são cruciais para sua sobrevivência (Mani 1964, 1992), pois os tecidos da galha nutrem e abrigam esses insetos durante seu desenvolvimento endofítico.

A maioria das plantas hospedeiras abriga um único inseto indutor, porém as super-hospedeiras merecem especial atenção por abrigarem uma comunidade de insetos galhadores, respondendo diferentemente aos estímulos promovidos por cada *taxa* de inseto. Neste caso, as galhas são entendidas como o fenótipo estendido dos seus indutores, dadas as características conservativas de sua morfogênese. As super-hospedeiras constituem modelos interessantes de estudo, devido à possibilidade de se comparar as modificações específicas induzidas por cada indutor em uma mesma planta hospedeira. Essas modificações são produzidas por uma série de reações celulares que ocorrem em resposta ao estímulo contínuo produzido pelo galhador (Mani 1964, Oliveira *et al*. 2010, Formiga *et al*. 2011, Isaias *et al*. 2011, Oliveira *et al.* 2011, Formiga *et al.* 2012).

O padrão de expansão e divisão celular nos diferentes tecidos vegetais tem papel preponderante na determinação do formato final dos órgãos vegetais. Sendo as galhas consideradas órgãos neoformados, o estudo de sua ontogênese permite definir quais são as células do órgão hospedeiro que mais se modificam e como a alteração de seus destinos leva à variedade de formas observadas na natureza (Moura *et al.* 2009, Oliveira & Isaias 2009, Isaias *et al.* 2011). Estas formas são determinadas por meio de diferentes graus de complexidade, sendo consideradas galhas mais simples aquelas que mais se assemelham ao órgão hospedeiro na condição não-galhada (Formiga *et al.* 2013). Além das alterações estruturais específicas de cada galha, a análise comparativa entre morfotipos induzidos em um mesmo hospedeiro permite traçar padrões de similaridade estrutural entre as galhas, dependentes de suas plantas hospedeiras e *taxa* de indutores (Rohfritsch 1992).

Apesar de as galhas serem consideradas como um fenótipo estendido do galhador (Dawkins 1982, Stern 1995, Crespi *et al.* 1997, Stone & Cook 1998, Stone & Schonrogge 2003, Raman 2011), as plantas hospedeiras também são determinantes para a estrutura final da galha, uma vez que estas são formadas exclusivamente por células vegetais e se desenvolvem dentro de limites morfogênicos restritivos da planta hospedeira (Isaias & Oliveira 2011, Isaias & Oliveira 2012, Formiga *et al.* 2013). Os diferentes insetos galhadores associados às super-hospedeiras atuam dentro de limites morfogênicos impostos por elas, induzindo, contudo, o desenvolvimento de diferentes morfotipos de galhas. Isto indica que os indutores têm capacidades distintas de estimular o *pool* gênico de suas hospedeiras, mas são incapazes de expressar caracteres que não estejam previamente determinados pelo genoma vegetal, o que determina o aparecimento de padrões conservativos. Um dos gêneros com muitas hospedeiras de galhas é *Baccharis*, cujas espécies têm sido objeto de estudos ecológicos (Burkhardt *et al*. 2004; Fagundes *et al.* 2005; Carneiro *et al.* 2006; Fernandes *et al.* 2007), morfológicos (Arduin & Kraus 2001; Arduin *et al.* 2005) e anatômicos (Oliveira & Bastos 1998; Arduin & Kraus 2001; Pegorini *et al.* 2008). *Baccharis reticularia* DC. (Asteraceae) é uma super-hospedeira de insetos galhadores, e possui cinco morfotipos de galhas induzidas por diferentes insetos.

Diagnoses morfológicas sobre as galhas na região neotropical tiveram seu início com os trabalhos de Tavares (1906, 1915, 1917a, 1917b, 1918, 1920, 1921, 1922, 1925) que apresentavam desenhos esquemáticos das galhas, identificação da planta hospedeira, muitas vezes em nível de família ou gênero, e o galhador comumente não identificado. Desde estes primeiros esforços, os trabalhos sobre galhas neotropicais avançaram para abordagens ecológicas (Lara *et al.* 2008; Carneiro *et al.* 2009; Coelho *et al*. 2009; Maia *et al*. 2009), morfológicas e anatômicas (Kraus *et al*. 1993; Arduin *et al.* 1994; Kraus *et al.* 1996; Kraus *et al*. 1998; Kraus & Tanoue 1999; Souza *et al.* 2000) durante o século XX. No século XXI, o interesse no desenvolvimento anatômico (Formiga *et al.* 2009, Oliveira & Isaias 2009, Sá *et al.* 2009), aliado a investigações bioquímicas e fisiológicas ajudaram na compreensão do metabolismo das galhas (Oliveira & Isaias 2009, Campos *et al.* 2010, Oliveira & Isaias 2010, Oliveira *et al.* 2011).

O estudo de alterações estruturais e metabólicas decorrentes da indução de galhas avança também, neste século, pela análise de variações da composição e

orientação dos componentes da parede celular (*sensu* Baskin 2005). Estudos recentes têm demonstrado que a imunolocalização de compostos das paredes celulares permite uma nova abordagem para a compreensão dos padrões de desenvolvimento de tecidos vegetais, utilizando galhas de insetos como modelo (Formiga *et al.* 2013). A parede celular é uma estrutura complexa, formada por uma matriz de microfibrilas de celulose e hemiceluloses, polissacarídeos pécticos e glicoproteínas. Alterações pécticas podem ocorrer durante o processo de diferenciação celular e desenvolvimento da planta (Knox *et al.*1990, Albersheim *et al.* 2010) e mesmo quando as celuloses e hemiceluloses estão ausentes, as pectinas podem manter a integridade das paredes celulares (Dolan *et al.* 1997, Albersheim *et al.* 2010). Tais alterações podem determinar mudanças morfológicas e/ou funcionais na parede possivelmente ligadas ao estresse sofrido pela planta no momento da indução da galha. Sendo assim, a composição das paredes celulares vegetais é fator determinante do formato e das novas funções dos tecidos das galhas (Formiga *et al.* 2013).

O tempo de permanência do inseto galhador no interior de sua galha pode ser variável, o que acaba por determinar padrões característicos de respostas celulares vegetais e de dinâmicas populacionais dos insetos. A ocorrência das galhas nos Neotropicos pode estar relacionada a diferentes fatores ambientais, tais como temperatura e pluviosidade (Pinheiro *et al.* 2002), frequentemente determinando sua sazonalidade. A alternância de síndromes sazonais parece ser uma estratégia dos galhadores associados à super-hospedeiras, como demonstrado por Oliveira *et al*. (2012).

Outro fator ecológico determinante para a dinâmica populacional de insetos galhadores é a interação com outros níveis tróficos, estabelecendo relações tritróficas (Cuevas-Reyes *et al.* 2007). Além de predadores e parasitoides, insetos galhadores podem ter relação com fungos endofíticos, presentes em diferentes espécies vegetais (Fernandes & Price 1992; Wilson 1993, 1995; Sinclair & Cerkauskas 1996; Oki *et al.* 2008, 2009). Fungos são organismos heterotróficos e sua sobrevivência depende das associações que conseguem fazer com outros organismos, principalmente com plantas (Zoberi 1972), sendo parasitismo ou mutualismo as relações mais comuns (Richardson 1999). No primeiro caso, os fungos vivem dentro das plantas, obtendo alimento e abrigo, podendo causar prejuízo para as plantas, pelo consumo de nutrientes e síntese de

substâncias tóxicas. Em associações mutualísticas, a presença dos fungos é assintomática e ambos podem ser beneficiados pela associação. Os fungos obtêm nutrição e proteção das plantas, e estas, por sua vez, aumentam sua resistência contra fatores bióticos (Clay 1988) e abióticos (Saikkonen *et al.* 1998). No caso das galhas, podemos observar diferentes espécies de fungos em plantas não galhadas e com galhas, sugerindo que a micota se adapta diferentemente à presença destas. Quando presentes em galhas, os fungos podem ser utilizados como alimento para o galhador (Arduin 2001), Sá *et al.* 2009) ou protegê-lo de possíveis predadores através da produção de substâncias tóxicas (Oki *et al.* 2008, 2009). O estudo da associação tritrófica entre *B. reticularia*, os insetos galhadores e os fungos endofíticos permite evidenciar que a presença de galhas não altera a riqueza de fungos endofíticos, mas pode causar grandes mudanças na composição dos morfotaxa.

Esta tese foi diagramada na perspectiva dos resultados do nível microscópico para o macroscópico. As similaridades anatômicas entre as galhas são discutidas com enfoque nos padrões morfogenéticos da super-hospedeira *B. reticularia*, e a análise comparativa entre os morfotipos é discutida em função da plasticidade dos órgãos hospedeiros, caules e folhas. As observações anatômicas são complementadas pela detecção imunocitoquímica de epitopos pécticos da parede celular, que controlam modificações nas formas e funções celulares. No nível macroscópico, é feita a análise da flutuação sazonal de cada morfotipo e a influência da precipitação e da temperatura na geração de síndromes sazonais que parecem ser cruciais para a colonização das super-hospedeiras. Por fim, a presença de micota diferencial entre plantas de *B. reticularia* não galhadas e com galhas influencia principalmente no aspecto nutricional das plantas.

Os cinco estudos que compõem essa tese foram escritos em formato de artigos científicos, relacionados abaixo.

1 - *Respostas estruturais e histoquímicas de* ***Baccharis reticularia*** *DC. (Asteraceae) a diferentes herbívoros galhadores* - Formatado de acordo com a Acta Botanica Brasilica.

2 - *Phenotypic plasticity and similarity among galling species on a multihost,* ***Baccharis reticularia*** - submetido à revista Plant Biology.

3 - *The role of pectic composition of cell walls in the determination of the new shape-functional design in galls of* ***Baccharis reticularia*** *(Asteraceae)* - publicado na revista Protoplasma.

4 - *Seasonal fluctuation of the gall morphotypes under the stability of the tropical climate on the superhost* ***Baccharis reticularia*** - submetido à Arthropod Plant Interactions.

5 - *Tritrophic interactions among host plant, galling herbivores and fungal endophytes* - submetido ao Journal of Plant Interactions.

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Respostas estruturais e histoquímicas de *Baccharis reticularia* DC. (Asteraceae) a diferentes herbívoros galhadores

Anete Teixeira Formiga[1] Renê Gonçalves da Silva Carneiro[1] G. Wilson Fernandes[1] Rosy Mary dos Santos Isaias[1*]

1 - Universidade Federal de Minas Gerais – Instituto de Ciências Biológicas Av. Antônio Carlos, 6627 - Pampulha - Belo Horizonte – MG CEP 31270-901 - Fone: +55 (31) 3409 2675, Fax number: +55(31) 3409 2671.

*Autor para correspondência: rosy@icb.ufmg.br

RESUMO

(Respostas estruturais e histoquímicas de *Baccharis reticularia* DC. (Asteraceae) a diferentes herbívoros galhadores). Galhas são estruturas anômalas formadas pelos vegetais a partir do estímulo de um indutor, na maioria das vezes um inseto. Tais estruturas resultam da perfeita sincronia do inseto indutor com a planta hospedeira, estas por sua vez, podem abrigar uma única interação ou várias interações com diferentes insetos galhadores. No segundo caso, são chamadas de super-hospedeiras e merecem especial atenção por responder diferentemente aos estímulos promovidos por cada *taxa* de inseto. Especialmente em super-hospedeiras, o estudo dos perfis estruturais e histoquímicos de galhas é interessante, pois permite delimitar os padrões anatômicos e químicos impostos pelas potencialidades da planta hospedeira, bem como as mudanças específicas induzidas em cada morfotipo pelo galhador. *Baccharis reticularia* apresenta cinco morfotipos de galha, cuja caracterização morfológica, anatômica e histoquímica comparativa foi realizada. Este estudo visa evidenciar as alterações induzidas pelos estímulos dos galhadores detectando similaridades estruturais e histoquímicas entre os morfotipos de galhas em *B. reticularia* que reflitam restrições impostas pela morfogênese padrão dos órgãos hospedeiros. As maiores modificações estruturais induzidas pelos galhadores foram observadas nos sistemas de revestimento e fundamental. A diferenciação de periderme e ductos secretores nas galhas globóide e reniforme representam uma potencialização da capacidade morfogenética da planta hospedeira. A detecção de polifenois exclusivamente nas galhas mostrou ser um potencial inerente à espécie hospedeira, já relatado para outras espécies do mesmo gênero.

Palavras-chave: *Baccharis*, anatomia, galhas, super-hospedeiras, padrões estruturais.

ABSTRACT

(Histochemical and structural responses of *Baccharis reticularia* DC. (Asteraceae) at different galling herbivores). Galls are abnormal structures formed by plants through the stimulus of an inducer, oftentimes an insect.These structures result from the perfectly synchronized relationship between the gall makers and host plants, which can bear one or multiple galling insects. In case they host more than one insect, the plants are called super-hosts and deserve special attention for they respond differently to the stimuli promoted by each insect *taxa*. In these cases, the study of structural and histochemical profiles of galls is interesting because it allows defining the anatomical patterns and chemical potential imposed by the host plant, as well as the specific changes induced by each gall morphotype. *Baccharis reticularia* presents five gall morphotypes whose morphology, anatomy and histochemistry were comparatively analyzed. This studyaims to highlight the changes induced by the stimuli of galling herbivores, detecting similarities between the gall morphotypes in *B. reticularia* that reflect the constraints imposed by the standard organ morphogenesis of the host. The major structural modifications induced by the galls were observed in thedermic and fundamental tissues. The differentiation of periderm and secretory ducts in the globular andkidney-shaped galls represent an enhancement of the morphogenetic capabilities of the host plant. The detection of polyphenols exclusively in galls was proven to be an inherent potential of the host species,which has been reported for other species of the same genus.

Key words: *Baccharis*, anatomy, galls, super-host, structural patterns.

Introdução

Os insetos indutores de galhas mantêm uma relação espécie-específica com suas plantas hospedeiras, fazendo com que cada sistema seja único e característico da interação. Dentre as plantas portadoras de galhas, existem aquelas que abrigam uma única interação e aquelas que abrigam um *pool* de interações com insetos galhadores, as super-hospedeiras. Estas configuram modelos de estudo interessantes, pois respondem diferentemente aos estímulos promovidos por cada *taxa* de inseto. A expressão visual de cada uma dessas interações constitui um morfotipo de galha que, devido à sua morfogênese conservativa (Raman 2007), pode ser interpretado como o fenótipo estendido do seu indutor (*sensu* Dawkins 1982). Nas super-hospedeiras, o estudo da morfogênese e do perfil histoquímico de galhas permite delimitar padrões estruturais e químicos impostos pelas potencialidades da planta hospedeira, bem como as mudanças específicas de cada morfotipo de galha, dependentes do galhador.

Dentre os biomas Neotropicais, especialmente os brasileiros, o Cerrado é tido como um cenário ambiental propício ao sucesso evolutivo dos insetos de hábito galhador (Gonçalves-Alvin & Fernandes 2001; Urso-Guimarães *et al*. 2003; Araújo *et al.* 2007; Ferreira *et al.* 2007; Coelho *et al.* 2009) abrigando várias super-hospedeiras. Essas plantas têm sido estudadas à luz de suas respostas teciduais diferenciadas (Drummond 2005, Oliveira *et al.* 2008, Oliveira & Isaias 2009, Oliveira *et al.* 2011), sendo uma delas *Baccharis reticularia*, foco do presente estudo. Na Serra do Caraça, *B. reticularia* apresenta 5 diferentes morfotipos de galhas, cuja caracterização morfológica, anatômica e histoquímica comparativa é aqui abordada. Esta caracterização visa evidenciar as alterações induzidas pelos estímulos dos galhadores de modo a responder as seguintes questões: (1) há similaridades estruturais e histoquímicas entre os morfotipos de galhas em *B. reticularia* que reflitam restricões impostas pela morfogênese padrão dos órgãos hospedeiros? (2) Os níveis de complexidade estrutural estão relacionados ao impacto dos galhadores sobre os tecidos hospedeiros e determinam o fenótipo das galhas?

Metodologia

Área de Estudo e planta hospedeira

A área de estudo constitui-se de quatro afloramentos rochosos dispostos em uma área de campo rupestre denominada Campo de Fora, que encontra-se a 1.511m de altitude nas coordenadas 20º07´035 Sul e 43º31´201 Oeste, na Serra do Caraça, MG. A planta escolhida para este estudo, *Baccharis reticularia*, é dióica, tem ramos lenhosos com folhas verdes claras, espiraladas, caule de aspecto lenhoso e altura entre 50cm e 5m para os indivíduos observados (Fig. 1A).

Amostragem

Coletas de galhas foliares e caulinares, além de folhas e caules não galhados ocorreram mensalmente, no período de maio de 2009 a dezembro de 2011. Parte das galhas ($n \geq 12$) foi fixada em solução de Karnovsky (O´Brien & McCully 1981) ou FAA (Formalina, ácido acético e álcool etílico) e parte ($n = 60$) foi acondicionada em sacos plásticos e transportada em bolsa térmica ao laboratório para realização de testes histoquímicos e dissecção em estereomicroscópio para caracterização morfológica e coleta dos indutores e fauna associada. Os insetos foram fixados em etanol 70% e enviados a especialistas para identificação.

Ramos floridos foram coletados, prensados, montados em exsicatas, identificados por taxonomistas e incorporados ao Herbário BHCB do Instituto de Ciências Biológicas da UFMG sob os números 161554 e 161555.

Caracterização anatômica e histoquímica dos morfotipos de galhas

Amostras de galhas foliares e caulinares, folhas e caules não galhados foram desidratadas e incluídas em PEG (polietilenoglicol 6000) ou em Paraplast®. O material foi seccionado em micrótomo rotatório Reichert-Jung® (10-20µm), corado com safranina-azul de astra 8:2 (v/v) e montado em gelatina glicerinada de Kaiser (Kraus & Arduin 1997) ou em verniz vitral incolor® (Paiva *et al.* 2006). Testes histoquímicos para detecção de substâncias do metabolismo primário e secundário (Tab. 1) foram realizados em seções à mão livre com auxílio de lâmina de barbear. As lâminas

permanentes e aquelas com os testes histoquímicos foram analisadas em microscopia de luz e fotodocumentadas.

Resultados

Descrição dos morfotipos

A galha ***reniforme*** é formada pelo enrolamento e dobramento das margens foliares, é aberta, séssil, pilosa, possui coloração verde, e forma uma câmara larval que pode conter de 1 a 4 indutores da ordem Hemiptera (Fig. 1B). A câmara é revestida internamente por cera branca, produzida pelo(s) indutor(es) (Fig. 1C). Ocorre isoladamente, tomando toda a área foliar e, na senescência, apresenta coloração preta e mecanismo de deiscência por afastamento das margens foliares. A galha de e***nrolamento*** ocorre por meio do enrolamento de uma das margens da folha, sendo aberta, séssil e pilosa. Possui coloração verde e ocorre isoladamente (Fig. 1D); a câmara larval abriga de 1 a 2 insetos indutores da ordem Hemiptera. Na senescência, as galhas tornam-se amarronzadas, ocorrendo abscisão foliar subsequente. A galha e***m bolso*** é formada pelo abaulamento da superfície foliar, sendo aberta, séssil e pilosa. Possui coloração verde e ocorre isoladamente, tomando toda a folha (Fig. 1E). Na senescência, torna-se amarronzada e quebradiça e sofre abscisão. A galha ***fusiforme*** é induzida no ápice caulinar, isolada, possui formato afilado nas extremidades apical e basal, e maior intumescimento na região mediana, onde se encontra a câmara larval. Possui cor esverdeada desde a indução até a maturidade, quando adquire coloração castanha, à semelhança do caule não galhado. É fechada, séssil, glabra, apresentando folhas em sua superfície (Fig. 1F). O caule cessa seu desenvolvimento acima da galha. Foi registrada a presença de um endoparasitóide não identificado, que oviposita dentro do corpo da larva, gerando várias larvas cujo desenvolvimento mata seu hospedeiro (Fig. 1G). O inseto indutor é um Lepidoptera não identificado (Fig. 1H). Na fase de senescência, esta galha permanece no caule. A galha ***globóide*** é induzida no ápice caulinar e forma uma estrutura fechada, séssil e glabra, que ocorre isolada ou agrupada. Possui coloração verde na maturidade e amarronzada, à semelhança do caule, na senescência. A estrutura caulinar e os primórdios foliares continuam a se desenvolver acima do sítio de desenvolvimento da galha (Fig. 1I). É induzida por um Diptera não identificado. Na fase senescente, permanece no ramo e apresenta coloração amarronzada característica da estrutura lenhosa.

Anatomia de órgãos não galhados e galhas

Lamina foliar não galhada **-** Em secção transversal, a folha apresenta epiderme unisseriada, com células de sessão transversal poligonal sobre as regiões internervurais e (Fig. 2A) papilosas sobre as nervuras. A cutícula é delgada. As células epidérmicas apresentam paredes anticlinais retilíneas com estômatos do tipo anomocítico, localizados em ambas as faces no mesmo nível das demais células epidérmicas (Fig. 2B). Tricomas glandulares podem ser observados em ambas as faces epidérmicas, inseridos em depressões individualmente ou em agrupamentos de 2-15 (Fig. 2C-D). Nos bordos, as células têm paredes retilíneas. O mesofilo é homogêneo, composto por 7-8 camadas de parênquima clorofiliano com maior quantidade de cloroplastos concentrados nas regiões adjacentes à epiderme (Fig. 2A). O feixe vascular da nervura mediana possui arranjo colateral e é circundado por fibras pericíclicas (Fig. 2E). Feixes vasculares de menor calibre encontram-se dispostos ao longo de toda a lâmina foliar, imersos entre as células de parênquima clorofiliano, sem tecidos de sustentação associados. Ductos secretores estão distribuídos ao longo da lâmina associados aos feixes vasculares.

Caule não galhado **-** Em secção transversal, o caule apresenta formato semicilíndrico com 6-8 costelas revestidas por epiderme uniestratificada papilosa (Fig. 2F-G). Tricomas glandulares pluricelulares ocorrem isolados ou agrupados, inseridos em leves depressões na epiderme, enquanto os estômatos são protuberantes em relação as demais células epidérmicas. A região cortical é formada por 6-7 camadas de células parenquimáticas de dimensões variadas em meio às quais distinguem-se 6-7 traços foliares com ductos secretores associados (Fig. 2G). O sistema vascular possui cerca de 16 feixes colaterais, em início de crescimento secundário, com presença de parênquima interfascicular. As fibras do floema primário formam calotas em formato de meia lua. A região medular possui células parenquimáticas isodiamétricas de paredes delgadas.

Galha reniforme **-** Esta galha é formada por hiperplasia e hipertrofia dos sistemas fundamental e de revestimento. A epiderme é uniestratificada, com células maiores e de formato quadrangular na face adaxial e de formato retangular na face abaxial. Nos bordos foliares, as células epidérmicas são papilosas. O córtex da galha, na região adjacente à nervura principal é formado por 14-20 camadas celulares homogêneas em

meio às quais se observa a proliferação de ductos secretores hipertrofiados não estritamente associados aos feixes vasculares e que ocupam grande parte do córtex da galha (Fig. 3A, detalhe). O sistema vascular não sofre alterações. As margens foliares não se fundem, permanecendo uma fenda (Fig. 3B). Os bordos são mais afilados quando comparados aos da folha não galhada, possuindo 2-3 camadas celulares (Fig. 3B).

Galha de enrolamento **-** O tecido de revestimento da face abaxial apresenta células hipertrofiadas, estômatos e tricomas glandulares. A região do bordo torna-se afilada, com 3 a 6 camadas de células hipotrofiadas homogêneas (Fig. 3C). Na região mediana da galha, o córtex é composto por 6-7 camadas de parênquima homogêneo, com ductos hipertrofiados que se mantêm associados aos feixes vasculares (Fig. 3D, detalhe). O sistema vascular não apresenta alterações em relação ao padrão não galhado.

Galha em bolso **-** As células do sistema de revestimento na face adaxial são hipertrofiadas, com estômatos e tricomas entremeados. O córtex é homogêneo, composto por 6-8 camadas de células alongadas anticlinalmente. Os ductos secretores localizados ao longo da lâmina possuem grande calibre, por vezes chegando a ocupar grande parte do córtex, diferentemente dos ductos associados aos feixes vasculares que permanecem inalterados (Fig. 3E-F, detalhe). O sistema vascular não apresenta alterações em relação ao padrão não galhado.

Galha Fusiforme - A indução ocorre no ápice caulinar e causa divisões celulares ao redor da larva, formando tecido nutritivo por toda a extensão da câmara (Fig. 4A-C). Em estágio de maturidade, o sistema de revestimento é formado por periderme descontínua. A região cortical é formada por 8-9 camadas de células parenquimáticas de dimensões variadas. O sistema vascular é formado por cerca de 30 feixes colaterais, com presença de parênquima interfascicular. A região medular apresenta hiperplasia e hipertrofia celular (Fig. 4D-E). Na região basal da galha, a medula apresenta-se hiperplásica com células necrosadas e excrementos da larva (Fig. 4D-E). Na região mediana, forma-se uma grande câmara larval revestida por 3-5 camadas celulares remanescentes da medula. As células nessa região apresentam divisões frequentes e em vários planos (Fig. 4E).

Galha Globóide - o sistema de revestimento é formado por periderme descontínua que se instala na camada mais externa do córtex (Fig. 4F). O córtex é formado por 16-18 camadas de células parenquimáticas em meio às quais se distinguem 5-6 traços foliares com ductos secretores associados. O sistema vascular possui cerca de 21 feixes colaterais com ductos secretores associados a alguns feixes (Fig. 4G, detalhe). A região medular apresenta hiperplasia e hipertrofia celular e é ocupada pela câmara larval, revestida por tecido nutritivo (Fig. 4G).

Comparação morfológica e histoquímica entre os morfotipos - os cinco morfotipos de galhas ocupam órgãos hospedeiros diferentes, caules e folhas, têm formas finais e mecanismos de abertura distintos, com as principais alterações anatômicas induzidas no sistema fundamental (Tab. 2). Caules e folhas não galhados e galhas apresentam acúmulo de substâncias dos metabolismos primário e secundário apenas nos tecidos parenquimáticos (Tab. 3, 4). Destaque-se a detecção de polifenóis exclusivamente nas galhas (Tab. 5). Além disso, podemos definir, empiricamente, que indutores como os das galhas de enrolamento, em bolso, globóide e fusifome têm baixo impacto sobre os tecidos dos órgãos hospedeiros, enquanto que o indutor da galha reniforme atua com maior impacto, gerando um morfotipo com um maior nível de complexidade (Fig. 5).

Discussão

Baccharis reticularia é uma super-hospedeira de insetos galhadores, na qual foram registrados 5 morfotipos recorrentes de galhas que apresentam similaridades e diferenças morfológicas, anatômicas e histoquímicas. As galhas foliares comprometem quase totalmente a estrutura foliar, sendo a reniforme aquela com maior impacto sobre os tecidos vegetais. As galhas caulinares, por sua vez, apresentam os mesmos padrões estruturais diferindo quanto à forma final.

O **sistema de revestimento**, em todas as galhas possui modificações quanto à forma e dimensões das células, exceto para a galha fusiforme, cujo sistema de revestimento mantém-se similar ao caule não galhado. Logo, os aspectos funcionais do sistema de revestimento parecem não terem sido alterados pelo desenvolvimento desta galha. Um dos aspectos relevantes observados na galha reniforme é a diferenciação de células papilosas no bordo foliar, antes restritas à região da nervura principal. Às papilas

é atribuída a função de reflexão da luz (Kay *et al.* 1981, Monteiro *et al*. 1985), diminuindo a perda de água, o que auxilia no equilíbrio fisiológico da planta. *B. reticularia* encontra-se em um local de alta radiação solar e o fato da galha reniforme permanecer aberta poderia causar perda de água e possível dessecação. A ocorrência de células papilosas nesse local pode representar um mecanismo para evitar a perda de água, mantendo o microclima no interior da galha com bons níveis de umidade. Na galha caulinar globóide, por sua vez, observa-se a formação de periderme descontínua, numa posição apical onde o órgão hospedeiro deveria manter o revestimento formado por epiderme. A periderme confere maior proteção mecânica (Meyer & Maresquelle 1983) e contra a dessecação (Esaú 1974), tendo sido relatada por Krishnan & Franceschi (1988), Arduin *et al.* (1989) e Kraus *et al.* (1996) para diversas galhas. Tecidos suberizados ou mesmo lignificados foram considerados por Kraus *et al.* (2002) como estruturas anti-herbivóricas, que podem beneficiar o galhador protegendo-o da ação de cecidófagos e parasitoides.

Tricomas glandulares e estômatos anomocíticos foram mantidos em todos os morfotipos foliares. Estômatos anomocíticos e anisocíticos são comumente relacionados ao gênero *Baccharis* (Ortins & Akisue 2000, Espinar 1973) e já foram relatados para várias espécies, como *B. articulata* (Budel *et al.* 2003, Cortadi *et al.* 1999, Espinar 1973), *B. crispa* (Cortadi *et al.* 1999, Espinar 1973), *B. trimera (*Alquini & Takemori 2000, Budel *et al.* 2003), *B. dracunculifolia* (Budel *et al*. 2004), *B. retusa* (Silva & Grotta 1971), *B. gaudichaudiana* (Budel *et al.* 2003) e *B. myriocephala* (Sá & Neves 1996). O padrão encontrado para os tricomas glandulares é o mesmo descrito para outras espécies do gênero, tais como *B. gaudichaudiana, B. crispa, B. trimera, B. dracunculifolia* e *B. myriocephala* (Budel *et al.* 2003, 2004). Desde modo, pode-se concluir que a diferenciação de estômatos e tricomas parece ser um limite morfogênico imposto pela planta hospedeira o qual os galhadores associados não foram capazes de manipular. Com relação às divisões celulares, as mudanças observadas no sistema de revestimento em *B. reticularia* configuraram-se em transformações direcionadas para a proteção mecânica e necessárias ao acompanhamento do desenvolvimento das formas finais das galhas.

O **sistema fundamental** mantém-se homogêneo nas galhas foliares, contudo suas células perdem em grande parte o alongamento anticlinal, tendendo a isodiamétricas. Nas galhas reniformes, observou-se marcante hiperplasia, os ductos mostraram-se hipertrofiados e não necessariamente associados aos feixes vasculares.

Nestas galhas, os Hemiptera indutores possuem aparelho bucal sugador, que perfura as células sem destruir suas estruturas completamente, deste modo não há formação de tecido nutritivo. Nas galhas caulinares, o córtex manteve-se similar aos caules não galhados e as maiores modificações ocorrem na medula, devido à formação da câmara larval. Nesta região, observam-se sítios hiperplásicos relacionados à diferenciação do tecido nutritivo, região que sofre constante estímulo pela alimentação do galhador. As galhas fusiformes são induzidas por um Lepidoptera, cuja larva possui aparelho bucal mastigador e um hábito alimentar voraz. As galhas globoides são induzidas por um Diptera, que possuem aparelho bucal primariamente adaptado para sugar alimentos liquefeitos, com grande variedade morfológica e funcional nas estruturas da probóscide (McAlpine 1981). Em ambas as galhas caulinares, observa-se a diferenciação do tecido nutritivo, o qual é necessário ao desenvolvimento das larvas (Rohfritsch & Shorthouse 1982, Rohfritsch 1992) sendo essencial que as células sejam acessíveis aos aparelhos bucais em questão. Células em divisão e crescimento possuem paredes delgadas, portanto, acessíveis aos modos alimentares dos indutores Diptera e Lepidoptera. As células da medula e do parênquima de *B. reticularia* são capazes de se dividir, crescer e se transformar em um tipo celular diferente, características descritas por Fosket (1994) para as células totipontes. Desta forma, parecem apresentar a competência necessária à formação dos tecidos nutritivos em resposta ao estímulo de indução ou alimentação. Os ductos secretores apresentaram-se hipertrofiados e tiveram sua diferenciação maximizada nas galhas reniformes, tendo sido diferenciados independentemente dos feixes vasculares. Ductos secretores de epitélio uniestratificado associados ao floema ou aos feixes vasculares são usualmente descritos para o gênero (Budel *et al.* 2003, Espinar 1973) tendo sido observados por Cortadi *et al.* (1999), Ortins & Akisue (2000) e Espinar (1973), para *B. articulata*, e por Budel *et al.* (2004) e Sá & Neves (1996) para *B. dracunculifolia*. Embora as informações acerca da natureza química de sua secreção sejam escassas, alguns autores reportam que os ductos em *Baccharis* geralmente acumulam substâncias lipofílicas (Budel *et al.* 2003, Budel & Duarte 2010, Souza *et al.* 2011), as quais não foram detectadas histoquimicamente em *B. reticularia*. A detecção histoquímica de lipídios em galhas de *Lonchocarpus muehlbergianus* foi relacionada a reserva de nutrientes, pois embora o galhador não possa utiliza-los diretamente, esses compostos podem ser metabolizados e convertidos em componentes estruturais e metabólicos importantes ao desenvolvimento da galha (Oliveira *et al.* 2006). A

metabolização das substâncias lipídicas durante o desenvolvimento das galhas pode explicar sua não detecção em *B. reticularia*.

Tendo em vista a observação de hipertrofia celular, sítios hiperplásicos e potencialização da diferenciação de ductos secretores, o sistema fundamental mostrou ser o mais plástico aos estímulos oriundos dos galhadores. Tal característica é esperada, devido à natureza parenquimática deste sistema no gênero *Baccharis*. O estímulo do inseto para o desenvolvimento da galha dispara gatilhos de crescimento e diferenciação celular em células totipotentes e as novas células produzidas farão parte da estrutura da galha (Dreger-Jauffret & Shorthouse 1992). Esse redirecionamento morfogênico é totalmente dependente da presença e estímulo do inseto indutor (Formiga *et al.* 2011) e cessa na ausência do mesmo, levando à necrose da galha.

O **sistema vascular** apresenta poucas modificações em todas as galhas estudadas, sendo os feixes de pequeno porte localizados em torno das câmaras larvais mantidas as posições relativas dos órgãos não galhados. A manutenção dos padrões vasculares parece ser mais um dos limites morfogênicos característicos da planta hospedeira, haja visto que muitos herbívoros são capazes de induzir neoformações vasculares nos sítios de desenvolvimento de suas galhas, tais como os indutores de galhas em *Lantana camara* (Moura *et al.* 2009), *Lonchocarpus muehlbergianus* (Isaias *et al.* 2011) e *Copaifera langsdorffii* (Oliveira et al. 2009).

Todos os morfotipos de galhas apresentaram uma única câmara larval, contudo, nas galhas reniforme, de enrolamento, em bolso e globóide, foi possível observar mais de um inseto por câmara larval. Nestes casos, os insetos aparentemente dividem os recursos alimentares drenados dos sítios de produção para os sítios de desenvolvimento das galhas, até que pupem ou atingam a forma adulta, quando deixam as câmaras larvais. Para tanto, os insetos cavam canais de fuga, como os indutores das galhas caulinares de *B. reticularia*, ou devem contar com mecanismos de abertura das galhas. No caso de galhas fechadas, há necessidade de mecanismos para deiscência, o que não ocorre com as galhas foliares de *B. reticularia*, nas quais o escape é facilitado, pois não há soldadura dos tecidos. Em contrapartida os galhadores estão mais expostos às intempéries ambientais e ao ataque dos inimigos naturais.

A maior hiperplasia da galha reniforme somada a neoformação de ductos define este morfotipo como o de maior complexidade estrutural, sendo o mais distante do padrão estrutural das folhas hospedeiras e dos demais morfotipos de galhas.

As neoformações e restrições impostas pela planta hospedeira comumente se estendem aos seus perfis químicos. Como resposta aos estímulos dos indutores, a produção e atividade de compostos químicos pré-existentes nas plantas hospedeiras podem ser potencializados.

Em *B. reticularia,* os testes histoquímicos permitiram observar acúmulo de proteínas, amido, alcaloides e flavonoides. Os flavonoides foram relatados por Abad & Bermejo (2007) para o gênero *Baccharis*, como sendo os compostos químicos de maior frequência. A presença destes compostos, tidos como defesas químicas, não impede a associação da planta com herbívoros galhadores. Tal fato indica alta especialização dos insetos indutores os quais possuem meios de manipular as defesas químicas de suas plantas hospedeiras. Aos flavonoides são atribuídas outras funções, além da defesa química, tais como proteção contra incidência de raios ultravioleta, o ataque de insetos, fungos, vírus e bactérias, ação antioxidante, controle hormonal e inibição de enzimas (Zuanazzi & Montana 2004). Deste modo, uma vez estabelecidos em suas galhas, a presença de flavonoides pode conferir maior proteção para os insetos galhadores. Outra classe de compostos com função de defesa é a dos alcaloides (Price *et al.* 1987) cuja distribuição varia entre diferentes espécies vegetais (Harbone 1999). Além disso, sua quantidade e localização podem variar de acordo com os insetos que atuam em uma determinada planta (Adler & Kittelson 2004). Em *B. reticularia,* os alcaloides foram detectados no mesofilo das galhas foliares e na região cortical das galhas caulinares. A presença de alcaloides em plantas galhadas representa uma lacuna no entendimento de sua atuação inibidora, a qual já foi provada para outros fitófagos (Adler & Kittelson 2004). Cordell (1993) já havia proposto que apenas insetos especialistas são capazes de suportar níveis elevados de alcalóides, o que certamente é o caso dos galhadores associados à plantas alcaloídicas.

A reação para polifenóis foi negativa nos órgãos não galhados o que pode ser indicativo de que os testes histoquímicos utilizados não foram capazes de detectá-los devido a sua baixa concentração. Sabe-se que o desenvolvimento das galhas pode ser o gatilho que desencadeia o aumento de fenóis (Purohit *et al.* 1979, Abrahamson & Weis

1997, Hartley 1998, Formiga *et al.* 2009), tornando-os em níveis detectáveis nas galhas de *B. reticularia.* Esta mudança no perfil histoquímico dos órgãos não-galhados para as galhas merece destaque, pois as relações dos fenólicos com os sistemas galhador-planta hospedeira são controversas. Espírito-Santo & Fernandes (1998), por exemplo, não encontraram nenhuma relação entre a presença de taninos e a indução de galhas, enquanto Soetens *et al*. (1991) encontraram uma relação positiva entre a concentração de glicosídeos fenólicos e a abundância de galhas.

Outro fator nem sempre considerado nos estudos é a histolocalização das substâncias tidas como tóxicas. Insetos galhadores parecem não ter dificuldades para manipular pequenas quantidades de polifenóis e, dessa forma, os tecidos que circundam a câmara tendem a apresentar menor concentração dessas substâncias (Nyman & Julkunen-Tiitto 2000). A presença de substâncias fenólicas na região de revestimento interno da câmara larval das galhas fusiformes denota que o galhador tem mecanismos de garantir a palatabilidade das células nutritivas. Fenólicos podem atuar, ainda, como manipuladores da expressão gênica agindo diretamente no crescimento dos tecidos vegetais (Hartley 1998). Uma função interessante atribuída aos polifenóis é a inibição das AIA-oxidases, levando ao aumento de auxinas envolvidas no processo de hipertrofia celular durante o desenvolvimento da galha (Fosket 1994, Hori 1992). Esta associação pode levar à aceleração do crescimento das galhas e, consequentemente, proteger o galhador.

Proteínas foram detectadas tanto nos órgãos não-galhados quanto nas galhas, denotando o potencial de produção dessas substâncias na planta hospedeira o qual foi mantido nas galhas. Alguns tipos de proteína são associados a locais onde há maior atividade metabólica (Schönrogge *et al.* 2000), enquanto outros são associados à nutrição (Harper *et al*. 2004). No caso das galhas, a produção de proteínas tem lugar em tecidos onde há grande atividade metabólica, ou seja, tecidos em processo de rediferenciação (*sensu* Lev-Yadun 2003). Em *B. reticularia*, os resultados encontrados para detecção de proteínas denotam que as células corticais e principalmente as medulares, estão sobre forte impacto dos estímulos oriundos do galhador e, portanto, há alta atividade metabólica.

A presença de amido foi detectada tanto nos tecidos não galhados do caule e da folha quanto em todas as galhas, em tecidos diversos. O amido é uma forma de carboidrato que pode ser utilizado no crescimento de tecidos vegetais (Larcher 2000). De modo geral, carboidratos podem ser armazenados em galhas e utilizados na

alimentação dos galhadores, para tal há necessidade de atividade enzimática de modo a torná-lo acessível à alimentação do galhador como relatado por Oliveira & Isaias (2010A, 2010B). Bronner (1992) propõe o acúmulo de carboidratos como um padrão para galhas de Cecidomyiidae, contudo, nas galhas de *B. reticularia,* tal acúmulo ocorreu independentemente do taxa de galhadores, neste caso, hemípteros, dípteros e lepidópteros. Deste modo, o potencial para acúmulo de carboidratos parece ser intrínseco a *B. reticularia* sem ligação com o padrão induzido pelos galhadores.

O acúmulo de substâncias tipicamente anti-herbivóricas, tais como flavonoides, alcaloides e fenólicos em *B. reticularia* não impede a formação das galhas, nem a sobrevivência da prole do inseto indutor. Tal fato pode ser explicado pela grande especificidade entre o galhador e a planta hospedeira. Uma das formas de estabelecimento dessas relações espécie-específicas é a utilização de janelas de oportunidade (Stone & Schonrögge 2003) quando o indutor se estabelece na planta hospedeira em momentos de baixa produção dessas substâncias. Em *B. reticularia*, a interação química estabelecida entre o potencial da planta e os estímulos do galhador está direcionado para a produção de substâncias que conferem aos galhadores proteção contra inimigos naturais.

Considerações Finais

As maiores modificações foram induzidas no sistema de revestimento pelo indutor das galhas globóide e fusiforme e no sistema fundamental pelo indutor da galha reniforme. Ambos foram capazes de potencializar uma capacidade morfogenética já presente em seus órgãos hospedeiros, ou seja, a diferenciação de periderme nas duas primeiras e ductos secretores, na galha reniforme, sendo esta última considerada a galha de maior complexidade estrutural. A não observação de neoformações denota a imposição de limites morfogênicos impostos por *B. reticularia* ao desenvolvimento das galhas, tanto caulinares quanto foliares. Cada morfotipo de galha desenvolvido apresenta uma forma final característica, as quais não obedecem a um padrão estrutural, denotando peculiaridades ligadas ao taxa indutor. Quanto aos perfis histoquímicos, a detecção de polifenóis exclusivamente nas galhas mostrou um potencial inerente a *B. reticularia,* já relatado para *B. trimera* (Mendes *et al.* 2007), *B. dracunculifolia*, *B. grisebachii*, *B.latifolia*, *B. illinita*, *B. pseudotenuifolia*, *B. ligustrina, B. gaudichaudiana e B. rufescens* (Abaj & Bermejo 2007), não expresso nos órgãos não galhados.

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Legendas das figuras

Figura 1. A-I – Ramo não galhado e morfotipos de galhas em *Baccharis reticularia*. A – Ramo não galhado. B- Galha reniforme. C – Galha reniforme aberta contendo um Hemiptera indutor envolto em cera. D – Galha de enrolamento. E – Galha em bolso. F – Galha fusiforme. G – Ovos de endoparasitóide na larva de lepidoptera. A cabeça da larva ainda preservada (seta). H – Galha fusiforme aberta contendo larva de Lepidóptera (seta). I – Galha globóide (cículo pontilhado).

Figura 2. Folha e caule não galhados de *Baccharis reticularia*. A, E-H = Secções transversais. B-C = Secções paradérmicas. A - Folha não galhada evidenciando ducto secretor associado a feixe vascular. B – Face abacial da epiderme evidenciando estômatos anomocíticos. C – Tricoma glandular isolado. Em detalhe agrupamento de tricomas. D – Tricomas glandulares agrupados. E – Nervura principal. Seta=ducto associado ao feixe vascular. F - Aspecto geral do caule. G – Caule em detalhe evidenciando parte da medula, parênquima interfascicular e feixes vasculares. Du=ducto secretor, Es=estômatos, FV=feixe vascular, Md=medula, Pi=parênquima interfascicular, TrG=tricoma glandular.

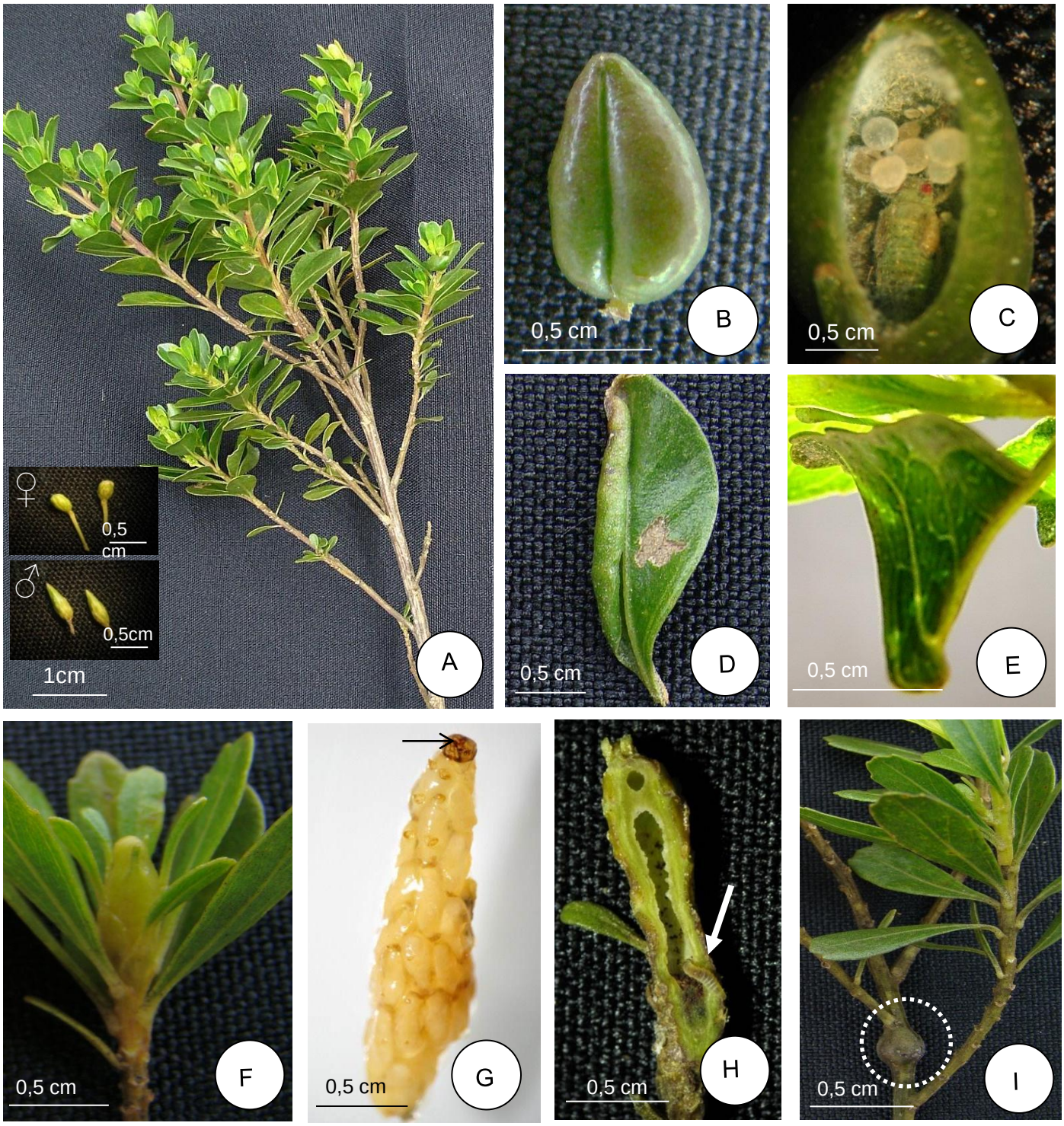
Figura 3. Secções transversais das galhas foliares. em *Baccharis reticularia.* A - Aspecto geral da galha reniforme evidenciando a proliferação de ductos secretores. Em detalhe galha reniforme na porção mediana. B – Região do bordo da galha reniforme evidenciando o afilamento da região, células papilosas e a abertura permanente da estrutura. C – Região do bordo da galha de enrolamento evidenciando ducto hipertrofiado. D – Aspecto geral da galha de enrolamento. Em detalhe galha de enrolamento na porção mediana. E – Aspecto geral da galha em bolso. Em detalhe secção transversal à nervura principal. F – Galha em bolso evidenciando ducto secretor hipertrofiado. Du=ducto secretor, ES=estômato, FV=feixe vascular, TrG=tricoma glandular.

Figura 4. A-C = Secções longitudinais da galha fusiforme em *Baccharis reticularia.* D-G = Secções transversais das galhas caulinares. A - Indução da galha fusiforme. Notar células em divisão ao redor da larva do indutor. B – detalhe da câmara larval evidenciando células em divisão. C – Região basal da câmara larval com excrementos

679 da larva. D - Aspecto geral da galha fusiforme na região basal, notar a presença de
680 excrementos da larva. E – Detalhe da galha fusiforme, evidenciando as células em
681 divisão na região da câmara larval. F–G = Galha globóide. F - Detalhe evidenciando o
682 início da formação de periderme (células com *). G - Região da câmara larval. Em
683 detalhe aspecto geral da galha. CD= células em divisção, CL= câmara larval, EX=
684 excrementos da larva.

685 **Figura 5.** Diagrama representativo dos níveis de complexidade das galhas de
686 *Baccharis reticularia.* CNG = caule não galhado, FNG = folha não galhada, GB =
687 galha em bolso, GE = galha de enrolamento, GF = galha fusiforme, GG = galha
688 globóide, GR = galha reniforme.

689 O aumento da quantidade de sinais positivos indica maior nível de complexidade.



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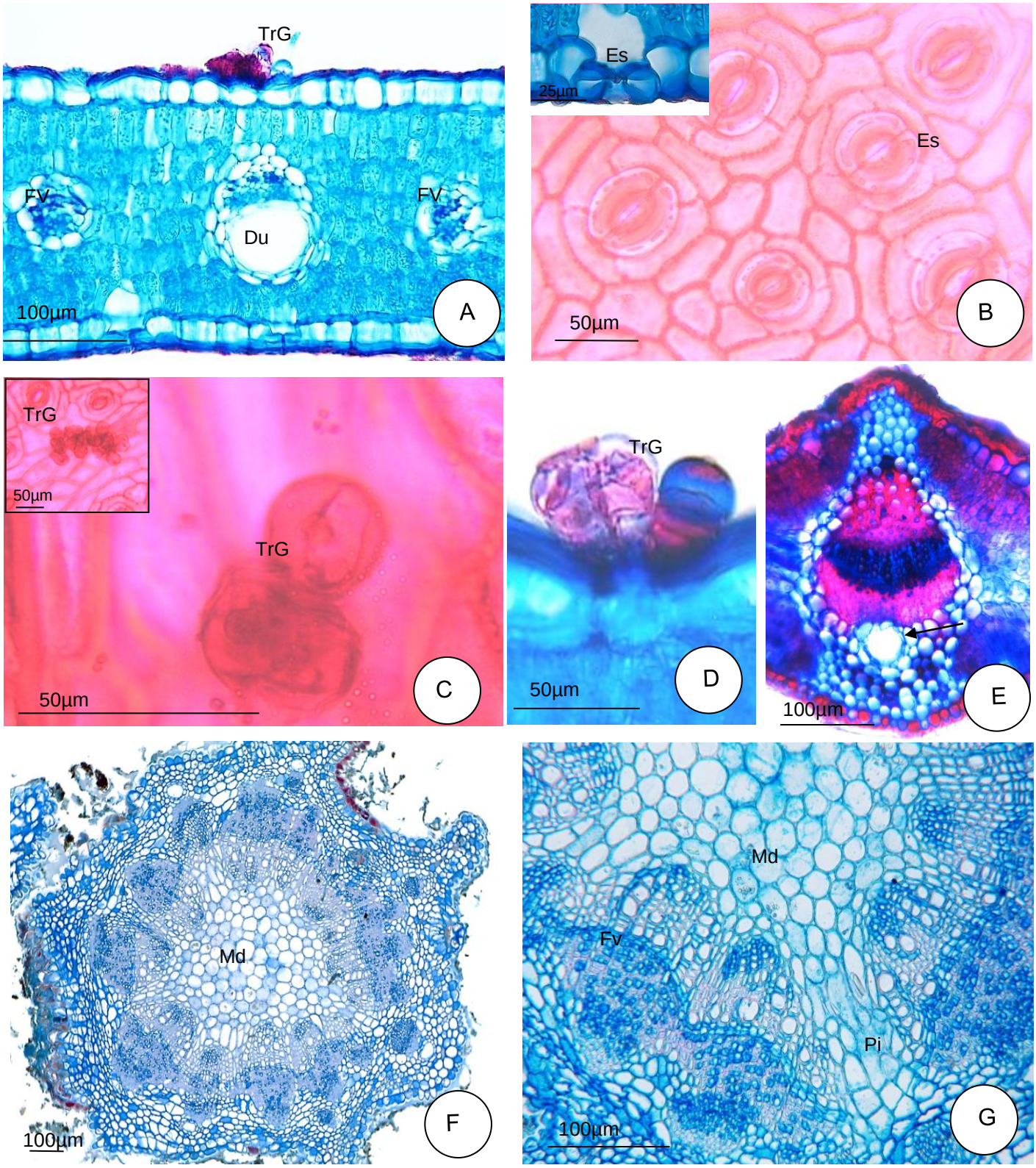

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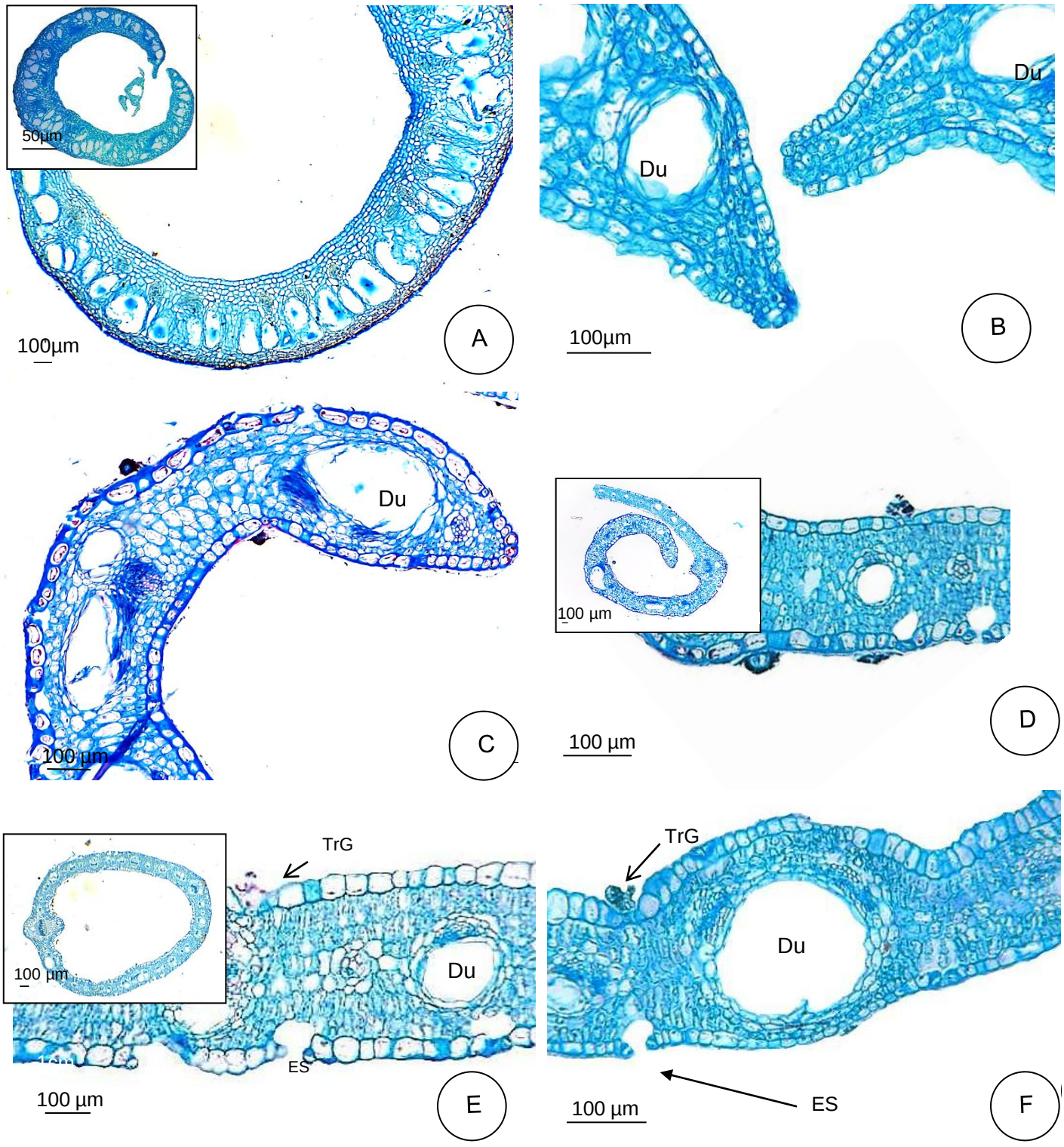

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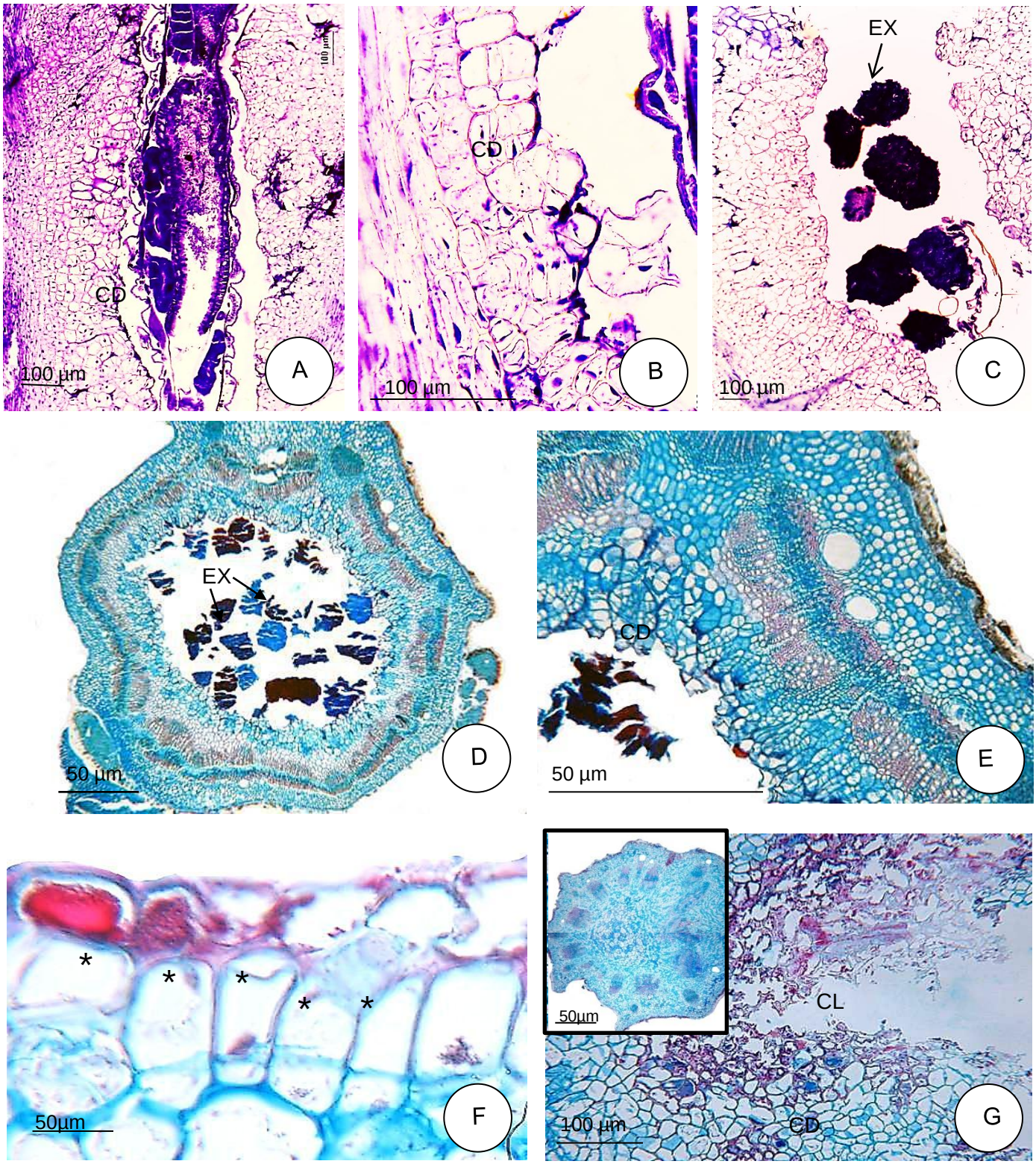

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Tabela 1. Testes histoquímicos para detecção de substâncias do metabolismo primário e secundário.

Grupo de metabólitos	**Testes aplicados**	**Referências**
Alcalóide	Reagente de Jeffrey	Johansen 1940
Flavanóis	DMACA (pdimetilaminacinamaldeído)	Feucht et al. 1986
Polifenóis	Sulfato ferroso 2% e formalina 10%	Johansen 1940
Amido	Reagente de Lugol (solução de iodeto de potássio em iodo (0.2%)	Baker 1958
Substâncias lipídicas	Sudan red B	Brundett et al. 1992

Tabela 2 – Comparação entre os morfotipos de galhas desenvolvidos em *Baccharis reticularia* (Asteraceae) na Serra do Caraça-MG

Tipos morfogênicos	***Reniforme***	***Enrolamento***	***Em bolso***	***Fusiforme***	***Globóide***
Características					
Órgão hospedeiro	Folha	Folha	Folha	Caule	Caule
Tipo	Enrolamento	Enrolamento	Em bolso	Cobertura	Cobertura
Abertura	aberta	aberta	aberta	fechada	Fechada
Posição	séssil	séssil	séssil	séssil	Séssil
Indumento	glabra	glabra	glabra	glabra	Glabra
Agrupamento	isolada	isolada	isolada	isolada	Isolada/agrupada
Cor	verde	verde	verde	Verde/marrom	marrom
Sist. Revestimento	Hiperplasia e hipertrofia. Papilosidade na região do bordo	Hipertrofia da face abaxial	Hipertrofia da face adaxial e hiperplasia na face abaxial.	Formação de periderme descontínua	Formação de periderme descontínua
Sist.Fundamental	Hiperplasia celular na região do córtex e ductos hipertrofiados	Hipertrofia celular na região do córtex interno e ductos hiperplasia celular na região do córtex externo dos bordos.	Hipertrofia celular na região do córtex interno	Hiperplasia e hipertrofia celular na região da medula	Hiperplasia e hipertrofia celular na região da medula
Sist. Vascular	Sem alterações	Sem alterações	Sem alterações	Sem alterações	Sem alterações
Nº câmaras	1	1	1	1	1
Nº insetos	1-4	1-2	1-2	1-2	1
Indutor	Hemíptera	Hemiptera	Hemiptera	Lepidóptera	Diptera

Tabela 3 – Histoquímica de substâncias dos metabolismos primário e secundário nos tecidos foliares não galhados (FNG) de *Baccharis reticularia* (Asteraceae) em 2009/2010 na Serra do Caraça – MG.

FNG	***Lipídios***	***Proteínas***	***Amido***	***Polifenóis***	***Alcalóides***	***Flavanóis***
Cutícula	+	-	-	-	-	-
Epiderme	-	-	-	-	-	-
Parênquima	-	+	+	-	+	+
Feixe Vascular	-	-	-	-	-	-
Ducto secretor	-	-	-	-	-	-

(+) reação positiva; (-) reação negativa.

Tabela 4 – Histoquímica de substâncias dos metabolismos primário e secundário nos tecidos caulinares não galhados (CNG) de *Baccharis reticularia* (Asteraceae) em 2009/2010 na Serra do Caraça – MG.

CNG	***Lipídios***	***Proteínas***	***Amido***	***Polifenóis***	***Alcalóides***	***Flavanóis***
Cutícula	+	-	-	-	-	-
Epiderme	-	-	-	-	-	-
Córtex	-	+	-	-	+	+
Medula	-	+	-	-	-	-
FV	-	-	-	-	-	-
Ducto secretor	-	-	-	-	-	-

(+) reação positiva; (-) reação negativa.

Tabela 5 – Histoquímica de substâncias dos metabolismos primário e secundário nas galhas de *Baccharis reticularia* (Asteraceae) em 2009/2010 na Serra do Caraça - MG

Tipos morfogênicos	***Lipídios***	***Proteínas***	***Amido***	***Polifenóis***	***Alcalóides***	***Flavanóis***
Reniforme	cutícula	Epiderme e parênquima	Ducto secretor	Parêquima	Parêquima	Parêquima
Enrolamento	cutícula	Parênquima e Ducto	Parênquima e Ducto	Parêquima	Parêquima	Parêquima
Em bolso	cutícula	Parênquima	Parênquima	Parêquima	Parêquima	Parêquima
Fusiforme	Cutícula, tecido que reveste a câmara internamente	Córtex, medula, floema, tecido que reveste a câmara internamente, ducto.	Tecido que reveste a câmara internamente	Cortex	Cortex	Cortex
Globóide	cutícula	Medula e Tecido que reveste a câmara internamente	Tecido que reveste a câmara internamente	Cortex	Cortex	Tecido que reveste a câmara internamente

plant biology

Phenotypic plasticity and similarity among gall morphotypes on a superhost, Baccharis reticularia

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Keyword:	Gall anatomy, multihost Baccharis, morphological patterns, multivariate analyses, phenotypic plasticity

Belo Horizonte, January 31st, 2013.

From Rosy M S Isaias
To Editorial board of Plant Biology

Dear Editor,

Please find attached the manuscript: "Phenotypic plasticity and similarity among gall morphotypes on a superhost, *Baccharis reticularia*", by Anete Teixeira Formiga, Fernando Augusto Oliveira Silveira, Geraldo Wilson Fernandes, and Rosy Mary dos Santos Isaias, to be submitted for consideration of publication. All authors have contributed, seen and agreed with the contents of the manuscript and, as an original work, it is not under review at any other type of publication vehicle. All the works cited are properly acknowledged and we believe that this new article best fits in the "Plant Biology".

The model plant species, *Baccharis reticularia,* has currently been the focus of several studies on morphogenetical potentialities of their specific host organs (Formiga et al. 2013. Protoplasma, doi: 10.1007/s00709-012-0473-8), seasonal fluctuation of her galling herbivores (Formiga et al. 2013. Arthropod Plant Interactions. *Submitted*) and tritrophic interaction involving host plant-galling herbivores-fungal endophytes (Formiga et al. 2013. Journal of Plant Interactions. *Submitted*). In this paper, we innovate by employing a multitude of qualitative and quantitative approaches to understand the patterns of growth and differentiation in four gall morphotypes in a single host plant. We compared two leaf galls induced by sap-sucking Hemiptera and stem galls induced by a moth and a gall midge. The following hypotheses were tested: (1) the more complex the galls are, the more distinct they are from non-galled organs, and (2) galls induced on less plastic host organs, such as stems, develop under more morphogenetical constraints and, therefore, should be more similar than galls induced on more plastic organs. Our results strongly suggest that both tissue plasticity and gall inducer identity interact to determine plant developmental patterns, and therefore, final gall shape/structure.

We hope this manuscript is adequately presented and look forward to your positive response.

Sincerely yours,

Professor Rosy M S Isaias & col.

Phenotypic plasticity and similarity among gall morphotypes on a superhost, *Baccharis reticularia*

Anete Teixeira Formiga[1] Fernando Augusto Oliveira Silveira[1] G. Wilson Fernandes[1] Rosy Mary dos Santos Isaias[1*]

1 - Universidade Federal de Minas Gerais – Instituto de Ciências Biológicas

Av. Antônio Carlos, 6627 - Pampulha - Belo Horizonte – MG CEP 31270-901 - Fone: +55 (31) 3409 2675, Fax number: +55(31) 3409 2671.

*corresponding author: rosy@icb.ufmg.br

ABSTRACT

Understanding the factors that modulate plant development is still a challenging task in plant Biology. Although research has highlighted the role of abiotic (light, soil fertility, among others) and biotic (competition, herbivory, among others) factors in determining final plant structure, we poorly know how these factors combine to produce specific developmental patterns. Here, we studied the patterns of cell and tissue organization in galled and non-galled organs of *Baccharis reticularia*, a Neotropical shrub that hosts more than 10 species of galling insects. We employed a multitude of qualitative and quantitative approaches to understand the patterns of growth and differentiation in its four most abundant galls. We compared two leaf galls induced by sap-sucking Hemiptera and stem galls induced by a moth and a gall midge. The following hypotheses were tested: (1) the more complex the galls are, the more distinct they are from non-galled organs, and (2) galls induced on less plastic host organs, such as stems, develop under more morphogenetical constraints and, therefore, should be more similar than galls induced on more plastic organs. Simple galls were qualitative and quantitatively more similar to non-galled organs than to complex galls, thereby supporting the first hypothesis. Stem galls were more similar between them than in relation to their host organ, hence proceeding only partial support for the second hypothesis. The similarity between stem galls may have been caused by the restrictive pattern of the host stems. The opposite trend was observed for the host leaves, which may generate either similar or distinct gall morphotypes due to their greater phenotypic plasticity. Our results strongly suggest that both tissue plasticity and gall inducer identity interact to determine plant developmental patterns, and therefore, final gall shape/structure.

Keywords: Gall anatomy, multihost *Baccharis*, morphological patterns, multivariate analyses, phenotypic plasticity.

Introduction

Galls are morphological structures differentiated through the responsiveness of host plant tissues to mechanical and/or chemical stimuli of an external biotic agent, producing hyperplasia and/or cell hypertrophy (Mani 1964). Depending on the degree of tissue reactiveness, the host plant develops different gall morphotypes with distinct degrees of similarity to their host organ, due to the genotypes of the involved organisms. The phenotypes of the host organs do not reveal all their genetic potentialities, which guarantee the flexibility for the development of the anomalous structure, the gall. Galls may be induced either in mature or already differentiated cells, with established functions (e.g., Oliveira and Isaias 2010), through a process of redifferentiation (*sensu* Lev-Yadun 2003).

The final gall structure may be complex and present a refined tissue organization with neoformations, while the more simple ones have poor tissue organization, and are structurally similar to non-galled organs (Floate 2010). Some galls may present an intermediate complexity pattern with a refined metabolism. This is the case of some galls induced by sap-sucking Hemiptera , such as that induced by a Pseudophacopteronidae on *A. australe* (Oliveira and Isaias 2010). The plasticity of host plant tissues is determinant to final gall morphology because of morphogenetical constraints imposed by host tissues to gall development. This particular aspect may be observed in superhosts of galling herbivores (see Fernandes and Price 1988, Espírito-Santo *et al*. 2010) due to their potential of responding to the stimuli of the different associated galling herbivores. Even though galls are considered extended phenotypes of the galling herbivores (Abrahamson and Weis 1991), the gall morphogenesis may be constrained by the tissue plasticity of the gall induction site. Therefore, stronger constraints on gall morphogenesis are expected in less plastic organs, such as stems compared to leaves (Pugnaire and Valladares 2007), and therefore, stem galls must be more similar to non-galled stems than leaf galls when compared to non-galled leaves.

An investigative approach that moves from anatomical description towards a more quantitative approach can help unraveling the patterns and mechanisms of tissue-specific differentiation to external stimuli. Quantitative and multivariate approaches have been recently used at both cell- and tissue-levels (Gasson *et al.* 2010; Oliveira & Isaias 2010; Dunham *et al.* 2007; Rossel *et al.* 2007) to improve our understanding on tissue responsiveness to varying abiotic (Bedetti *et al.* 2011) and biotic conditions (Isaias *et al.* 2011; Oliveira & Isaias 2009; Moura *et al.* 2008). These approaches have not been used in studies of gall diversity, and their use joins confidence to the interpretation of the morphological and anatomical data.

Galls represent phenomena under the influence of which distinct external conditions, and hence may provide an important scenario to address many unexplored developmental issues. The comparative study of distinct gall morphotypes induced by distinct galling species on a single superhost represents an elegant model for checking the constraints imposed by the host plant to gall development. Moreover,

insect-induced galls represent an ideal model to study phenotypic plasticity *stricto sensu* under natural conditions, and approach rarely carried out (Gianoli & Valladares 2012). A superhost and its galls constitute an appropriate model for this kind of study because it involves a unique plant genome under the influence of several insect stimuli. An important factor to gall development is the feeding habit of the insect. Sap-sucking insects, such as the Hemiptera, insert their stylets directly into the phloem, and induce minor modifications in host plant tissues, producing simple galls (Oliveira & Isaias 2009). Chewing insects, such as the Lepidoptera, may inflict severe damage and in turn induce more complex galls with more specialized tissues (Meyer 1987).

Here, we employed a quantitative approach to study gall morphology in *Baccharis reticularia*. The genus *Baccharis* hosts a large number of galling insect taxa (Fernandes *et al.* 1996). *B. reticularia* is a superhost, with somewhat 10 gall morphotypes induced by several distinct gall-inducing insects. Hence, this system offers the opportunity to test the hypotheses regarding the morphogenetical potentialities and constraints of the host organs. We focus on morphological, anatomical, cytometric, and histometric data of four gall morphotypes on *B. reticularia* through multivariate analyses and phenotypic plasticity tests. Specifically, we addressed the following questions: (1) are more complex galls more distinct from their host organ tissue patterns when compared to less complex galls? And (2) are galls induced on less plastic organs more morphogenetically constrained than galls induced on more plastic organs?

Materials and Methods

Sampling

This study was performed at the Campo de Fora, at Serra do Caraça, Minas Gerais, southeastern Brazil (20° 07'S and 43º 31'W, 1.497m altitude above sea level). The main vegetation is called *campos rupestres* (altitudinal rocky grasslands) under nutrient-poor soils and a mesothermic climate with rainy summers and dry winters (Benites *et al.* 2003). The study system consists of *Baccharis reticularia* DC. (Asteraceae) (Fig. 1a) and its gall-inducing insect fauna. The host plant is a 1-2m tall, dioecious shrub commonly found in the highlands of Cerrado vegetation in southeastern Brazil, extending from South to Northeast (Borges & Forzza 2008). This species hosts several gall-inducing insects and here, we focused on the two more common leaf galls, and the two more common stem galls. We comparatively studied these four galls, non-galled leaves and non-galled stems (1st internode from the apex). Three samples of each gall morphotype, non-galled leaves, and non-galled stems were randomly collected from each specimen (n = 30). Male and female plants were sampled at a 1:1 ratio. All gall morphotypes were collected at the maturation stage based on anatomical analyses.

Insect Sampling – The collection of insects were performed by three methods, and monitored during 2 months. (1) In field conditions, galls were involved in a bag of fine-mesh net. (2) Galled stems and leaves were put in plastic pots with wet substrate, covered with fine-mesh net, and maintained in laboratory conditions. (3) Mature galls (n = 60) were dissected under stereomicroscope and the insects were directly

fixed in ethanol 70°C

As adult gall inducers were not observed, and their identification to the species level was not possible, the widely accepted concept of gall morphotypes was used, as it has been extensively used in inventories of gall diversity and richness in the Neotropics (Maia & Fernandes 2004, Maia *et al* 2008, Carneiro *et al* 2009, Maia & Oliveira 2010, Maia 2012, Santos *et al* 2011a, b, Malves & Frieiro-Costa 2012, Santos *et al* 2012). Also, by gall dissection in the laboratory, the two leaf galls were associated to two distinct sap-sucking nymphs, and each of the two stem galls was associated to larva or pupa of a Lepidoptera or of a gall midge. The leaf rolling gall (Fig. 1b) is brown and brittle in senescence, with a wide, single chamber comprising one or more nymphae of unidentified Hemiptera. The kidney-shaped gall (Fig. 1c) is dark in senescence; its chamber is internally coated with white wax produced by 2-4 inducing Hemiptera. The fusiform gall is a stem gall which presents conspicuous necrotic cells and feces of 1-2 inducing Lepidoptera larvae (Fig. 1d) at the base of the chamber. The globoid gall (Fig. 1e) is induced by a gall midge (Diptera: Cecidomyiidae) on the stems of the host plant. It contains a single larvae per chamber and turns into brown in senescent phase

Anatomical and histometric analyses

Samples of galls and non-galled tissues were fixed in FAA, dehydrated in *n*-butyl series and embedded in Paraplast®. The samples were sectioned (20µm) in a Reichert-Jung microtome rotary®, stained with Astrablue:safranin (8:2, v/ v) (Kraus & Arduin 1997), and mounted on stained colorless varnish® (Paiva *et al.* 2006). The images were obtained with a digital camera coupled to the microscope. Tissues and cells, common to the four morphotypes, were measured using the AxioVision LE program, version 4.8 (CarlZeiss MicroImaging GmbH). The areas of secretory ducts and vascular bundles, and the linear measurements of the other cells and tissues were taken. For all the samples, the linear measurements were the width and height of epidermal cells at the abaxial and adaxial epidermal surfaces for non-galled leaves and leaf galls, and the cortical layers (inner and outer cortex), identified by the shape and size of the cells. For each of the 30 individuals sampled, three different sections of all the samples were used. For each structure or tissue, five measurements were taken. The mean values on each individual were used in the statistical analyses to avoid pseudoreplication (Bedetti *et al.* 2011).

Statistical Analyses

Morphological and anatomical comparisons of the non-galled host organs and the gall traits were performed by cluster analysis using only qualitative traits. We excluded traits specifically associated with galls because they inflated the numbers of zeros in non-galled tissues and artificially grouped leaves and stems (data not shown). A dendrogram of similarity was calculated using indexes of morphological and anatomical similarity between non-galled leaves, non-galled stems, leaf galls, and stem galls on *B. reticularia* in a matrix of Manhattan distance. Principal Component Analysis (PCA) and

Relative Distance of Plasticity Index (RDPI) were applied to examine whether the anatomical differences and/or similarities were supported on quantitative basis. The Principal Component Analysis (PCA) was based on the correlation matrix between morphometric variables (Tables 1, 2). The data were log-transformed to meet the assumptions of parametric analyses (Zar 1996). For the multivariate analyses (PCA and Cluster), the software PAST – Paleontological Statistics Software Package (Hammer *et al*. 2001) was used.

The extent of variation between non-galled and galled tissues was determined by an estimation of phenotypic plasticity *sensu stricto* (Valladares *et al.* 2006, Gianoli & Valladares 2012). The relative distance plasticity index (RDPI) measures the distances between the values of the chosen variables for all pairs of comparisons between different tissues. Even though leaves and stems belong to the same plant module, the shoot, and their tissues form a *continuum* (White 1979, Heard & Cox 2009), leaves and stems have different structures and, therefore, the RDPI for NL and leaf galls, and non-galled stems and stem galls were calculated separately. The variables (Tables 3, 4) on cells and tissues measurements were chosen based on their occurrence either in the host organs or in the gall morphotypes. The distances among the non-galled tissues (non-galled leaves and non-galled stems) and those of the four gall morphotypes were calculated according to the following equation:

$$RD_{ij \to i'j'} = d_{ij \to i'j'} / (x_{i'j'} + x_{ij})$$

(1) where j and j' are the non-galled tissues, i e i', the galled ones and x_{ij} refers to the trait value of a given individual. The RDPI ranges from zero (no plasticity) to one (maximum plasticity) and was calculated as:

$$RDPI = \sum (d_{ij \to i'j'} / (x_{i'j'} + x_{ij}) / n$$

(2) where *n* is the total number of distances. The RDPI values were submitted to ANOVA followed by Tukey test for multiple comparisons. For all analyses α values of 0.05 were established.

Results

Non-galled leaves and leaf galls

The non-galled leaf has a homogeneous 7-8 layered mesophyll (Fig. 2a). It is amphistomatic, hairy, with uniseriate epidermis on both surfaces, and cells larger on the adaxial surface than on the abaxial one. The cuticle is thin. The vascular bundle of the midrib is collateral with 3-4 layers of fibers next to the conducting cells, and 5-6 layers of angular collenchyma adjacent to the epidermis on both sides of leaf lamina (Fig. 2b). The minor vascular bundles are interspersed to the mesophyll cells. The secretory ducts are associated with the phloem bundles (Fig. 2a, b). The epidermal cells are isodiametric with straight anticlinal walls. The stomata are anomocytic and located at the same level of epidermal cells or slightly protruding. The trichomes are individual or 2-5 grouped, disposed in depressions on both epidermal surfaces.

Rolling Gall

It is formed by the rolling of one of the leaf margins (Fig. 2c). It is permanently open, sessile, hairy, and green. The epidermal cells are hypertrophied, and periclinally elongated. Stomata and glandular trichomes are arranged on the abaxial surface. The leaf margin is acute at gall site, and the cell layer adjacent to the epidermis is periclinally elongated. The 2-3 median layers are hypertrophied, and the 2 inner layers are hyperplasic. The cortex is homogeneous. The inner cortex is 2-3 layered with small isodiametric cells, and the outer cortex is 4-6 layered with larger cells. The secretory ducts and the vascular system are little altered (Fig. 2d).

Kidney-shaped Gall

It is formed by the folding of the two leaf margins (Fig. 2e). The dermal and ground tissue systems are hyperplastic and hypertrophic. The epidermis is uniseriate, with large and round cells at the adaxial surface (Fig. 2f). The epidermal cells of the gall margin are papillose. The feeding sites are close to the midrib, where lysis of some epidermal cells is frequent. The cell layer adjacent to the adaxial epidermis in the mid rib region is hypertrophic, and the cells of the adjacent cortex are hyperplasic. The hypertrophied secretory ducts occupy a large portion of the cortex (Fig. 2e, f). The gall is permanently opened along the leaf margins (Fig. 2e).

Non-galled Stems and stem galls

The non-galled stems are semi-cylindrical in cross section, with 6-8 ribs (Fig. 3a). The epidermis is uniseriate, papillose, with polyhedral cells, and thick cuticle. Multicellular glandular trichomes are isolated or grouped into slight depressions. The stomata protrude in relation to the other epidermal cells. The cortical region has 6-7 layers of parenchyma cells of various dimensions, and 6-7 vascular bundles associated with the secretory ducts. The vascular cylinder has 16 collateral bundles with xylem and phloem in equivalent amounts and in early secondary growth. The interfascicular parenchyma is present. The primary phloem fibers form a half moon-shaped cap. The pith cells are isodiametric with thin walls (Fig. 3b).

Fusiform gall

This gall morphotype resembles a spindle. It is greenish when young and brown at maturity, with the major swelling in the mid portion, around the larval chamber (Fig. 3c, d). It is closed, sessile, glabrous, with small leaves on its surface. It is isolated, in the first internode of the lateral branches, which continue to develop above the gall initiation site. The dermal system is constituted of periderm, and the ground system is parenchymatic similar to that of non-gallwd stem. The vascular system is in secondary growth

and is arranged around a parenchymatic pith where the larval chamber is located.

Globoid galls

This gall morphotype is cylindrical, and induced at the shoot apex (Fig. 3e). The stem and leaf primordia continue to develop above the gall site. It is closed, sessile and glabrous, isolated or coalescing, and green. The dermal system is formed by a discontinuous periderm that differentiates into the outer cortex (Fig. 3f). The vascular system has 16 bundles similar to the non-galled stem, arranged arund the parenchymatic pith where 1-2 larval chambers develop. In the basal region, the pith has hyperplasic cells, the larval chamber is limited by some intact and some necrotic cells. In the mid portion, the larval chamber and the escape channel are limited by necrotic cells, in the middle of the vascular bundles which are disorganized.

Phenotypic similarity

The qualitative morphological and anatomical characters (Table 1) separated two groups, one composed by non-galled leaves and leaf galls and the other composed by non-galled and galled stems. The stem galls were more similar among them than with non-galled stems, and the leaf-rolling gall was more similar to non-galled leaves than to the kidney-shaped gall (Fig. 4).

In relation to the quantitative assessment, the kidney-shaped gall was separated from the rolling gall RG and non-galled leaves by the principal component 1 (PC1) (Fig. 5A). The non-galled leaves was separated moderately from the rolling gall by the principal component 2 (PC2), with little overlap between the two (Fig. 4A). All histometric variables except the adaxial epidermis thickness were positively correlated with the PC1. All histometric variables, except the thickness of the mesophyll, were also positively correlated with the PC2. The first two axes of PCA explained 71.6% of the total variance (Table 1).

The two stem gall morphotypes were separated from the non-galled stems by the PC1 (Fig. 5B). Globoid and fusiform galls overlapped both in the PC1 and in the PC2 (Fig. 5B). The vascular bundles and the height of the epidermis were positively correlated by the PC1, while the area of the secretory ducts and width of the epidermal cells were negatively correlated with PC1. The PC2 was also correlated with the width of the epidermis. The first two axes of PCA explained 72.7% of the total variance (Table 2).

Morphological Plasticity

The values of RDPI for non-galled leaves and leaf galls ranged from 0.01 to 0.25. There were significant differences among paired-comparisons for all histometric traits, except for the adaxial epidermal cell height. Consistently to our previous analyses, tissue plasticity was higher in kidney-shaped

gall when compared to the rolling gall. The induction of the kidney-shaped gall resulted in a higher production of vascular bundles, secretory ducts and thicker mesophyll than the rolling gall (Table 3). Regardless of gall morphotype, cell plasticity was lower (ranging from 0 to 0.08) than that of tissue plasticity (0 to 0.25).

The values of RDPI for non-galled stems and stem galls ranged from 0.02 to 0.42. Secretory ducts plasticity was higher either for FG and GG. All other tissue traits were not significantly plastic (Table 4).

Discussion

Our results strongly suggest that both tissue plasticity and gall inducer identity determine plant developmental patterns, and therefore, final gall shape/structure. The qualitative and quantitative approaches consistently showed that irrespective of the taxa of gall inducer, stem galls were morphologically more similar to non-galled stems than the leaf galls to the non-galled leaves. On the other hand, the pattern of similarity found for stem galls do not hold for the leaf galls. Despite both leaf galls are induced by sap-sucking Hemiptera, the leaf rolling gall was consistently more similar to non-galled leaves than to the kidney-shaped gall. Current results respectively show the determinant role of tissue plasticity and gall inducer identity in defining the patterns of development. Given the importance of the control of organ development in plants (Gonzalez *et al*. 2012), these results together indicate that both internal and external factors may modulate the patterns of cell and tissue growth and differentiation. The competence of cells and tissues of the superhost *B. reticularia* seems to vary according to the vegetative organ, and so their ability to respond to the insect's stimuli.

The morphological and anatomical study of the gall morphotypes on *B. reticularia* compared to the non-galled host organs demonstrates that the host plant morphogenesis imposes constraints that determine distinct structural patterns. Also, gall inducers have different capacities to explore plant plasticity. This conclusion is supported either by the cluster analysis using qualitative data, or the principal components analysis using quantitative data, which allowed similar groupings. Both analyses have highlighted which host organ features were altered by the feeding action of the gall inducing insects, and therefore greatly influenced the patterns of similarity.

Similarity between non-galled leaves and leaf galls

The leaf rolling galls were anatomically more similar to the non-galled leaves than to the kidney-shaped galls. Because of its predominant parenchymatic structure and similarity with the non-galled leaves, the rolling gall may be considered less complex. Accordingly, there are no statistically significant differences between the area of the secretory ducts; the vascular system presents few changes, and the hyperplasia, common process in various galls (Rohfritsch 1992, Abrahamson & Weis 1997), was

restricted to leaf margin. In the kidney-shaped gall, hyperplasic sites are detected in the mesophyll and the secretory ducts are associated with the vascular bundles and hypertrophied. Since the cellular organization of a differentiated plant tissue reflects the pattern of cell division which occurred during its development (Muller *et al.* 2009), and thus determine its function, the changes reported herein strongly influence the change in form and function of cells and tissues due to the formation of galls. In the leaf galls of *B. reticularia*, the changes lead to different forms, but with similar functions.

The secretory ducts, vascular bundles, and mesophyll constitute the sites with greater phenotypic plasticity, being therefore composed of cells and tissues which were more susceptible to the feeding activity of the gall-inducing insects. The characteristics of these sites, despite the low plasticity index found, along with the height of the cells of the adaxial epidermis were important for the PC 1 of the PCA, reinforcing its role in the similarity or dissimilarity between the morphotypes.

Both rolling and kidney-shaped galls are induced by sap-sucking Hemiptera, which directly introduce the stylets in the phloem (Meyer 1987), causing a few modifications in the cellular structure of their galls as a whole. In these galls, the feeding sites are located near the secondary veins or midrib, which are associated to the secretory ducts. The alterations in the dimensions of the secretory ducts may be explained by cellular responses either of the secretory or vascular parenchyma. In fact, changes in the vascular system are only likely to be induced in the parenchymatic cells, due to their totipotency (Fosket 1994) and the absence of nuclei in the tracheal and sieve cells. The expression of the totipotency of the parenchymatic cells indicates their competence to assume distinct developmental pathways as has been reported in other galling herbivore-host plant systems (Souza *et al*. 2000, Arduin *et al*. 2005, Moura *et al.,* 2009, Sa *et al.,* 2009). . Moreover, due to the storage of lipids of the secretory ducts, the access to this energy resource could be maximized by the induced changes.

Similarity between non-galled stems and stem galls

The stem galls, either globoid or fusiform, were more similar among themselves and differed from the non-galled stems. Both galls have a central larval chamber located in the pith region which is surrounded by disorganized vascular bundles. Around the larval chamber, the cortex and pith have hyperplasic sites in both gall morphotypes.

The secretory ducts and the vascular bundles were the more plastic structures within stem galls. This group of traits plus the height of the epidermal cells define the characteristics of the PC1 of the PCA. This last characteristic has a low plasticity index (RDPI $\leq$ 1) and was not considered relevant to the determination of the morphotypes. The constant alteration in the secretory ducts of the stem galls denotes that the gall inducing insects explore the morphogenetical potential of the host plant despite the taxon or feeding habit.

A unique trait of the globoid gall is the formation of periderm, the secondary tissue of the dermal

system which confers great mechanical protection to the gall inducer (Meyer & Maresquelle 1983) as well as protection against desiccation (Esau 1974). According to Enstone & Peterson (1998), the deposition of suberin in cell walls can be accelerated by moisture. Hawkins and Boudet (1996) and Biggs &Miles (1985) relate this deposition to a plant response to the attack of pathogens. So, this trait can provide protection from abiotic stress (see Price *et al.* 1987, Fernandes & Price 1988, Nicholson & Hammerschimidt 1992, Boudet 2003). In addition to environmental influence, and structural patterns, the chemical composition of the cell walls are coordinated by the expression of numerous genes involved in the biosynthesis, and deposition of the different constituents of the cell wall (Plomion *et al.* 2001). Thus, the gall-inducing insect of the globoid gall should impose a larger impact on the morphogenesis of the dermal system of the host organ than the gall-inducing insect of the fusiform gall, maximizing the potential for the formation of periderm common to stems, even in young internodes.

Comparisons between leaf and stem galls

The different gall-inducing insects on *B. reticularia* have different feeding habits and therefore impact the plant cells in varying degrees. Both leaf galls studied here are induced by Hemiptera, which typically induce simple galls (Mani 1964, Rohfritsch 1992). However, hemipteran induced leaf galls on *B. reticularia* were very different from each other, with the rolling gall similar to the host organ and the kidney-shaped gall somewhat dissimilar. The stem galls on *B. reticularia* were induced by a Lepidoptera and a gall midge, with very distinct feeding habits (Mani 1964, Meyer 1987), capable of inducing complex galls (Redfern & Askew 1992; Rohfritsch 1992), expected to present low morphological similarity. However, contrary to that expectation, these galls showed high levels of similarity between them and with their host stems.

The levels of similarity found for the leaf galls support the hypothesis that less complex galls are statistically similar to the morphogenetic pattern of their host organ (non-galled leaves). The distance between the kidney-shaped gall, and the group formed by the rolling gall and the non-galled leaves denotes that its gall-inducing Hemiptera caused greater alterations in its host organ, the leaf. As it is more plastic, less morphogenetic constraints were imposed to the associated herbivore. In contrast, the galls induced in the host organs which were considered to be less plastic, the stems, develop into major morphogenetic restrictions, corroborating the second hypothesis proposed. In this case, both kinds of stem galls deviate from the host organ pattern (non-galled stems) keeping higher similarity between themselves.

Concluding remarks

Most research on phenotypic plasticity relies on the use of experimental studies to determine the

extension of environmentally-induced phenotypic variation (Gianoli & Valladares 2012). However, the system comprising *B. reticularia* and its galling herbivore fauna, represents a good model for the comparative study of the morphogenetic potentialities of plant tissues and their ability to handle the biotic components with the advantage of being studied under natural conditions. Studying phenotypic plasticity under natural conditions is rarely performed and this work represents a first attempt to combine qualitative and quantitative analyzes to demonstrate in what degree the cells and host tissues fates are altered by the action of different galling insects on the same host plant. As future perspectives, these cells and tissues may be the target for cytological and metabolic studies to elucidate cell-specific and tissue-specific patterns of stimuli-response involving insect-plant biotic interactions.

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Figure Legends

Fig. 1. General aspect of *Baccharis reticularia* DC. (Asteraceae) stem and the galls studied here. Arrows indicate the galls a. Branch with male and female flowers in detail. b. Leaf rolling gall. c. Kidney-shaped gall. d. Fusiform gall. e. Globoid gall (dotted circle).

Fig. 2. Transverse sections of non-galled leaf and leaf galls of *Baccharis reticularia* (Asteraceae). a. Non-galled leaf, evidencing leaf lamina organization. b. Midrib. Arrow = Secretory duct associated to a vascular bundle. c. General aspect of rolling leaf gall (RG). d. Detail of RG. e. General aspect of the kidney-shaped gall (KG). f. Detail of the KG. GT = glandular trichome, SD = secretory duct, ST = stomata, VB = vascular bundle.

Fig. 3. Transverse sections of non-galled stem and stem galls of *Baccharis reticularia* DC. (Asteraceae). a. General aspect of non-galled stem. b. Detail of the non-galled stem pith. Instalation of secondary growth in the vascular system. c. Mid region of fusiform gall (FG). Arrow = feces from the gall-inducing larvae. d. Detail of the FG evidencing cells in division (DC) around the larval chamber. e. General aspect of the globoid gall (GG) in the mid region. f. Instalation of periderm (*). DC=division cells, FE = feces, LC = larval chamber, Md= Medule, VB = vascular bundles.

Fig. 4. Dendogram showing the relationship among leaf and stem galls in *Baccharis reticularia* based on histometric traits.

Fig. 5. Representation of trait scores of the first two axes of the PCA of the cytometrical and histometrical traits of non-galled and leaf galls (A), and non-galled and stem galls (B) of *Baccharis reticularia* DC. (Asteraceae). Circles represent leaf rolling galls and fusiform stem galls; squares represent leaf kidney-shaped gall and non-galled stem, and triangles represent non-galled leaves and stem globoid gall. BEH= abaxial epidermis height, BEW= abaxial epidermis width, DEH= adaxial epidermis height, DEW= adaxial epidermis width, EH = epidermis height, EW= epidermis width, VB= vascular bundles.

Tables

Table 1. Results of the principal components 1 (PC1) and 2 (PC2) from the matrix or correlation based on the histometrical data of the leaf galls of *Baccharis reticularia* DC. (Asteraceae).

Table 2. Results of the principal component 1 (PC1) and 2 (PC2) from the matrix of correlation obtained from the histometrical data of the stem galls of *Baccharis reticularia* DC. (Asteraceae).

Table 3. Values of RDPI of the histometrical variables of non-galled leaves and leaf galls of *Baccharis reticularia* DC. (Asteraceae).

Table 4. Values of RDPI of the histometrical variables of non-galled stems and stem galls of *Baccharis reticularia* DC. (Asteraceae).

Supplementary documents

1 – Table of the average values of the measured tissues and cells in non-galled organs and galls of *Baccharis reticularia* DC. (Asteraceae).

(1)

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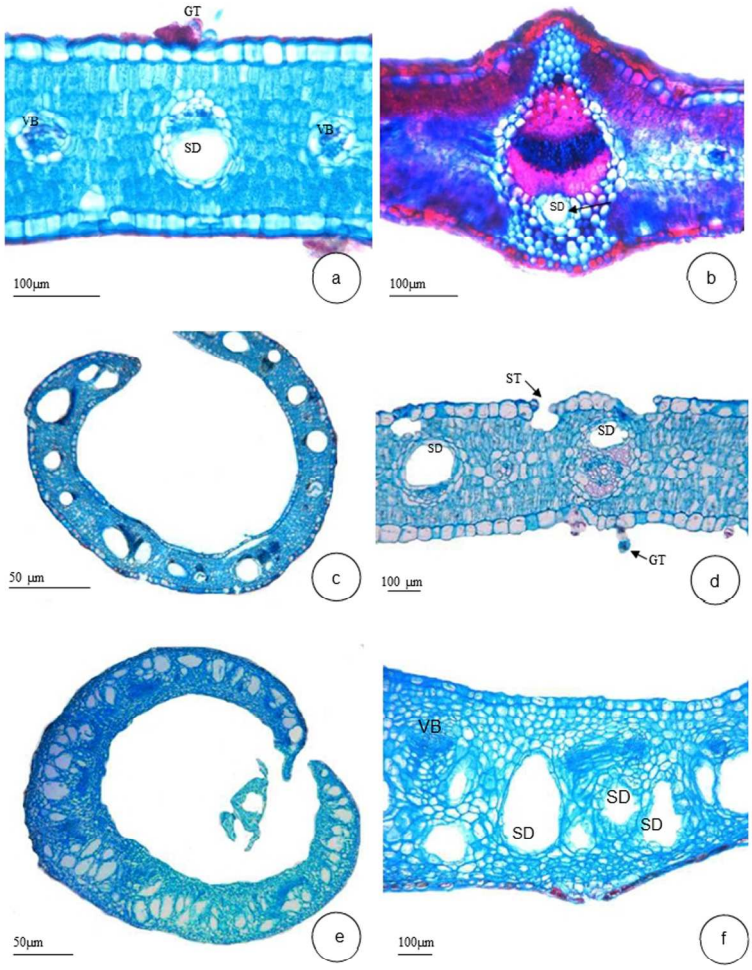

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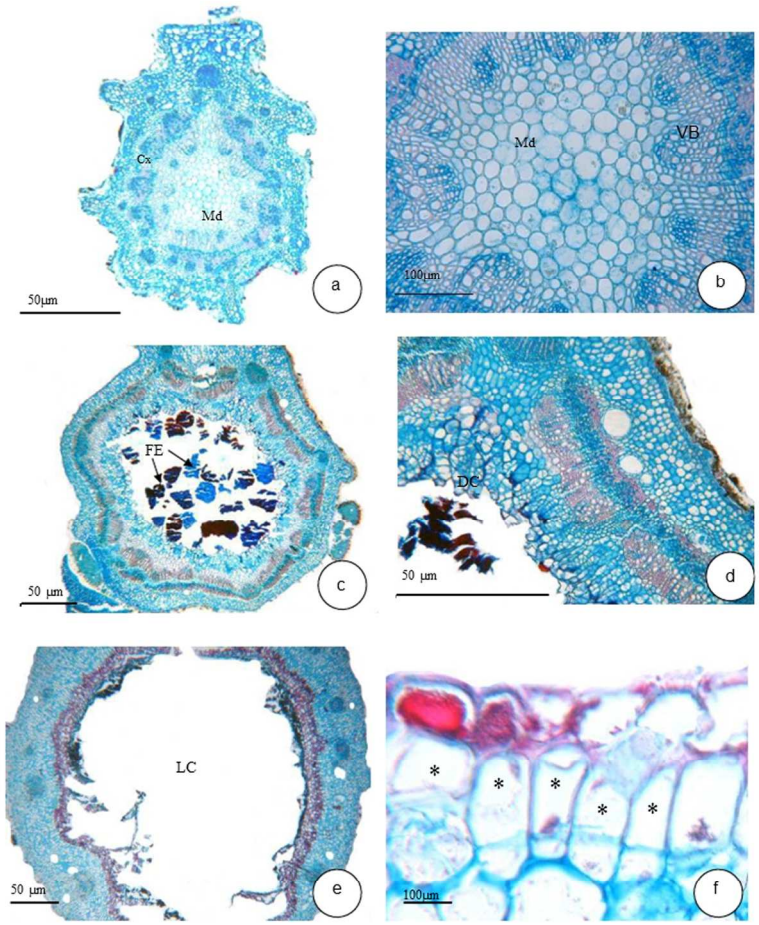

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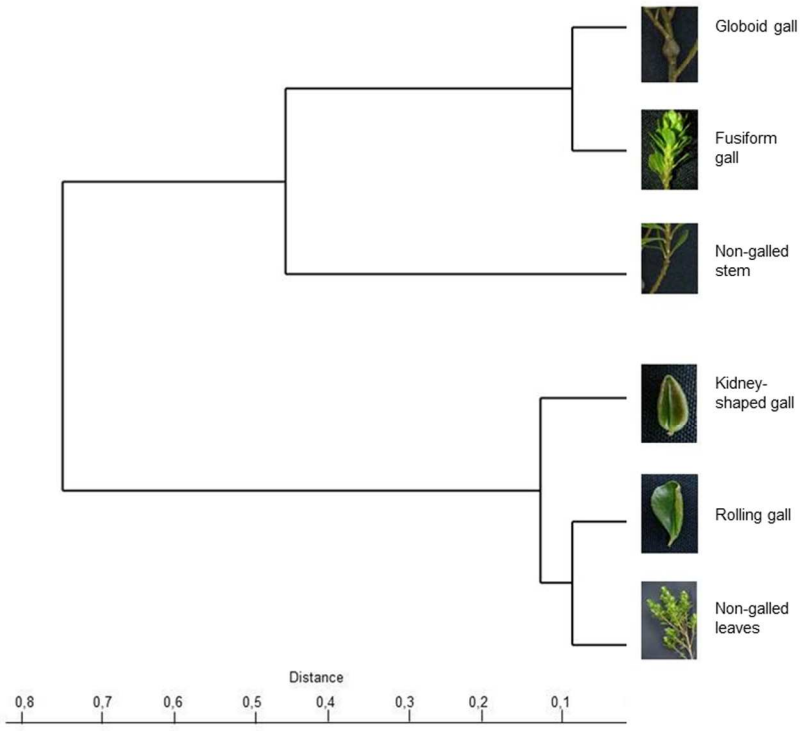

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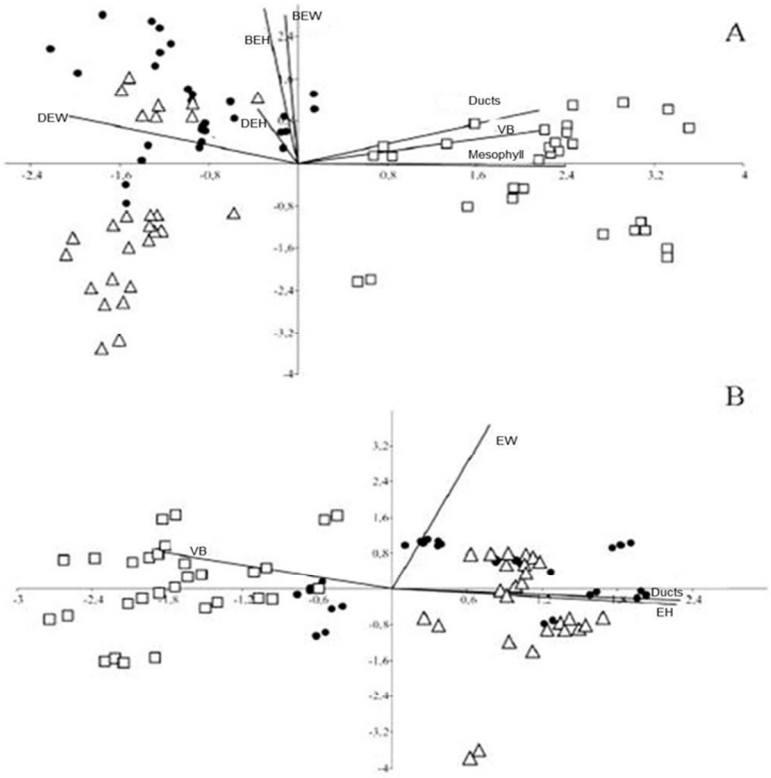

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Table 1 Results of the principal components 1 (PC1) e 2 (PC2) obtained from the correlation matrix of histometric characters of leaf galls in *Baccharis reticularia* DC. (Asteraceae)

Histometry	Axes	
	PC1	PC2
Secretory ducts	0.48	0.23
Vascular bundles	0.49	0.14
Mesophyll	0.54	-0.02
Adaxial epiderm cells width	-0.46	0.20
Adaxial epiderm cells height	0.08	0.23
Abaxial epiderm cells width	0.03	0.63
Abaxial epiderm cells height	0.06	0.66
Explained variance (%)	43.3	28.3

Table 2 Results of the principal components 1 (PC1) e 2 (PC2) obtained from the correlation matrix of histometric characters of stem galls in *Baccharis reticularia* DC. (Asteraceae)

Histometry	Axes	
	PC1	PC2
Secretory ducts	0.61	-0.06
Vascular bundles	-0.48	0.22
Epiderm cells height	-0.21	0.97
Epiderm cells width	0.60	-0.09
Explained variance (%)	48.6	24.1

Table 3 RDPI values of histometric variables from non-galled leaves and leaf galls in *Baccharis reticularia* (Asteraceae)

Histometric variable	Rolling-Kidney-Shaped	Rolling-Non-galled Leaves	Kidney-shaped-Non-galles Leaves	F
Secretory ducts	0.18a	0.07b	0.24c	6.6**
Vascular bundles	0.11a	0.20b	0.25b	30.1***
Mesophyll	0.24a	0b	0.23a	73.1***
Adaxial epiderm cell width	0.08a	0.01b	0.05c	30.2***
Adaxial epiderm cell height	0.04	0.01	0.01	1.22
Abaxial epiderm cell width	0.04a	0.12b	0.05a	12***
Abaxial epiderm cell height	0.05a	0.09b	0.01c	25.4***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Means followed by different letters in the same lines indicate significantly different values by Tukey test.

Table 4 RDPI values of histometric variables from non-galled stems and stem galls in *Baccharis reticularia* (Asteraceae)

Histometric variable	Globoid-Fusiform	Globoid-Non-galled Stem	Fusiform-Non-galled Stem	F
Secretory ducts	0.42a	0.26b	0.23c	62.4***
Vascular bundles	0.14a	0.09b	0.15a	32.8***
Epiderm cell height	0a	0.06b	0.03ab	6.6**
Epiderm cell width	0.1a	0.07b	0.02c	35.8***

$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Means followed by different letters in the same lines indicate significantly different values by Tukey test.

Supplementary Documents

Table 1. Averages measured in tissues and cells of non-galled orgns of *Baccharis reticularia* DC. (Asteraceae).

Organ or morphotype	MI	DS	VB	ME	IC	OC	EW	EH	DEW	DEH	BEW	BEH
NGL	46403,5	11166,83	9555,44	292,05					39,26	31,85	27,49	27,49
NGS		2534,72	63315,92				32,80	25,45				
RG	53572.82	24998,69	46675,02	460,91	411,23	61,31			41,37	32,44	33,91	33,91
KG		8919,80	7584,60	230,64	325,63	167,61			31,55	33,62	28,97	28,97
FG		7337,86	31605,23		657,34	195,79	38,09	29,44				
GG		14449,95	39977,8		665,40	211,66	34,34	33,00				

DS = secretoryu ducts, BEH = Abaxial epiderm cells height, BEW = Abaxial epiderm cells width, DEH = Adaxial epiderm cells height , DEW = Adaxial epiderm cells width, EH = epiderm cells height, EW = epiderm cells width, FG = fusiform gall, GG = globoid gall, IC = inner cortex, KG = Kidney-Shaped gall, ME = mesophil, MI = midrib, NGL = non-galled leaves, NGS = non-galled stem, OC = outer cortex, RG = rolling gall, VB = vascular bundle.

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ORIGINAL ARTICLE

The role of pectic composition of cell walls in the determination of the new shape-functional design in galls of *Baccharis reticularia* (Asteraceae)

Anete Teixeira Formiga · Denis Coelho de Oliveira · Bruno Garcia Ferreira · Thiago Alves Magalhães · Ariane Chagas de Castro · G. Wilson Fernandes · Rosy Mary dos Santos Isaias

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Abstract The pectic composition of cell wall is altered during the processes of cell differentiation, plant growth, and development. These alterations may be time-dependent, and fluctuate in distinct regions of the same cell or tissue layer, due to the biotic stress caused by the activity of the gall inducer. Among the roles of the pectins in cell wall, elasticity, rigidity, porosity, and control of cell death may be crucial during gall development. Galls on *Baccharis reticularia* present species-specific patterns of development leading to related morphotypes where pectins were widely detected by Ruthenium red, and the pectic epitopes were labeled with specific monoclonal antibodies (LM1, LM2, LM5, LM6, JIM5, and JIM7) in distinct sites of the non-galled and the galled tissues. In the studied system *B. reticularia*, the epitopes for extensins were not labeled in the non-galled tissues, as well as in those of the rolling and kidney-shaped galls. The high methyl-esterified homogalacturonans (HGA) were labeled all over the tissues either of non-galled leaves or of the three gall morphotypes, while the intense labeling for arabinogalactans was obtained just in the rolling galls. The pectic composition of non-galled leaves denotes their maturity. The kidney-shaped gall was the most similar to the non-galled leaves. The pectic dynamics in the gall tissues was particularly altered in relation to low methyl-esterified HGA, which confers elasticity and expansion, as well as porosity and adhesion to cell walls, and are related to the homogenization and hypertrophy of gall cortex, and to translocation of solutes to the larval chamber. Herein, the importance of the pectic dynamics of cell walls to the new functional design established during gall development is discussed for the first time. The repetitive developmental patterns in galls are elegant models for studies on cell differentiation.

Keywords Pectins · Cell development · Extensins · Insect galls

Abbreviations

AGPs	Arabinogalactans
FAA	Formaldehyde–acetic acid–ethanol
HGAs	Homogalacturonans
HR	Hypersensitive response
MAbs	Monoclonal antibodies
PBS	Phosphate buffered saline
PCD	Programmed cell death

Introduction

Plant cell wall is a complex structure formed by a crystalline matrix of cellulose microfibrils and a cellular matrix of hemicelluloses, pectic polysaccharides, and glycoproteins. Even in the absence of cellulose and hemicelluloses, the pectins may keep the integrity of the structure (Dolan et al. 1997; Albersheim et al. 2011) but may be altered during cell

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A. T. Formiga · B. G. Ferreira · T. A. Magalhães · A. C. Castro · G. W. Fernandes · R. M. S. Isaias (✉)
Instituto de Ciências Biológicas,
Universidade Federal de Minas Gerais - UFMG–ICB,
Belo Horizonte, MG CEP: 31270-901, Brasil
e-mail: rosy@icb.ufmg.br

D. C. Oliveira
Instituto de Biologia, Universidade Federal
de Uberlândia - UFU–INBIO, Uberlândia, MG, Brasil
e-mail: denisoliveira@inbio.ufu.br

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differentiation and plant development (Knox et al. 1990; Albersheim et al. 2011). Changes in pectic composition may be time-dependent or fluctuate in function of cell or tissue regions (Dolan et al. 1997). The elasticity, rigidity, porosity, and control of programmed cell death may also be related to the pectic composition (Gao and Showalter 1999; Willats et al. 2001). Biotic stresses induced by galling herbivores (Fernandes 1990; Fernandes et al. 2000) could also represent a potential source for such changes in cell wall composition. These herbivores, mainly insects, develop inside plant tissues and alter their host plant cells not only by its feeding habit but also by the oxidative stress that they generate (Sá et al. 2009; Oliveira and Isaias 2010; Formiga et al. 2011; Isaias et al. 2011; Oliveira et al. 2011). The pectic composition of the cell walls may also respond to the galling insect's stimuli configuring a limiting factor that determine the gall shape and the new functions of gall tissue layers.

Pectins are a group of polysaccharides rich in galacturonic acid (GalA). The GalA forms the backbone of three domains that can be found in all pectin species: homogalacturonan (HGA); rhamnogalacturan I; and rhamnogalacturan II (Willats et al. 2001). Pectins form approximately 35 % of the dry weight of dicot cell walls. They are polymerized in the cis Golgi, methyl-esterified in the medial Golgi, and substituted with side chains in the trans Golgi cisternae. The most abundant class of pectins, the HGA, is a linear homopolymer of (1-4)-α-linked-D-galacturonic acid with some 100–200 GalA residues located in the primary cell wall matrix of all land plants (Willats et al. 2001). They are involved in cell division, expansion, and adhesion (Xu et al. 2011). They are present since the formation of the primary cell walls, influencing cell porosity and elongation (Verhertbruggen et al. 2009). The arabinogalactan proteins (AGPs) constitute a family of high-molecular weight proteoglycans associated with the plasma membrane, the cell wall, and the intercellular spaces of plants (Albersheim et al. 2011). The AGPs contribute to several aspects of plant development (Gao and Showalter 2000; Rumyantseva 2005), including cell divisions (Serpe and Nothnagel 1994), expansion (Willats and Knox 1996; Ding and Zhu 1997), and programmed cell death (PCD) (Chaves et al. 2002; Guan and Nothnagel 2004). The AGPs can form a gel plug in sites of cell injury constituting a physical barrier to cell invasion (Cassab 1998), specifically the glycosylations made with (1→4)-β-galactans and (1→5)-α-arabinans. (1→4)-β-galactans are formed by flexible polymers of D-galactose in β configuration (Albersheim et al. 2011). When they are glycosylating the AGPs, they permit cell expansion (Jones et al. 1997). (1→5)-α-arabinans are polymers of L-arabinose in α configuration (Albersheim et al. 2011). In glycosylations of AGPs, they contribute to cell wall flexibility and also to intercellular adhesion (O'Donoghue and Sutherland 2012). The loss of these two cell wall components may confer opposite functionality to cell walls, i.e., cell wall rigidity and loss of adhesion (Brummell et al. 2004).

Another important component of cell walls, the extensins, are evidenced at the end of cell expansion and gets cell final shape (Cassab 1998). They are strengthening proteins present during cell growth and development (Leroux et al. 2011) and, together with the pectins, can assume a structural role in cell walls (Sabba and Lulai 2005), keeping cell shape in the absence of cellulose. As the structure and functionality of insect galls are constantly repeated in nature, gall systems constitute excellent models for the study of the new structural and functional design related to the pectic domains assumed during the development of the cell layers in galls. The use of antibody probes permits relating pectin chemistry and its variations with their biological functions at the level of tissues and cells (Verhertbruggen et al. 2009). The detection of HGAs by monoclonal antibodies together with that of glycoproteins represented by AGPs, galactan, and arabinan and extensins on galls should lead to a new perspective on the understanding of cell wall dynamics related to the function and maturity of plant tissues. This study conducted in three gall morphotypes and in non-galled leaves of a superhost constitutes an elegant model for comparative and developmental studies.

The comparative study of different insect galls in the non-galled leaves of *Baccharis reticularia* aims to elucidate the spectrum of responses of similar host cells to diverse galling herbivore inductions. Monoclonal antibodies are excellent tools for the location of cell wall pectic components (Jones et al. 1997; Cassab 1998), and insect galls are efficient models to relate the dynamics of these components with plant cells and tissues functioning. The specific monoclonal antibodies (MAbs) (JIM 5, JIM7, LM1, LM2, and LM5) are here used to the comparative screening of the distinct pectic composition of non-galled cell walls of *B. reticularia*, as well as those of tissues of three leaf gall morphotypes, the rolling gall, the pocket gall, and the kidney-shaped gall. Our purpose is to relate the role of each investigated epitope with the transformations of structure and function in the three distinct morphotypes over the same pool of host cell responses.

Material and methods

Sampling and fixation

Samples of non-galled leaves and three gall morphotypes of *B. reticularia* (Asteraceae) in maturation phase, defined by size and anatomical features (Table 1), were collected at Campo de Fora, Serra do Caraça, Minas Gerais, Brazil (20°07′035″ S, 43°31′201″ W, 1.511 m). Due to the specificity of interactions, each morphotype represents one host plant-galling herbivore system (Carneiro et al. 2009). The

Table 1 General features of three leaf gall morphotypes of *B. reticularia* (Asteraceae)

Morphotypes/ gall inducer	Host organ	Position	Attachment/ ostiole	Surface	Relation larvae/ chamber	Color	Texture/features	Anatomical features
Rolling gall/Psylloidea (Hemiptera)	leaf	Adaxial epidermis	Intralaminar/ open	hairy	≥1/1	green	Membranaceous, formed by rolling of the leaf margin.	Hypertrophied epidermis with thick walls, homogeneous cortex with hypertrophied secretory ducts and vascular bundles neoformed
Kidney-shaped gall/ Psylloidea (Hemiptera)	leaf	Adaxial epidermis	Intralaminar/ open	hairy	2–4/1	green	Membranaceous, fleshy consistency	Severe hypertrophy of epidermal cells, homogeneous parenchyma with hypertrophied secretory ducts
Pocket gall/Cecidomyiidae (Diptera)	leaf	Adaxial epidermis	Intralaminar/ open	hairy	1/1	green	Membranaceous, formed by bulging of the adaxial epidermis	Adaxial epidermis hypertrophied, homogeneous parenchyma with hypertrophied secretory ducts

rolling and kidney-shaped galls are induced by unidentified species of Hemiptera, while the pocket gall is induced by an undescribed species of Diptera: Cecidomyiidae (Fig. 1). The samples of non-galled tissues and of tissues of rolling galls, pocket galls, and kidney-shaped galls were fixed in FAA (1:1:18), dehydrated in *n*-butyl series, and embedded in Paraplast® (Johansen 1940).

Histochemistry

Sections (10–12 μm) of all samples were stained with ruthenium red for 15 min to pectins detection (Jensen 1962).

Immunocytochemistry

Sections (15–20 μm) of all samples were incubated in MAbs JIM5, JIM7, LM1, LM2, LM5, and LM6 (Table 2) produced at the Centre for Plant Sciences, University of Leeds, UK. The sections were hydrated in phosphate buffered saline (PBS) pH 7.1, blocked with a 3 % solution of powder milk for 30 min, and incubated with the primary antibodies in PBS for 1 h in room temperature. For control, the primary antibody was suppressed. The sections were washed in PBS, and incubated in the secondary antibody anti-rat IgG - FITC (Sigma) in PBS for 2 h in the dark. After washing in PBS, the sections were mounted in 50 % glycerin. The analyses were performed in a Confocal Zeiss 510 META microscope, with excitation wavelength of 488 and 505–530-nm emission filter.

Results

General features of the galls

The three gall morphotypes, the rolling, pocket, and kidney-shaped galls, on *B. reticularia* are easily distinguished by its morphological features (Fig. 1, Table 1).

Histochemistry of pectins

The non-galled leaves presented positive reaction for ruthenium red in the mesophyll, mainly in the outer periclinal cell walls of epidermis either in the adaxial or abaxial surfaces (Fig. 2a, b). Similar results were detected for the parenchymatic cortical cells of the three gall morphotypes. Also, both the external and internal periclinal epidermal cell walls were thicker than those of the non-galled leaves, with intense positive reaction to the ruthenium red (Fig. 2c–h).

Immunocytochemistry

There were some alterations in the distribution of the pectic epitopes from non-galled leaves to galls (Fig. 3, Table 3).

Fig. 1 General aspect of *B. reticularia* **a** Herbaceous specimen (Campo de Fora, Serra do Caraça, Minas Gerais, Brazil). **b** Rolling gall. **c** Kidney-shaped gall. **d** Pocket gall. *Bars*=1 cm

The non-galled leaves of *B. reticularia* showed a weak reaction to the extensin epitope in the epidermal cells, vascular bundle, and secretory ducts. In the pocket gall, the extensin epitope was labeled in the parenchymatic cells of the cortex and ducts (Fig. 3a, Table 3).

The labeling of AGP glycans epitope was moderate in the cells of the secretory duct (Fig. 3b, Table 3) and weak in the epidermis and vascular bundles of non-galled leaves. In the rolling and pocket shaped galls, the AGPs were marked in all tissue layers (Fig. 3c, Table 3), while in the kidney-shaped galls, the LM2 labeled AGPs only in the epithelium of the ducts (Fig. 3d, Table 3).

The epitopes of (1→4)-β-D-galactan were weakly marked in the epidermis and vascular bundles of non-galled leaves. In the rolling gall, it was moderately marked in all tissue layers. In the pocket galls, the cell walls of the epidermis and of the vascular bundles were moderate and weakly labeled, respectively. In the kidney-shaped gall, there was no labeling (Fig. 3e, Table 3).

In the non-galled leaves, the epitopes of (1→5)α-L-arabinans were weakly marked in the cell walls of the epidermis, vascular bundles, parenchyma, and secretory ducts. On the other hand, the LM6 strongly labeled the epidermis (Fig. 3f, Table 3) and the vascular bundle of the rolling galls. The detection of arabinan chains was moderate in the parenchyma of the rolling galls, in the epidermis, and parenchyma of the kidney-shaped galls, and absent in all tissues of the pocket galls.

Table 2 Relation of the monoclonal antibodies and their epitopes

Monoclonal antibodies	Epitopes	References
LM1	Extensin	Smallwood et al. 1995; Cassab 1998; Sabba and Lulai 2005; Leroux et al. 2011.
JIM 5	HGA methyl-esterified up to 40 %	Vanderbosch et al. 1989; Knox et al. 1990; Willats et al. 2000; Clausen et al. 2004.
JIM 7	HGA methyl-esterified 15 – 80 %	Knox et al. 1990; Willats et al. 2000; Clausen et al. 2004.
LM2	AGP glycan	Yates et al. 1996; Smallwood et al. 1996.
LM 5	(1→4) β-D-galactan	Jones et al. 1997.
LM 6	(1→5) α-L-arabinans	Willats et al. 1998.

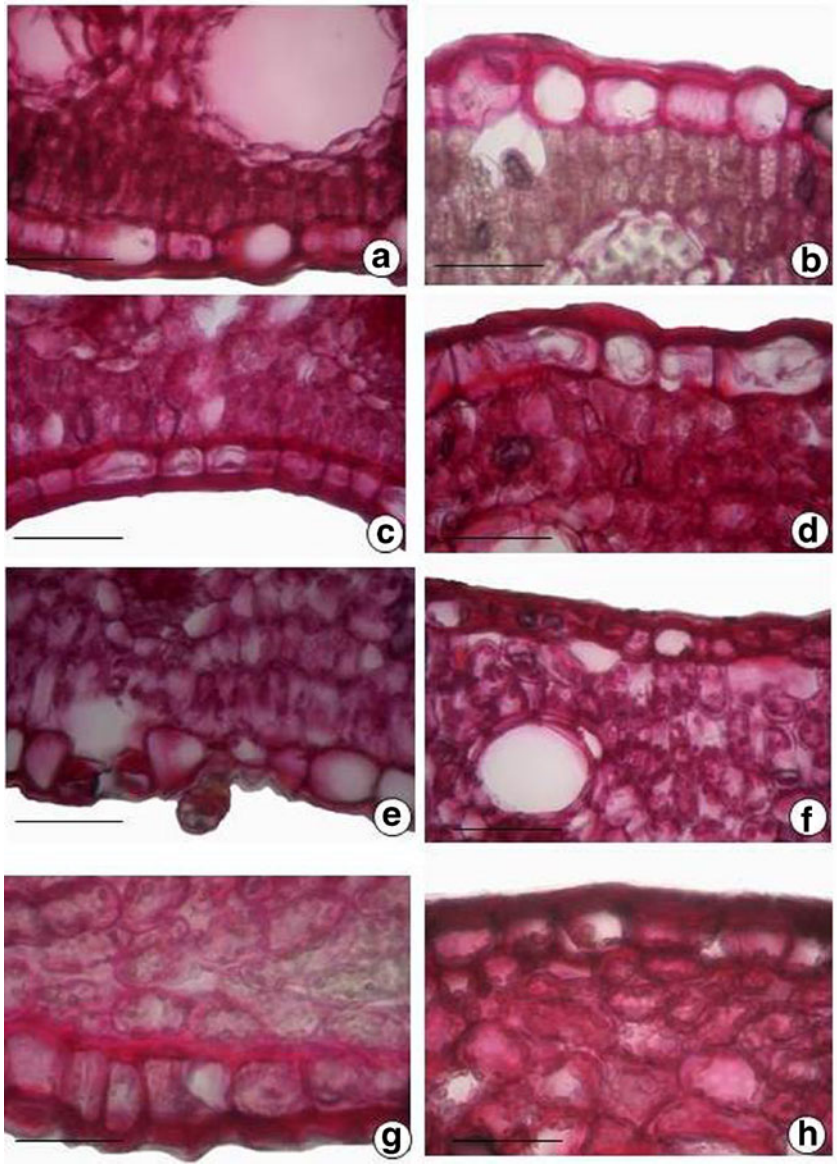

Fig. 2 Details of epidermis of *B. reticularia*. **a**–**b** Non-galled leaf. **a** Abaxial surface. **b** Adaxial surface. **c**, **d** Rolling gall. **c** Epidermis of larval chamber. **d** Outer epidermis. **e**, **f** Pocket gall. **e** Epidermis of larval chamber. **f** Outer epidermis. **g**, **h** Kidney-shaped gall. **g** Epidermis of larval chamber. **h** Outer epidermis. *Bars*=50 μm

The HGA up to 40 % methyl-esterified was weakly labeled in the epithelium of the secretory ducts of the non-galled leaves, intensely labeled in the epidermis of rolling and pocket galls (Fig. 3g, h, Table 3), moderate in the vascular bundle, parenchyma, and ducts of the rolling and pocket galls, and moderate in the parenchyma of kidney-shaped galls. Nevertheless, the HGA with 15–80 % methyl esterification were moderately labeled in the cell walls of epidermis, vascular bundle, and parenchyma and intense in the epithelium of the secretory ducts of non-galled leaves (Fig. 3i, Table 3). The JIM7 labeled all tissues in galls. The intense labeling can be visualized in the outer periclinal cell walls of epidermis and in the epithelium of the secretory ducts of the pocket (Fig. 3j, Table 3), rolling, and kidney-shaped galls. The intense detection was also observed in the vascular bundle of the rolling galls (Fig. 3k, Table 3) and in the epidermis and parenchyma of the kidney-shaped galls.

Discussion

The three tissue systems of the non-galled leaves of *B. reticularia* are altered by gall induction and development. These alterations are associated with the new structural and functional design established during the development of the three gall morphotypes. The distribution of the pectic epitopes in these galls corroborates the histochemical detection of pectins and reveals patterns for cell wall alterations. The high methyl-esterified HGA were the most ubiquitous pectins either in non-galled leaves or galled tissues, with the rolling galls presenting the greatest diversity of pectins among the analyzed morphotypes. Intriguingly, this morphotype is the most similar to the non-galled leaves (Formiga et al. in preparation), but the pectic constitution of its cell walls diverges either in relation to the non-galled leaves or to the other gall morphotypes.

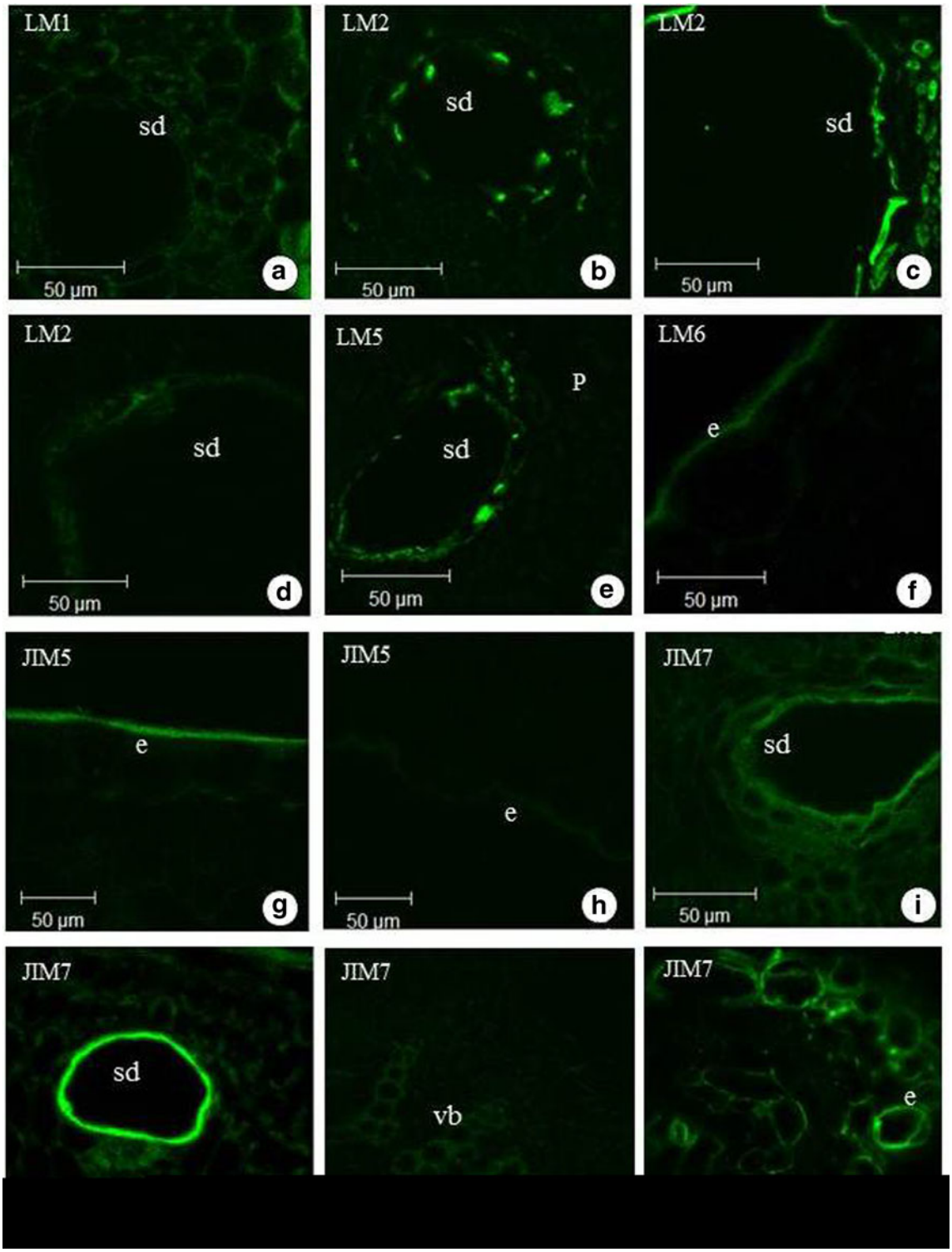

Fig. 3 Immunocytochemistry with monoclonal antibodies in non-galled leaves and galls of *B. reticularia*. **a** LM1 labeling extensins in a secretory duct of pocket gall. **b**–**d** LM2 labeling AGP glycan in secretory ducts. **b** Non-galled leaf. **c** Rolling gall. **d** Kidney-shaped gall. **e** LM5 labeling (1→4) β-D-galactan in secretory duct and parenchyma of rolling gall. **f** LM6 labeling (1→5) α-L-arabinans in the epidermis of rolling gall. **g**, **h** JIM5 labeling HGA<40 % methyl-esterified in epidermis. **g** Rolling gall. **h** Pocket gall. **i**–**l** JIM7 labeling HGA 15–80 % methyl-esterified. **i**, **j** Secretory ducts. **i** Non-galled leaf. **j** Pocket gall. **k**, **l** Rolling gall. **k** Vascular bundles. **l** Epidermis. *e* epidermis, *p* parenchyma, *sd* secretory ducts, *vb* vascular bundles. *Bars*=50 µm

Table 3 Distribution of pectic epitopes on non-galled leaves and galls tissues of *B. reticularia*

Monoclonal Antibodies	Tissue	Epidermis	Vascular bundle	Parenchyma	Secretory Duct
LM1	Non-galled leaves	+	+	−	+
	Rolling gall	−	−	−	−
	Pocket gall	−	−	++	++
	Kidney-shaped gall	−	−	−	−
LM2	Non-galled leaves	+	+	−	++
	Rolling gall	+++	+++	+++	+++
	Pocket gall	++	++	++	++
	Kidney-shaped gall	−	−	−	+++
LM5	Non-galled leaves	+	+	−	−
	Rolling gall	++	++	++	++
	Pocket gall	++	+	−	−
	Kidney-shaped gall	−	−	−	−
LM6	Non-galled leaves	+	+	+	+
	Rolling gall	+++	+++	++	−
	Pocket gall	−	−	−	−
	Kidney-shaped gall	++	−	++	−
JIM5	Non-galled leaves	−	−	−	+
	Rolling gall	+++	++	++	++
	Pocket gall	+++	++	++	++
	Kidney-shaped gall	−	−	++	−
JIM7	Non-galled leaves	++	++	++	+++
	Rolling gall	+++	+++	+++	+++
	Pocket gall	+++	+++	++	+++
	Kidney-shaped gall	+++	++	+++	+++

Labeling: absent (−), weak (+), moderate (++), or intense (+++)

Among the gall morphotypes, the epitope for extensin was exclusively labeled in the pocket galls. The extensin seems to keep the properties of reinforcing cell walls as proposed by Sabba and Lulai (2005). Otherwise, this function seems not to be corroborated in the other smaller galls, the rolling and kidney-shaped. The structure of pocket galls seems to require special reinforcement due to their distinct growth orientation, i.e., the major elongation towards the lateral portion and the protrusion to the abaxial leaf surface, which differs from the other gall morphotypes formed just by leaf rolling or folding.

The AGP proteins are developmentally regulated proteoglycans detected in high amounts at plant cell surfaces and in association with the cell walls (Samaj et al. 2000). The MAb for AGP epitopes labels the cell walls of all tissue layers of rolling galls and pocket galls. They are quickly synthesized, secreted, and then recycled (Gibeaut and Carpita 1991), and their cytochemical localization with monoclonal antibody LM2 in the rolling galls indicates a high dynamic functioning of various endomembrane systems including endoplasmic reticule, Golgi apparatus, and tonoplast in this gall morphotype. As the rolling galls have a high level of structural similarity with the non-galled leaves, the divergence in its pectic histochemical status is indicative of the high metabolism imposed by the galling insect larva on gall tissues (Oliveira and Isaias 2009; Oliveira et al. 2010). In these galls, the AGP epitope is strongly labeled in association with the cytoplasm and tonoplast, similarly to the results described by Samaj et al. (2000) for root nodules.

Even though the AGP glycan is commonly associated to young and meristematic tissues (Herman and Lamb 1992; Albersheim et al. 2011), it is plausible to assume that in the mature galls, it is associated to the prevention of the PCD, and consequently to the avoidance of hypersensitive responses (HR) in host plant tissues (Fernandes 1990). In the kidney-shaped galls, the labeling of AGP glycan was restricted to the epithelium of the secretory ducts. The intensity of this epitope and consequent prevention of the PCD in the cells of the secretory ducts was previously observed by Mastroberti and Mariath (2003, 2008) in *Araucaria angustifolia*, as well as by Gao and Showalter (1999) in cells of *Arabidopsis*, and by Letarte et al. (2006) in microspores of *Triticum aestivum*.

The galactan was intensely labeled in the endomembrane system, while the labeling of arabinan was very weak and

restricted to the outer anticlinal cell walls of the epidermis of rolling and kidney-shaped galls. Galactans and arabinans may be present either as independent polysaccharides or together as arabinogalactan I (O'Donoghue and Sutherland 2012). In the gall morphotypes on *B. reticularia*, the galactans are independent of the arabinans. The differences in labeling of cell wall epitopes indicate the modification towards a new functional, spatially significant design, as proposed by O'Donoghue and Sutherland (2012) for cell control and responses to expansion in epidermis and parenchyma of *Sandersonia* petals. The galactan and arabinan generally serve the same function as side branches of pectins, and were equally labeled in the epidermis and vascular bundles of the rolling galls. Nevertheless, the loss of arabinan has been connected to the loss of adhesion in cell walls (Brummell et al. 2004), which permits rapid cell expansion in the parenchymatic cortex of this gall morphotype. There seems to be equilibrium of these two AGP epitopes in the rolling gall, and so the dynamics of cell expansion and rigidity is guaranteed.

The arabinan chains labeled in the cell walls of the epidermis, parenchyma, and vascular bundles in the rolling gall suggest their association with flexibility (Jones et al. 1997; McCartney et al. 2000; McCartney and Knox 2002), which should permit the rolling of the host leaf without cracking the structure. The flexibility of cell wall structure given by the arabinan chains has been previously related to the guard cells in *Arabidopsis* (Jones et al. 2003) and to the development of mucilage cells in *A. angustifolia* (Mastroberti and Mariath 2008).

The low and high methyl-esterified HGA constitute the main pectic component of cell walls. These pectic domains are involved in several physiological processes during plant development, including gel formation and cellular adhesion (Albersheim et al. 2011). The significance and regulatory aspects of its methyl esterification is not fully understood (Verhertbruggen et al. 2009). Nevertheless, Willats et al. (2001) elegantly demonstrated the patterns of methyl esterification using MAbs. These authors have shown that the wall around one cell can contain microdomains, each one accumulating a particular type of HGA. Both JIM5 and JIM7 bind optimally to partially methyl-esterified epitopes of HGA and are used here to provide the variation in methyl esterification status during the development of the same pool of cells, namely, the host leaf cells of *B. reticularia*. The JIM7 binds the high methyl-esterified HGA epitopes similarly either on non-galled leaves or on the three gall morphotypes on *B. reticularia*. This result indicates that the methyl esterification in all tissue layers was maintained independently of gall development, reflecting the involvement of these pectic domains in the mechanical stability of cell walls during gall development (Albersheim et al. 2011). Nevertheless, the JIM5 weakly bound low methyl-esterified HGA epitopes in the epithelium of the secretory ducts of the non-galled leaves, but labeled in distinct degrees in all tissue layers of the rolling and pocket galls, and the cortex of the kidney-shaped galls. The decrease in the degree of methyl esterification detected in gall tissues may represent a cell response to the intrinsic oxidative stress generated throughout the interaction. The low methyl esterified HGA forms Ca^{2+} crosslinks (Albersheim et al. 2011), and the influx of this calcium should maintain the dynamics of the interaction, as proposed by Maffei et al. (2007). In galls, the calcium is available in the low methyl-esterified HGA, interacting with resultant gels of de-methyl-esterification and promoting the avoidance of loss of rigidity in cell walls (Jiang et al. 2005) due to oxidative stress.

The specific labeling pattern of pectic domains in gall tissues denotes the capacity of these cells to elongate and keep their shapes even in mature stages. These two properties may be consequence of the close association of extensins and pectins in plant cell walls (Swords and Staehelin 1993; Qi et al. 1995). However, the pectic labeling with the MAbs LM2, LM5, LM6, JIM5, and JIM7 indicates the changes in cell wall compounds of *B. reticularia* from non-galled condition towards gall maturation. The current results indicate the maintenance of the potential for elongation and flexibility throughout gall development. Besides, the exclusive labeling of extensins in pocket galls corroborates the more conspicuous changes necessary to this gall shape formation when compared to the other leaf galls on *B. reticularia*. The pectic dynamics of cell walls established due to the new functional design during gall development has been proved and is herein discussed for the first time. The repetitive developmental patterns in galls have been demonstrated to be elegant models for studies on cell differentiation.

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Conflict of interest The authors declare no conflict of interest in the manuscript titled "The role of pectic composition of cell walls in the determination of the new shape-functional design in galls of *B. reticularia* (Asteraceae)."

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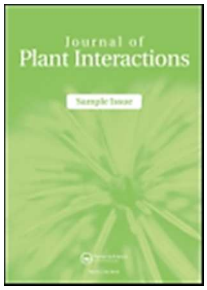

Seasonal fluctuations of the gall morphotypes under the stability of the tropical climate on the superhost Baccharis reticularia (Asteraceae)

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Seasonal fluctuations of the gall morphotypes under the stability of the tropical climate on the superhost *Baccharis reticularia* (Asteraceae)

Anete Teixeira Formiga[a], Cleber Cunha Figueredo[a], Cleber Juliano Neves Chaves[a], Geraldo Wilson Fernandes[b], Rosy Mary dos Santos Isaias[a*]

[a]Departamento de Botânica, Universidade Federal de Minas Gerais - UFMG, Belo Horizonte, MG, Brazil, [b]Departamento de Ecologia, Universidade Federal de Minas Gerais - UFMG, Belo Horizonte, MG, Brazil.

*Corresponding author - e-mail: rosy@icb.ufmg.br, Phone: +55-31-34092687, Fax: +55-31-34092671

Abstract

The occurrence of insect galls throughout the year is a common feature in the tropical areas due to the stability of the climate. Nevertheless, even in tropical areas, some superhosts of galling herbivores, such as *Baccharis reticularia* (Asteraceae), gall abundance may vary along the year in response to discrete climatic variations. Different strategies are necessary for the galling herbivores to share the superhosts, which may vary either due to climatic factors or to intrinsic features of the associated organisms. To check these premises, thirty individuals of *B. reticularia* (three leaf galls and two stem galls) were studied at Serra do Caraça, Minas Gerais, Brazil, along two consecutive years, where five gall morphotypes were monitored. The seasonal distribution of the galls on the superhost *B. reticularia* denotes that the associated galling herbivores used two different strategies for sharing the same host: asynchrony among the life cycles, and low infestation throughout the year. We argue that such strategies enable them to mobilize the resources offered by the host plant ensuring the completion of their life cycles.

Keywords: herbivore seasonality, insect galls, insect herbivory, population dynamics, superhost.

Introduction

Baccharis L. is one of the largest genus of the Asteraceae, with wide distribution from Mexico to Argentina (Nesom 1994). *Baccharis* species are abundant in the South and Southeast Brazil, amongst which there are many hosts of galling herbivores (Fagundes and Fernandes 2011). Galls are neoformed structures induced by specific associated insects (Stone and Schönrogge 2003), as the result of profound alterations in the host plant developmental patterns. Even though, these structures may also be induced by several other organisms, the insects are usually the most common inducing agents (1964). The galls induced by insects are quite different morphologically (Raman 2011), and highly specialized towards structural and chemical defenses for the galling agents (Nyman and Julkunen-Tiitto 2000). Thus, by promoting changes in plant tissues, the insects get an optimum microenvironment, ensuring food, protection against natural enemies and environmental stresses (Price et al. 1986-1987; Nyman and Julkunen-Tiitto 2000; Stone and Schönrogge 2003; Araújo et al. 2006).

The balance between the phenology of the host plants and the life cycle of the galling insects seems to vary either in temperate or tropical region. In temperate regions, there is a great synchrony between the life cycles of insects and the host plants phenology, demonstrated by the study of the genetic alterations which led insects to regulate their life cycles in function of the World's climatic changes (Moore and Allard 2008; Bale and Hayward 2010). Also, some variation in animal seasonal patterns due to light intensity in Temperate and Polar Regions was observed Bradshaw and Holzapfel (2001-2006-2010). Moreover, according to Wolda (1988), the environmental seasonality is an important factor for insect abundance in temperate regions, where it is considered as a common fact of life. Nevertheless, in the tropical regions, seasonal activities tend to be longer, the species richness is higher, and the seasonal peaks are less defined when compared to the temperate regions (Wolda 1988). At the Brazilian Cerrado,

Pinheiro et al. (2002) have described the relation between the climatic factors and the abundance of insects. However, Dalbem and Mendonça (2006) did not find any statistical relation between the richness and the abundance of galls, and the phenology of 84 host plant species in a Subtropical Seasonal Forest. Their data are indicative that the patterns of the establishment of the galling herbivores on their host plants are much more complex and needs more studies, especially in the Neotropics. Campos et al. (2010) reported the greatest abundance of gall induction just after leaf flushing in *Aspidosperma australe*, which denotes a synchronism between this host plant and its associated galling herbivore. Similar results were found for other two Neotropical species, *Aspidosperma macrocarpon* (Castro et al. 2013), and *Copaifera langsdorffii* (Oliveira et al. 2012). In fact, among these species, *C. langsforffii* is the only superhost of galling herbivores studied in such perspective. In this host plant, not all the gall morphotypes are induced at the same time of the year, and three seasonal syndromes related to climatic factors such as water stress were evidenced in a Cerrado area (Oliveira et al. 2012).

The occurrence of seasonal syndromes in a superhost of galling herbivores in the Neotropics is herein revisited in *Baccharis reticularia* and its associated galling insects guild as a model system. Commonly, the studies of the richness and/or abundance of galls in relation to the varying climate in the tropics do not consider the patterns of abundance with the fluctuation of the individual morphotypes (*cf.* Araújo and Santos 2009; Coelho et al. 2009; Tessinari et al. 2009; Silva et al. 2011). Five distinct galling insects associate to *B. reticularia*, and it is assumed that these insects should synchronize their life cycles or develop special strategies to establish in the same host plant on time basis. Current study aimed to analyze the seasonal fluctuation of the five galling herbivores associated to a population of *B. reticularia* checking if there is any relation of the abundance of each gall morphotype with temperature and rainfall. The following questions are addressed: (1) what are the peculiarities of the gall morphotypes on *B. reticularia*? (2) Do the gall morphotypes on *B. reticularia* have similar abundances? (3) Do these gall morphotypes repeat the patterns for the other superhosts previously described in the Neotropical region? And (4) what are the patterns of occurrence of the five gall morphotypes through a year-time?

Methods

Sampling area, plant species and gall morphotypes. Serra do Caraça is a mountain range located at the east side of the Quadrilátero Ferrífero, Southern of the Cadeia do Espinhaço (Falcão et al. 2003), in Minas Gerais State, Brazil. The climate at the area is Cwb of Köppen-Geiger (Peel et al. 2007) with rainy summers, and average annual precipitation above 1.500 mm (Brandão et al. 1994). The average annual minimum and maximum temperatures are 16 and 21^{o}C, and the altitude ranges from 750 - 2072m (Silveira 1924). The rainy season lasts from October to March, and the dry season lasts from April to September (Silva and Talamoni 2003). For this study, 30 individuals of *Baccharis reticularia* were randomly selected and marked in a rupestrian vegetation (20º07'035"S, 43º31'201"W, 1.511m. *B. reticularia* is a superhost of five distinct galling insects (Table 1, Fig. 1). The sampled individuals are 50cm to 4m high, grown on quartzite soil, partially shaded by small trees and shrubs. Flowering branches

were collected, identified by taxonomists and incorporated into the herbarium BHCB of the Instituto de Ciências Biológicas - UFMG under numbers 161554 and 161555.

Host plant phenology. The total number of leaves per terminal branch (n=3) of 10 individuals. The occurrence of flowering and fructification were registered monthly during 2009-2010.

Characterization of the gall morphotypes and galling insects. Gall morphotypes were described according to the following parameters: approximated shape, presence or absence of indumentum, color and precise position of the galls in their host organs (Table 1), as proposed by Isaias et al. (2013), assuming that each gall morphotype corresponded to a different species of gall inducing insect (Carneiro et al. 2009). Each 30 days, gall samples (n = 60) were dissected in a stereomicroscope in order to obtain the galling insects, which were fixed in ethanol 70%.

Seasonal fluctuation of the gall morphotypes. The fluctuation of the gall morphotypes was recorded monthly during 2009-2010. Non-galled and galled leaves were counted in three terminal branches of each plant to estimate the infestation levels. The density of galls on each terminal branch was calculated by the formula: gall density = n of galls / n of total leaves (galled + non-galled).

Precipitation and temperature data were obtained and kindly provided to our study by Reserva Particular do Patrimônio Natural Serra do Caraça, and by the environment team of the AngloGold Ashanti company (Fig. 2).

Statistical analyses. The relationship between the climatic variables and the abundance of the distinct gall morphotypes was analyzed by Spearman Rank Correlation using the software SAS JMP 5.0, once the data did not present normal distribution and homocedasticity. The temporal and spatial effects, as well as the effects of the presence of non-galled leaves on the abundance of leaf and stem galls were evaluated by models of Mixed Linear Effect (MLE), adjusted for longitudinal data (repeated measurements) with special net random effect, with the *software* R 2.15.2 (R Core Team 2012). Mixed effects model the possible correlations among grouped data (Buckley et al. 2003). Also, repeated measurements, as grouped data, may avoid pseudo-replication, and elevate the statistical power of the analysis (Crawley 2002).

The model was progressively simplified by removing the non-significant terms. These procedures were systematically performed, with the removal of the terms with higher significance values (p> 0,05) (Crawley 1993). The models were analyzed by variance, F-tests of maximum likelihood within and between them. Finally, the model of minimum adequacy was calculated and evaluated (Crawley 2002; Buckley et al. 2003).

Results

Host plant phenology. The higher amounts of total leaves were observed on June, July, and August 2009-2010, which corresponds to the dry season. Nevertheless, the monthly availability of leaves was similar along the year (Fig. 2), which implies in a relative constant potential for gall inducing. Flowering and

fructification occured two times a year, at the transition from rainy to dry season (March to May), and at the transition from dry to rainy season (August to October). No positive correlation was found between the phenophases of the host plant and gall abundance.

Gall morphotypes. Three leaf galls and two stem galls (Fig. 1) were observed on *B. reticularia*. Rolling, kidney-shaped and pocket galls are induced on mature leaves and differ in shape and size. Fusiform and globoid galls are induced on stems apices and differ by their shape and inducing *taxa* (Table 1). The pocket gall is unique amongst the gall morphotypes induced on the leaves of *B. reticularia*, due to its peculiar morphology. Its morphogenesis leads to the formation of a deep depression on the leaf lamina, thus affecting leaf expansion. The leaf rolling and kidney-shaped galls are formed by the rolling and folding of one or both leaf margins, respectively. The stem galls are structurally distinct, as the fusiform galls are elongated with acute apices and the globoid gall is round.

Gall morphotypes abundance and infestation levels. The abundance of the kidney and pocket galls was strictly opposite one another (ρ = -0.534; P= 0.007), and presented more evident inversed tendencies during August-December 2009/2010, with more representative peak (kidney) and a decline (pocket) on September 2009/2010 (Fig. 3). The kidney-shaped, leaf rolling, and fusiform galls were present throughout the year, while the pocket and the globoid galls presented a discontinuous occurrence. The pocket gall did not occur on March and April 2009, and from December to April 2010, except for an extremely low occurrence in January. The globoid gall was not observed in April, May and September on both years (Fig. 3d).

We have observed a high positive correlation between temperature and rainfall (Spearman ρ=0.76, P<0.0001), as expected for the Neotropical region. However, considering that temperature has exhibited a low range of variation, we focused our analysis and discussion on rainfall data, because of its wider temporal oscillation. The correlation between the abundance of the leaf rolling and globoid galls and the rainfall index was positive (ρ = 0.412; P=0.045, and ρ = 0.456; P=0.03, respectively). The correlation between the other gall morphotypes and rainfall was not significant (Table 2). The population dynamics of the fusiform gall did not present an evident seasonal pattern as it was constantly recorded with very small variation (Fig. 3e).

The levels of infestation of galls on *B. reticularia* were low all over the year especially during May-September 2009/2010 (dry season) and January-February 2009/2010 (rainy season) with peaks in the transitional periods (Fig. 2).

Seasonal fluctuation of the gall morphotypes. During the two consecutive years of observation (2009-2010), temperature ranged from 16°C to 24°C (medium values) with maximum peaks on November 2009 and January 2010. The higher levels of precipitation occurred from September to March with peaks on January and December 2009 (Fig. 2).

Although the total number of galls remained nearly constant throughout the year, a cyclic pattern could be observed with small density peaks during both periods of transition between dry and rainy seasons (September-October) and rainy and dry seasons (March-April) (Fig. 2). The infestation levels were low (5 to 12 galls per plant) in spite of the occurrence of gall induction and development all over the year, both on stems and leaves (Fig. 2).

Discussion

Gall morphotypes on B. reticularia. The shapes of the leaf rolling and kidney shaped galls were generated by two similar morphogenetical processes, strictly related to the taxa of the inducing insects. The two species of Hemiptera, and the similarities or their galls should be consequence of the triggering of plant morphogenetical responses in a convergent pattern. Nevertheless, these two gall morphotypes are distinguishable by the intumescence of the host leaf in the kidney-shaped gall, which is absent in the leaf rolling gall. According to Fernandes and Price (1992), leaf folders/rollers are the basal gall types. The simplicity of the rolling gall on *B. reticularia* is an indicative of the basal developmental pattern. This presumption is intensely discussed by Nymam and Julkunen-Tiitto (2000). The variations in shape of the stem galls have been attributed by Rohfritsch (1992) to the feeding patterns of the galling herbivores. This proposal fits for the stem galls of *B. reticularia*, whose distinct feeding habits of the Lepidoptera and the Diptera might determine the peculiar variations in shape (Formiga et al. in preparation). Nevertheless, the impact of the feeding behavior of the galling insects should be associated with the morphogenetical constraints imposed by the host plant cells to determine the final shape of the galls.

As expected for some interactions, several factors should act together during the life cycles of the associated organisms. The life cycle of the gall inducers, the morphogenetical constraints of the host plant (Oliveira and Isaias 2010a), as well as environmental factors such as type of soil, water availability, light intensity, and temperature are determinant for the induction and development of galls (Blanche 2000, Ogah et al. 2012). The fluctuation on the occurrence of the gall morphotypes on *B. reticularia* indicates that gall induction did not depend on variations in temperature and precipitation. Hence, successive generations of these insects were believed to successfully establish on mature leaves or stems of *B. reticularia* along the year. Moreover, Wolda (1988) affirmed that the seasonal activity of tropical species tend to be longer, and the seasonal peaks less well defined, what may be represented by the uncommon use of mature leaves of *B. reticularia* as sites of oviposition.

Gall abundance on B. reticularia. Each gall morphotype on *B. reticularia* presented different patterns of occurrence. Some patterns might adjust to the transition between dry and rainy season. The rolling and the pocket galls were induced at the end of the dry season, while the kidney-shaped gall was induced at the end of the rainy season. The fusiform gall was induced all along the year, while the globoid gall was induced during the transitional periods between rainy and dry seasons. The low rate of infestation during the year suggested that few galls were induced on each individual of *B. reticularia,* which was not a pattern for the Neotropics. Some galling herbivores, such as the *Pseudophacopteron* sp. associated to *Aspidosperma australe* (Oliveira and Isaias 2010b), had a distinct strategy, massively infesting few

individuals (Campos et al. 2010). The homogeneity observed on *B. reticularia* denoted that the host plant seems to have reactive sites for gall induction (*sensu* Weis et al. 1988), and nutritional resources for the galling herbivores all over the year. In relation to reactive sites, the galling herbivores of *B. reticularia* were capable of altering mature leaves, which are long living and can be used as sites of oviposition throughout the year. The oviposition in mature leaves was said to be uncommon, as young tissues are more reactive to galling stimuli (Rohfritsch 1992) but has been observed in some other host plants in Neotropical (Moura et al. 2009; Oliveira et al. 2009).

The leaf gall morphotypes occured in alternate periods of the year, which indicated a temporal strategy for using the host leaves on the same plant species. This strategy does not seem to be a pattern for superhosts, once the galling herbivores associated to *Copaifera langsdorffii* (Fabaceae) induced galls either on young or mature leaves, and also in different parts of the same plant organ, indicating the oviposition on different sites as an strategy (Oliveira et al. 2012). Among the stem galls, strategies of synchrony or asynchrony were not observed. The fusiform gall was constant throughout the year and presented no representative peaks of abundance, while the spherical gall alternated abundance peaks with gaps of occurrence.

Host plant phenology* vs. *Galling insects colonization. The synchrony between the host plant and the life cycle of the herbivorous insects is crucial for the success of the latter, and may be determined by the amount and quality of the available plant resources (Yukawa 2000). However, the phenology of *B. reticularia* did not influence the galling insect establishment, and consequently some other biotic or abiotic factors might determine the success of the interactions. These results were opposite to those observed by Campos et al. (2010) for *Aspidosperma spruceanum,* and *A. australe,* in which host plant phenology seems to be crucial for galling herbivores establishment. Castro et al. (2013) have additionally found a positive correlation between leaf flushing and nutrients availability with gall induction on *A. macrocarpon.* On the other hand, in *Copaifera langsdorffii,* Oliveira et al. (2012) observed either synchrony or asynchrony between the host plant phenology and the associated galling herbivores. In this superhost plant, the synchrony between the hydric potential of the plant and the abundance of the gall morphotypes was the most important factor for the establishment of the galls.

For *B. reticularia*, even though there were variations in leaf amount among the plant individuals, the total leaf availability in the population was similar in a year time, and the colonization occured along the whole year, with a slight seasonality of some morphotypes.

Seasonality of the galls. Even though the oscillation in temperature is low, the rainfall at Serra do Caraça is clearly seasonal. Peculiarly, the gall morphotypes on *B. reticularia* had distinct cycles. The kidney-shaped gall had a clear opposite seasonal variation in relation to the rolling and pocket galls, while the other gall morphotypes maintained constant population densities throughout the years of 2009 and 2010. Even though rainfall and temperature were considered to be important to determine the life cycles of some galling herbivores (Wolda 1988), the fluctuation of the population of galling insects associated to *B. reticularia* seemed to be influenced rather by rainfall than by temperature at Serra do Caraça.

The dry season lasted from April to September 2009, and the rainy season lasted from October 2009 to March 2010 at Serra do Caraça, similarly to the data reported by Silva and Talamoni (2003). The periods between March and April as well as September and November represented transitional periods between the dry and rainy seasons. The referred periods presented the highest gall abundance recorded on field conditions, probably due to the co-occurrence of mature and dehiscent galls (*sensu* Rohfritsch 1992) from the previous cycle together with recently induced galls. The distinct periods of maximum abundance of the kidney-shaped, pocket, and rolling galls indicated a divergent behavior of the gall inducers. All of them used the same host plant and the same host organs, but in different periods of time. The herbivores associated to these three gall morphotypes have asynchronous life cycles, and share the same host plants, as observed by Dalbem and Mendonça (2006) for 84 host plants in a Brazilian subtropical forest.

Conclusions

The five morphotypes of galls on *B. reticularia* presented two distinct syndromes of occurrence related to the dry and rainy seasons. Galls distribution on this superhost denoted distinct strategies to share the same host plants: asynchronous life cycles, and low levels of infestation along the whole year. The galling herbivores used the resources of the host plant to ensure the development of their galls, imposing minimum damages to the development of the host plants. Despite the abundance of oviposition sites an intriguing factor remained to be elucidated, *i.e.* the causes of the low abundance of galls on *B. reticularia.* Should it be an exclusive product of natural enemies attack? Or is there another factor which is successfully controlling the interaction, such as host plant silent cellular reactions?

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Figure captions

Figure 1. Gall morphotypes on *Baccharis reticularia*. (a) Kidney-shaped gall (b) Rolling gall (c) Pocket gall (d) Fusiform gall (e) Globoid gall.

Figure 2. Montlhy mean values of daily rainfall, air temperature on Serra do Caraça, MG, Brazil, and gall abundance on *Baccharis reticularia* during the studied period. Data supplied by the rain gauge of the Reserva Particular do Patrimônio Natural Serra do Caraça (rainfall), and by the environment team of the AngloGold Ashanti company (temperature). Periods of flowering and fructification are highlighted by the thick lines.

Figure 3. Gall abundance of the morphotypes on *Baccharis reticularia* during the studied period. (a) Rolling gall (b) Kidney-shaped gall (c) Pocket gall (d) Globoid gall (e) Fusiform gall.

Figure 1. Gall morphotypes on Baccharis reticularia. (a) Kidney-shaped gall (b) Rolling gall (c) Pocket gall (d) Fusiform gall (e) Globoid gall.
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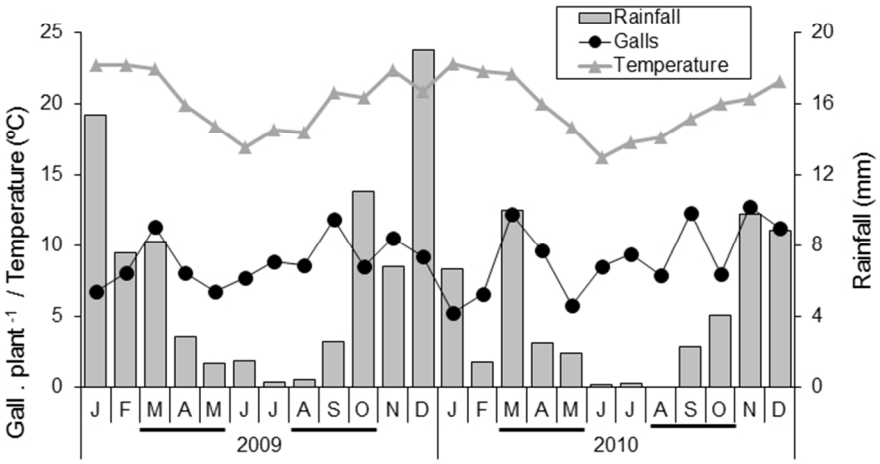


Figure 2. Montlhy mean values of daily rainfall, air temperature on Serra do Caraça, MG, Brazil, and gall abundance on Baccharis reticularia during the studied period. Data supplied by the rain gauge of the Reserva Particular do Patrimônio Natural Serra do Caraça (rainfall), and by the environment team of the AngloGold Ashanti company (temperature). Periods of flowering and fructification are highlighted by the thick lines.
60x81mm (300 x 300 DPI)

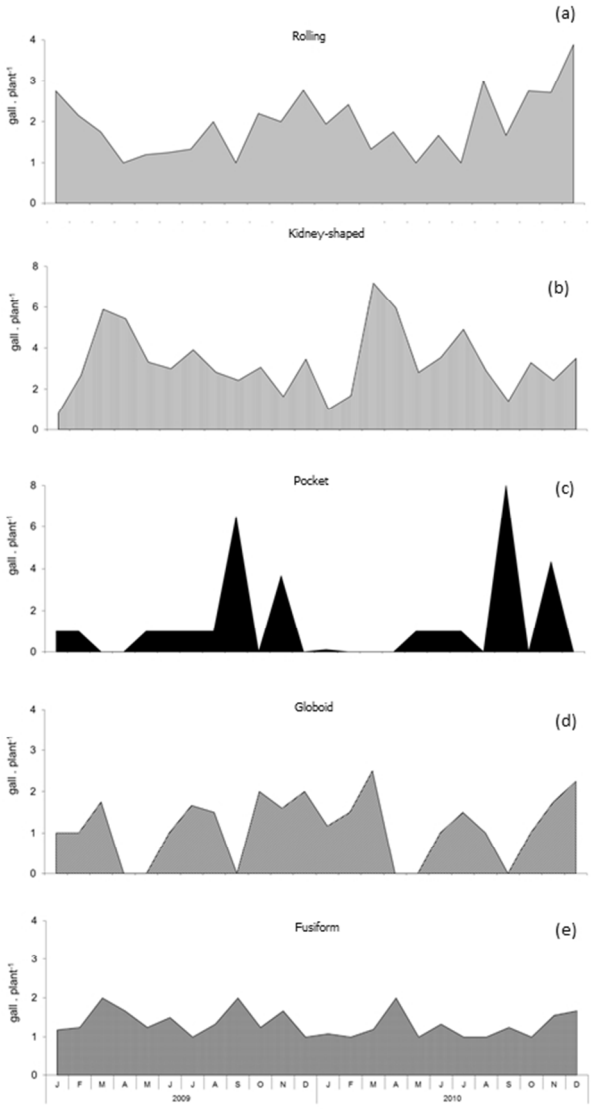


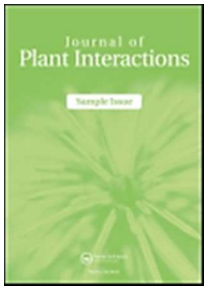
Figure 3. Gall abundance of the morphotypes on Baccharis reticularia during the studied period. (a) Rolling gall (b) Kidney-shaped gall (c) Pocket gall (d) Globoid gall (e) Fusiform gall.
60x81mm (300 x 300 DPI)

Table 1 – Caracterization of gall morphotypes on *Baccharis reticularia* (Asteraceae) in maturation phase

Morphotypes	Host organ	Position	Color	Indument	Dimensions (height X width) mm	Gall inducing taxa
Rolling	Leaf	Attachemnt	Green	Glabrous	14.2 x 2.0	Hemiptera
Kidney-shaped	Leaf	Attachemnt	Green	Glabrous	5.6 x 2.9	Hemiptera
Pocket	Leaf	Attachemnt	Green	Glabrous	9.5 x 7.4	Hemiptera
Fusiform	Stem	Medular	Green/Brown	Suberous	27.8 x 2.7	Lepidoptera
Globoid	Stem	Medular	Brown	Suberous	35 x 4.9	Diptera

Table 2 - Nonparametric correlation analysis (Spearman Rank Correlation) between the rainfall and the abundance of different gall morphotypes

Rainfall *vs.* gall morphotypes	**Spearman Rho**	**Prob>[Rho]**
Kidney-shaped	-0.0948	0.6595
Rolling	0.4122	0.0453
Pocket	-0.2193	0.3033
Fusiform	0.2033	0.3406
Globoid	0.4563	0.0250



Tritrophic interactions among host plant, galling herbivores and fungal endophytes

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Tritrophic interactions among host plant, galling herbivores and fungal endophytes

Anete Teixeira Formiga[a], Yumi Oki[b], Bárbara Iglesias de Mello Medeiros[c], Geraldo Wilson Fernandes[b] and Rosy Mary dos Santos Isaias[a*]

[a]Departamento de Botânica, Universidade Federal de Minas Gerais - UFMG, Belo Horizonte, MG, Brazil; [b]Departamento de Ecologia, Universidade Federal de Minas Gerais - UFMG, Belo Horizonte, MG, Brazil; [c]Departamento de Ecologia, Pontifícia Universidade Católica de Minas Gerais, MG, Brazil

*Corresponding author - e-mail: rosy@icb.ufmg.br, Phone: +55-31-34092687, Fax: +55-31-34092671

Abstract

Endophytic fungi may share the gall environment with the galling larvae, establishing tritrophic interactions also involving the host plant whose nutritional status may be influenced by the associated organisms. One part of a tritrophic interaction, the endophytic mycota of *Baccharis reticularia* was inventoried, and related to the presence of the kidney-shaped galls as well as to the host plant nutritional status. The high richness of endophytic fungi species (227 species) was not affected by the presence of galls, but there is a certain degree of specificity in non-galled and galled groups. The interaction with endophytic fungi seems to be beneficial to the galling herbivore, with an increase in nitrogen and phosphorous contents in galled leaves.

Keywords: *Baccharis*, enfophytic fungi, tritrophic interactions, gall, galling herbivores

Introduction

Galling insects are sophisticated herbivores (Shorthouse et al. 2005) that may share their microenvironment with many other organisms, either detrimental to them, such as their predators and parasitoids (Askew 1960, 1961; Price 2005), or beneficial such as the fungi of ambrosia galls, from which they feed (Arduin and Kraus 2001; Sá et al. 2009). Some other organisms, such as the endophytic fungi, are not directly related to the galling herbivore nutrition, but may contribute positively to the interaction.

In recent decades some studies reported the occurrence of many endophytic fungi that share the gall environment with the galling larvae (e.g., Fernandes and Price 1992; Wilson 1993, 1995; Sinclair and Cerkauskas 1996). Some of them synthetize substances that can prejudice the galling insect larvae (Butin 1992), such as *Paecilomyces lilacinus*, which can be used as a biological control agent of the nematodes *Meloidogyne incognita* (Jonathan and Rajendran 2000), and *Radopholus similis* (Devrajan and Rajendran 2001), both gall inducers on *Musa spp.* On the other hand, the fungi *Phomopsis juniperovora* and *Kabatina juniperi* may constitute food resource for *Juniperus scopulorum* (Namet et al 2012). In other cases, they may be the organisms which trigger hypersensitive reaction on plants, leading the galling larvae to death (see Fernandes 1990).

In tropical region, the high richness of endophytic fungi during the rainy season (Wilson and Carroll 1994; Bills 1996; Arnold et al 2003; Suryanarayanan and Thennarasan 2004) may form complex tritrophic interactions with host-plants and galling herbivores (Arnold and Lutzoni 2007). The association between the richness of fungi and precipitation could be explained by their mode of reproductive (spore propagation) (Faeth and Hammon, 1997 a, b), but humidity may not be a conditioning factor for this kind of reproductive behavior. Oki et al. (2008), for instance, found the highest richness of endophytic fungi in *Baccharis dracunculifolia* at Serra do Cipó (Minas Gerais, Brasil) during the dry season, and related this richness to the conservational state of the habitat (Oki et al. 2008, 2009).

Another factor that may influence both the endophytic fungi and the galling herbivore richness is the nutritional status of the host plant (Fernandes et al. 2011). This status may be influenced by the stimuli of the alling herbivores over the production of primary and secondary metabolites in their host plants (Formiga et al. 2011), indirectly

modulating the richness of endophytic fungi in galled plants. The higher number of species of endophytic fungi in galled plants, in the rupestrian field, may also be related to the high water and phenolic contents, as argued by Sanchez-Azofeifa et al. (2012) for *Coccoloba cereifera.* Therefore, the tritrophic relationship established among the host plant, the galling insect, and the endophytic fungi may be very intrincate, and deserves attention for it may not be simply interpreted either as parasitic or as mutualistic (Raman et al. 2012).

The relationship between fungi and gall inducers can be mutualistic when the fungi live in the intestines of herbivores and metabolize phytotoxic secondary compounds (Jones 1984; Dowd 1991). However, mycotoxins may be similar to plant allelochemicals, performing antagonistic roles such as attracting parasitoids, (Bragg 1974; Vinson 1975; Price 1981; Elzen et al.1983; Williams et al. 1988; Whitman 1988) or being toxic to herbivores, and consequently protecting the host plant (Oki et al. 2008, 2009). In fact, some studies have shown that grasses became unpalatable to certain insects when inhabited by endophytic fungi (Funk et al. 1983; Clay 1988, 1991; Latch et al. 1985; Wilson 1995). Even though the tritrophic associations among galling insects-host plants-endophytic fungi are difficult to visualize (Bixby-Brosi and Potter 2011), its comprehension may provide important information on the mechanisms and processes involved in the success of the galling habit.

In this study, we address the tritrophic interaction among the host plant *Baccharis reticularia* (Asteraceae), the gall inducer of the kidney-shaped gall, and the endophyte community, focusing on the following questions: (1) what is the endophytic mycota in *B. reticularia*? (2) Is there any correlation between the endophyte community and the presence of the kidney-shaped galls? (3) Is there any correlation between the endophyte community and the host plant nutritional status? And (4) does the presence of the kidney-shaped galls affect the nutritional status of the host plant?

Methods

***Sampling*.** Plant material was sampled twice a month in a rupestrian field vegetation, at Serra do Caraça, MG, in 1.511m (20º07'035" S and 43º31'201" W) of altitude from January to November, 2012. The local climate is Cwb of (Köppen-Geiger), with light and rainy summers, and annual average precipitation over 1.500 mm (Brandão et al. 1994). The dry season in this area lasts from April to September, and the rainy season

lasts from October to March (Silva and Talamoni, 2003). The model species, *Baccharis reticularia* DC. (Asteraceae), is a dioiceous shrub, with woody branches and green leaves with spiral phylotaxy, up to 4 m high, which predominates in the region landscape (Figure 1A, B) (Barroso 1976). It hosts a gall induced by an unidentified Hemiptera, which develops through the folding and hypertrophy of leaf both margins (Figure 1C, D).

Endophytic mycobiota. The influence of the galls on the endophytic mycobiota was evaluated on 3 branches from 10 non-galled, and 3 branches from 10 galled individuals of *B. reticularia,* randomly chosen in the field. All samples were taken to the laboratory within 24 hours. Three mature leaves were detached from each branch, sterilized and cut in 4 mm^2 fragments (n = 8) which were transferred to Petri dishes with PDA (potato-dextrose-agar) supplemented with chloramphenicol (1:1000 p/v) to suppress bacterial growth. The dishes were incubated at 25°C (±1°C), and monitored daily. After detecting the development of endophytic fungi, the colonies were isolated and separated on morphological basis. Each isolated fungus was microcultivated in glass slides for the identification at species level based on their reproductive structure (Oki et al. 2009). The majority of the fungi did not sporulate. Data on the morphotaxa and number of isolated fungi per plant individual were recorded. The frequency of leaf fragments in which fungi emerged was calculated by dividing the number of fungi in each fragment for the total number of fragments.

Nutrients vs. Galls vs. Endophytic fungi. The influence of the galls on plant nutrients (nitrogen, carbon, macro and micronutrients) and on the endophytic community was evaluated in the same non-galled (n = 10) and galled (n = 10) individuals sampled for the evaluation of endophytic mycobiota. Non-galled mature leaves (n = 3) were sampled from each individual, weighted, and dried at constant temperature (65^0C) during one week to achieve stable weight. The samples were grinded, and sent for the analyses of macro and micronutrients at the Departamento de Solos of Universidade Federal de Viçosa, MG.

Statistical analysis. Data on the richness of endophytic fungi (EF) of non-galled and galled samples, collected in dry and rainy seasons, had normal distribution. The comparison of the richness and frequency of endophytes between the samples and between the seasons was performed with the factorial ANOVA. The Jaccard index of

similarity was used to compare the similarity of the morphotaxa of EF between the samples (Mueller-Dombois and Ellenberg 1974). The nutrient contents (N, P, K, Ca, Mg, S, Zn, Fe, Mn, Cu, B, C) of non-galled and galled leaves were compared with the *t*-test. Pearson's correlation was used to evaluate the relationship between each nutritional parameter and the richness of endophytic in non-galled and galled leaves. Only the nutritional parameters with differences between non-galled and galled samples were used in Pearson's correlation.

SigmaStat for Windows Version 3.5 (Copyright© 2006, Systat software Inc.) was used for statistical tests and PAST - Paleontological Statistics Software Package (Hammer et al. 2001) for the analyses of similarity.

Results

The experiments of fungi isolation and microcultivation with non-galled and galled leaves of *B. reticularia* resulted in the recognition of 227 colonies, from which 102 morphotaxa of endophytic fungi (EF) were identified. The non-galled groups had 105 colonies of EF with 63 morphotaxa, while the galled ones had 122 colonies of EF with 68 morphotaxa. The richness of EF did not vary between the treatments (galled and non-galled) ($p= 0.18$), the seasons ($p= 0.06$), and between the two factors (treatment and season) ($p=0.8$). An average of two species of EF was found in each individual.

The frequency of EF did not vary between the treatments ($p= 0.18$), and between the interaction of treatment and season ($p = 0.9$), but varied between the dry and rainy seasons ($p = 0.02$). The average frequency of fungi either in non-galled or galled individuals was 0.3. This frequency per individual was greater in the dry season (0.35 ± 0.04) than in the rainy season (0.24 ± 0.03). The EF (Jaccard index) between non-galled and galled individuals had only 28% of similarity.

Nitrogen and phosphorous had higher concentration in galled samples (Fig 1). The average content of nitrogen was 1.34 ± 0.7 dag/kg (%) in non-galled leaves, and 1.863 ± 0.05dag/kg (%) in galled leaves. The average content of phosphorous was 0.07 ± 0.004 in non-galled leaves and 0.09 ± 0.004 in galled ones. The contents of the other macro- and micronutrients between galled and non-galled leaves did not differ statistically (Table 1).

The correlation between the N content and the diversity of EF morphotaxa in galled individuals was positive (r = 0.815, p = 0.048), but not significant for non-galled individuals (r = 0.617, p = 0.192) (Fig 2a). Similarly, the correlation between phosphorous content and the diversity of EF morphotaxa in galled individuals was positive (r = 0.949, p = 0.0383), and uncorrelated for the non-galled individuals (r = 0.644, p= 0.167) (Fig 2b).

Discussion

The richness of the endophytic fungi community may vary greatly according to the plant species and its area of occurrence. *Baccharis reticularia* (Asteraceae) at Serra do Caraça has a high richness of EF when compared to *B. dracunculifolia*, which hosts only 8 species at Estação Ecológica da UFMG (Oki et al. 2008, 2009). The first plant species present 102 morphotaxa, while the latter almost 70 endophytic fungi species at Parque Nacional da Serra do Cipó (Oki et al. 2008, 2009). Some other Asteraceae such as *Viguiera robusta* has only 12 species of endophytic fungi (Momesso 2004), *V. arenaria* has 34 (Guimarães et al. 2008), *Smallanthus sonchifolius* has 32 morphotaxa (Gallo et al. 2009) and *Parthenium hysterophorus* has 125 species of EF (Romero et al. 2001).

The higher frequency of plants with endophytes during the dry season as observed for *B. reticularia* may be a pattern for *Baccharis* species for Oki et al. (2008, 2009) found similar values for *B. dracunculifolia.* It is also probable that the high frequency of endophytes in *B. reticularia* during the dry season may be dependent on their mode of transmission, either by herbivores or wind, and their site of occurrence.

Current data also evidenced that the presence of the galls caused a strong change in the composition of the morphotaxa of EF (almost 80%) in *B. reticularia*. Variations on the endophytic fungi community have been observed in other plant species and were believed to occur both due to plant structural features, namely, waxes, fibers, air space, and biomass, and chemical alterations, such as the accumulation of nutrients and secondary compounds (Fernandes et al. 2011; Sanchez-Azofeifa et al. 2012). Also, biotic factors, like herbivory and gall induction may affect the richness of EF (Fernandes et al. 2011). Moreover, the fungi may also increase the contents of secondary compounds, which indirectly protect the host plants from herbivores. In fact,

the chemicals produced by the fungus *Phomopsis oblonga* have repellent effect on the herbivore *Physocnemum brevilineum* (Coleoptera) (Webber 1981). Endophytes associated with *Baccharis megapotamica* (Kupchan et al. 1977), *B. cordifolia* (Busam and Habermehl 1982; Habermehl et al.1985) and *B. artemisioides* (Rizzo et al. 1997), as well as those associated to *Smallanthus sonchifolius* (Asteraceae) (Gallo et al. 2009) produce substances that are toxic to cattle (Jarvis et al. 1996).

The presence of galls altered the main plant nutritional status (nitrogen and phosphorous contents) of *B. reticularia*. These data are similar to those of Diamond et al. (2008) for *Eurosta solidaginis* (goldenrod gall fly) - *Solidago gigantea* (Asteraceae) system, where the high content of the macronutrients was important for the nutrition of the associated galling insects. The higher levels of nutrients in galled tissues of *B. reticularia* may benefit the galling herbivore with a higher supply of nutrients during gall development. These nutritional alterations, among others not examined herein, probably influenced the endophyte composition of *B. reticularia*. The positive correlation between endophytic richness and the content of nitrogen and phosphorous in galled plants suggests that: 1) endophyte richness increases the nutritional status of galled plants. In this case, the endophytes could be increasing the vigor of plant tissues, thus minimizing the negative effects of gall infestation; 2) alternatively, galled plants with higher nutritional status would hold a richer endophyte community.

Conclusion

B. reticularia presents a high richness of endophytic fungi species, which is not significantly affected by the presence of the kidney-shaped galls. Nevertheless, the presence of distinct endophytic fungi in non-galled and galled groups indicates a certain degree of specificity in the fungi-gall interaction for this host plant. This close interaction seems to be beneficial to the galling herbivore, with an increase in nitrogen and phosphorous contents in galled leaves. Further studies should be conducted with other superhosts-galling herbivores systems for checking the positive correlation as a pattern for this kind of tritrophic interaction.

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Table 1. Average (± standard error) of potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), iron (Fe), manganese (Mn), copper (Cu), boron (B), and carbon (C) contente of non-galled and galled leaves of *B. reticularia.* p > 0,05 indicates statistical similarity.

	galled			Nonngalled			P
K (dag/kg)	1,21	±	0,104	0,961	±	0,056	p= 0,06
Ca dag/kg	,32	±	0,015	0,295	±	0,015	p= 0,26
Mg dag/kg	,12	±	0,014	0,156	±	0,01	p= 0,06
S dag/kg	,27	±	0,043	0,332	±	0,029	p= 0,07
Zn mg/kg	34,2	±	1092	39,933	±	3,64	p= 0,39
Fe mg/kg	730,32	±	53,7	681,7	±	73,86	p= 0,60
Mn mg/kg	810,77	±	80,87	1055	±	1,179	p= 0,12
Cu mg/kg	14,3	±	1,44	12,13	±	1,208	p= 0,28
B mg/kg	41,4	±	2,86	47,73	±	1,709	p= 0,09
C mg/kg	52,6	±	0,1	52,258	±	0,142	p= 0,9

Figure captions

Figure 1. (A) General aspects of Serra do Caraça, Minas Gerais, Brazil. (B) Herbaceous habit of *Baccharis reticularia.* (C) Kidney-shaped gall in branch (dotted circle) (D) Kidney-shaped gall.

Figure 2. Average (± standard error) of nitrogen (N) and phosphorous (P) contents of non-galled and galled leaves of *Baccharis reticularia*.

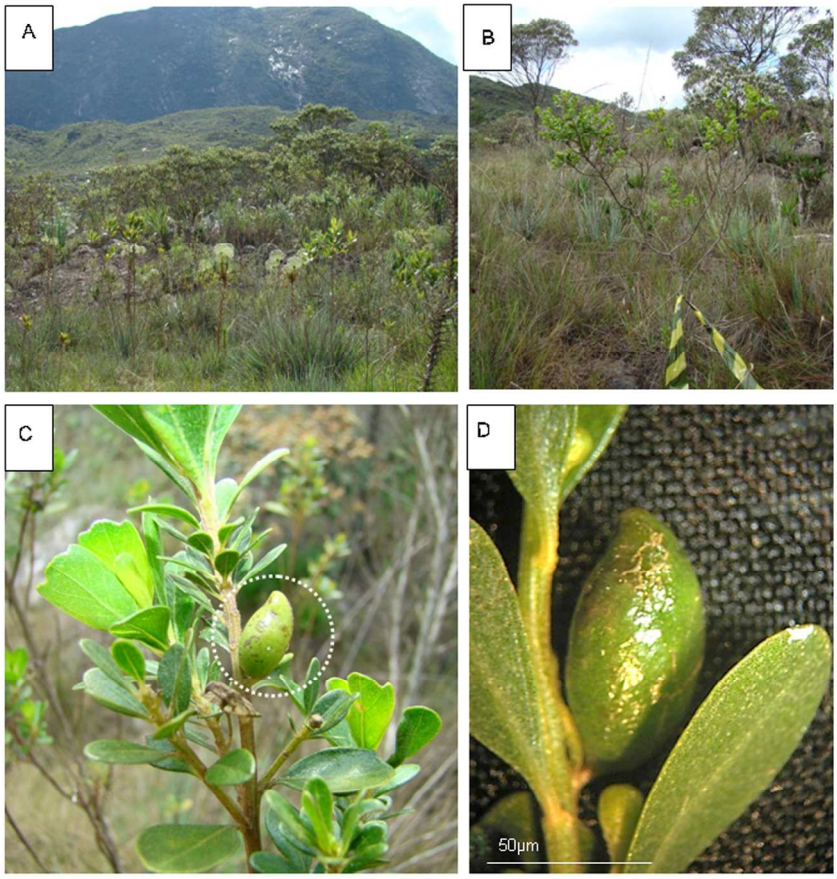

Figure 1. (A) General aspects of Serra do Caraça, Minas Gerais, Brazil. (B) Herbaceous habit of Baccharis reticularia. (C) Kidney-shaped gall in branch (dotted circle) (D) Kidney-shaped gall.
190x254mm (300 x 300 DPI)

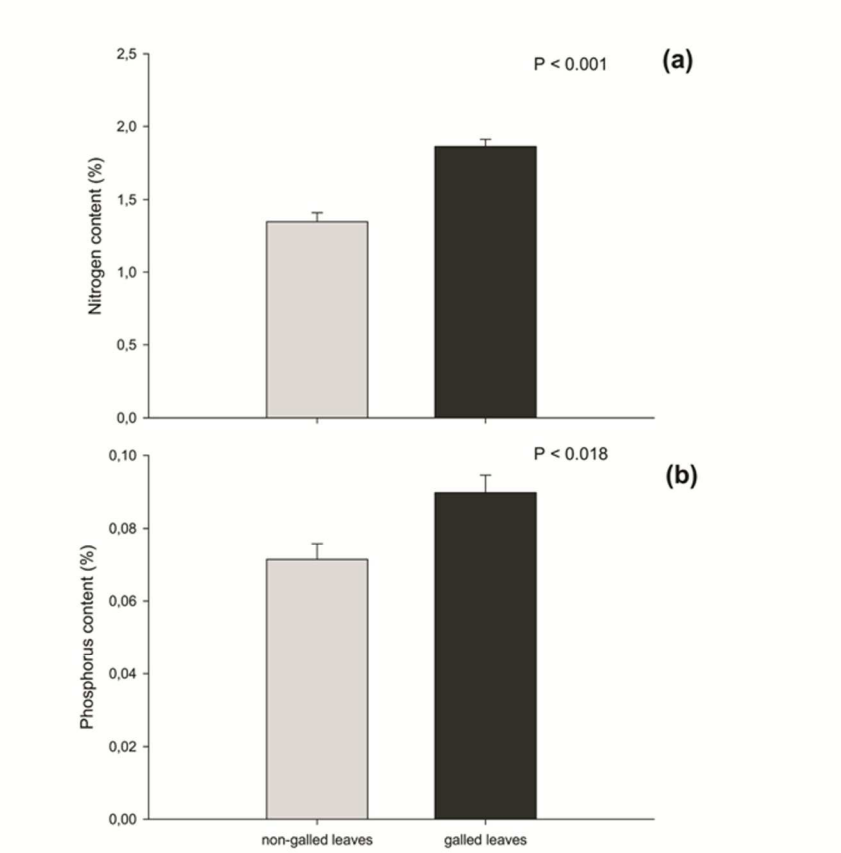

Figure 2. Average (± standard error) of nitrogen (N) and phosphorous (P) contents of non-galled and galled leaves of Baccharis reticularia.
254x338mm (300 x 300 DPI)

Considerações Finais

Esta tese conta com a diagnose anatômica e o perfil histoquímico de cinco morfotipos de galhas em *Baccharis reticularia,* o que permite evidenciar a manipulação do potencial da planta hospedeira por parte dos diferentes insetos galhadores. É observada a estrutura anatômica de diferentes galhas, sem, contudo haver neoformação de tecidos. Histoquimicamente, é notável a presença de fenólicos somente nos tecidos das galhas. Tais substâncias tanto podem estar relacionadas à defesa química da planta contra herbivoria, quanto com a interação metabólica com reguladores de crescimento, o que propicia o desenvolvimento do novo órgão, a galha. A comparação morfológica qualitativa e quantitativa das galhas constitui um primeiro passo no estudo de um novo sistema galhador-planta hospedeira. Tal abordagem permite corroborar o conceito das folhas como órgãos mais plásticos a ação dos herbívoros galhadores. A análise de componentes principais e o índice de plasticidade permitem evidenciar a proximidade estrutural dos morfotipos de galhas estudados e as características morfológicas que mais contribuíram para a determinação da forma final das galhas. Os morfotipos caulinares são mais próximos entre si e não diferem grandemente do órgão hospedeiro, enquanto os morfotipos foliares possuem uma plasticidade fenotípica maior, sendo a galha reniforme aquela que mais se afasta do padrão foliar. As galhas de enrolamento e em bolso foram consideradas de menor complexidade estrutural e, portanto, mais similares às folhas hospedeiras. Ainda, a análise de variações na composição da parede celular nos permite concluir que há o estabelecimento de uma dinâmica péctica nas paredes celulares de *B. reticularia* determinando um novo *design* de estrutura e funcionalidade nos tecidos da planta hospedeira durante os diferentes estágios de desenvolvimento das galhas.

Em um nível macroscópico, a análise do comportamento das galhas ao longo do tempo e do espaço permitiu detectar que as galhas foliares possuem flutuações distintas no tempo, por vezes estritamente opostas entre si. As galhas caulinares ocorrem simultaneamente, não apresentando flutuações tempo dependentes. Ainda, duas galhas, uma caulinar e outra foliar, possuem períodos de ausência durante o ano. As flutuações dos morfotipos responderam a influência dos fatores climáticos (temperatura e

precipitação). A despeito da grande quantidade de plantas hospedeiras no local, os níveis de infestação são considerados muito baixos quando comparados a outras super-hospedeiras já estudadas. Desta forma, os cinco insetos galhadores de *B. reticularia* apresentaram duas diferentes estratégias de ocupação dos sítios de oviposição na planta hospedeira: 1) ciclos de vida assincrônicos; 2) baixos índices de infestação ao longo do período amostral (dois anos). O padrão disperso de distribuição populacional dos insetos galhadores relacionados a *B. reticularia* diverge do que é usual para outros sistemas galhador-planta hospedeira.

O presente trabalho encerra-se com o estudo da relação tritrófica estabelecida entre plantas, insetos galhadores e fungos. Uma grande diversidade da micota foi detectada em *B. reticularia*, diversidade essa mantida com o desenvolvimento da galha reniforme, o morfotipo mais abundante. Em plantas onde se estabelece este morfotipo, a micota mantém sua diversidade, porém, os morfotaxa ocorrentes são diferentes daqueles encontrados nas plantas não galhadas, denotando uma especificidade de 80% para plantas com galhas. A presença dos fungos endofíticos em galhas parece ser benéfica ao galhador, pois há um aumento dos níveis de nitrogênio e fósforo das plantas com galhas.

O estudo das variáveis estruturais, histoquímicas e ecológicas no sistema insetos galhadores – *Baccharis reticularia* evidencia mecanismos responsáveis pelo estabelecimento e desenvolvimento das galhas. As alterações celulares induzidas pelos galhadores, bem como as relações ecológicas , são cruciais para o seu sucesso reprodutivo. De modo semelhante, as complexas relações ecológicas estabelecidas entre plantas – insetos – fungos endofíticos beneficiam os organismos envolvidos, aumentando o valor nutricional dos tecidos vegetais. Apesar de extensivamente explorado neste trabalho, o sistema insetos galhadores – *B. reticularia* possui ainda muitos aspectos morfogenéticos, fisiológicos e bioquímicos a serem explorados, representando um modelo elegante de investigações das respostas teciduais a influência de um organismo externo, o galhador.