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

REISILA SIMONE MIGLIORINI MENDES

GALHAS FOLIARES EM *BYRSONIMA* spp. KUNTH (MALPIGHIACEAE): do laboratório para a sala de aula

Belo Horizonte
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"GALHAS FOLIARES EM *Byrsonima* SPP. KUNTH (MALPIGHIACEAE): DO LABORATÓRIO PARA A SALA DE AULA"

REISILA SIMONE MIGLIORINI MENDES

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Dedico este trabalho a minha mãe (in memoriam)

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RESUMO

Esta tese está dividida em duas seções: a seção I é constituída por três capítulos que tratam dos procedimentos, resultados e implicações da pesquisa científica sobre *Byrsonima* spp. e suas galhas e a seção II, constituída por dois capítulos e faz a ponte entre a pesquisa científica, a divulgação científica e o ensino de Botânica com foco na formação inicial de professores da Educação Básica. *Byrsonima coccolobilofia* Kunth, *B. variabilis* A. Juss. e seus galhadores associados foram aqui estudados do ponto de vista fenológico, anatômico, histométrico, histoquímico e imunicitoquímico para investigar as estratégias dos galhadores associados voltadas a manipular o potencial morfogenético das plantas hospedeiras no sentido do sucesso de seus ciclos de vida. Os caracteres das duas galhas estudadas e peculiaridades de sua história de vida serviram, ainda, de inspiração para ampliar metodologias capazes de tornar o conhecimento botânico atualizado e acessível aos futuros professores da educação básica e ao grande público. A criação de cada estratégia de ensino e divulgação teve o objetivo de trazer de volta a *Scientia amabilis* de Carolus Linneus ao riquíssimo campo de novas descobertas e teve a parceria de alunos da licenciatura do curso de Ciências Biológicas da UEMG/Ibirité. O objeto de estudo se constitui nas galhas, ou seja, estruturas que se desenvolvem em resposta a organismos parasitas que podem ser vírus, bactérias, fungos, nematódeos, ácaros ou insetos. A capacidade de induzir galhas é uma estratégia eficiente de utilização dos vegetais pelos herbívoros que encontram nessas estruturas um microambiente adequado ao seu estabelecimento e nutrição, assim como proteção contra seus inimigos naturais e intempéries ambientais. O desenvolvimento das galhas nos tecidos vegetais é influenciado e coordenado pelo organismo galhador por meio de fatores ainda não elucidados que redirecionam as células para uma morfogênese característica resultando na diferenciação ou rediferenciação das células no local de formação da galha. Os estudos fenológicos no sistema *B. coccolobifolia*-Cecidomyiidae galhador revelaram a dinâmica de ocupação temporal e as estratégias desenvolvidas pela planta hospedeira para escapar de ciclos sucessivos de indução. O sincronismo entre a fase de indução da galha e a fenofase de brotamento foliar da planta hospedeira foi evidenciado. As fases vegetativa e reprodutiva de *B. coccolobifolia* são assíncronas com o desenvolvimento da galha caracterizando uma possível estratégia de escape à competição com o Cecidomyiidae. Os estudos anatômicos e histométricos revelaram que as galhas de inseto apresentam grande especialização com a formação de dois compartimentos teciduais, com o compartimento tecidual externo assumindo a função de armazenamento e proteção do galhador e o compartimento tecidual interno sendo responsável pela nutrição direta do galhador. Já na galha de fungo da ferrugem, não ocorre alteração na estrutura do mesofilo de *B. variabilis* nem formação de tecidos especializados. O investimento estrutural é maior nas galhas de insetos e, praticamente nulo nas galhas de fungo, contudo, em termos de perfil químicos, os estudos histoquímicos e imunocitoquímicos revelaram semelhanças na localização e distribuição dos metabólitos nas folhas não galhadas das duas espécies de *Byrsonima* hospedeiras das galhas e revelaram um gradiente de distribuição dos metabólitos primários bem semelhantes entre as galhas de inseto em *B. coccolobifolia* e de fungo em *B. variabilis*. A dinâmica dos componentes das paredes celulares revelou alterações envolvidas no desenvolvimento da estrutura das galhas, e na nutrição e defesa dos indutores. A imunomarcação dos epítopos de arabinanos e galactanos nas paredes celulares, também revelaram semelhanças relativas ao aumento da porosidade das células próximas à galha favorecendo o transporte de substâncias em direção aos galhadores. Pressupomos que a origem-destino dos tecidos das galhas, alterações na distribuição dos metabólitos e dinâmicas dos

epitopos das paredes celulares em resposta ao desenvolvimento da galha seriam semelhantes nas plantas estudadas por serem espécies congenéricas. Nossa hipótese foi parcialmente corroborada, tendo em vista que a especialização tecidual foi evidenciada somente nas galhas induzidas pelo Cecidomyidae galhador. Entretanto, as alterações histoquímicas e imunocitoquímicas em função da instalação e desenvolvimento das galhas de inseto e de fungo da ferrugem são muito semelhantes no que diz respeito à distribuição dos metabólitos primários e à dinâmica das paredes celulares. Os dispositivos interpretativos criados permitiram a ponte entre o conhecimento produzido na academia e a formação de professores, configurando à pós-graduação um papel ativo na formação de cidadãos críticos e conscientes, capazes de compreender a importância da ciência e sua aplicação no cotidiano. Ademais, a divulgação fora dos ambientes acadêmicos faz a ponte academia-sociedade com a qual esperamos alcançar a grande meta do letramento científico.

Palavras-chave: anatomia, dispositivos educativos, divulgação científica, fenologia, histoquímica, imunocitoquímica de parede celular, interação planta-inseto.

ABSTRACT

This thesis is divided into two sections: section I consists of three chapters dealing with the procedures, results, and implications of the scientific research on *Byrsonima* spp. and their galls, and section II consists of two chapters that linnks the gap between scientific research, scientific dissemination, and the teaching of Botany with a focus on the initial training of Basic Education teachers. *Byrsonima coccolobilofia* Kunth, *B. variabilis* A. Juss., and their associated galling organisms were studied here from phenological, anatomical, histometric, histochemical, and immunocytochemical approaches. We investigated the strategies of the associated galling organisms to manipulate the morphogenetic potential of the host plants toward the success of their life cycles. The characters of the two studied galls and the peculiarities of their life histories also inspired methodologies capable of making botanical knowledge up-to-date and accessible to future teachers of basic education and the general public. The creation of each teaching and dissemination strategy had the goal of bringing back the *Scientia amabilis* of Carolus Linneus to the rich field of new discoveries and had the partnership of undergraduate students of the Biological Sciences course at Universidade do Estado de Minas Gerais/Ibirité. The object of study is the galls, i.e., the structure that develop in response to parasitic organisms that can be viruses, bacteria, fungi, nematodes, mites, or insects. The ability to induce galls is an efficient strategy for herbivores using plants toward finding a microenvironment suitable for their establishment and nutrition, as well as protection against their natural enemies and environmental hazards. The development of the galls in plant tissues is influenced and coordinated by the galling organisms by means of factors not yet elucidated that redirect the cells to characteristic morphogenesis resulting in the differentiation or redifferentiation of cells at the site of gall formation. Phenological studies in the *B. coccolobifolia*-Cecidomyiidae galling system revealed the temporal occupancy dynamics and the strategies developed by the host plant to escape successive induction cycles. The synchronism between the gall induction phase and the leaf budding phenophase of the host plant was evidenced. The vegetative and reproductive phases of *B. coccolobifolia* are asynchronous with gall development, which characterized a possible escape strategy from the competition with Cecidomyiidae. The anatomical and histometric studies revealed that the insect galls present great specialization forming two tissue compartments. The outer tissue compartment assumes the function of storage and gall protection, while the inner tissue compartment is responsible for the direct nutrition of the gall. In the rust fungi gall, there is no alteration in the structure of the mesophyll of *B. variabilis* or the formation of specialized tissues. However, regarding chemical profiles, the histochemical and immunocytochemical studies revealed similarities in the location and distribution of metabolites in the leaves of the two species of *Byrsonima* and revealed a similar gradient of primary metabolites between insect galls in *B. coccolobifolia* and rust fungi galls in *B. variabilis*. The dynamics of cell wall components revealed alterations in the development of the gall structure, and in the nutrition and defense of the inducers. The immunolabeling of the epitopes of arabinans and galactans revealed similarities concerning the increased porosity of cell walls near the gall, favoring the traffic of substances toward the galling organisms. We assumed that the origin-destination of gall tissues, alterations in the distribution of metabolites, and dynamics of cell wall epitopes in response to gall development would be similar in the studied plants because they are congeneric species. Such a hypothesis was partially corroborated, considering that tissue specialization was evidenced only in the galls induced by the galling Cecidomyidae. However, the histochemical and immunocytochemical alterations as functions of the insect and the rust fungi gall establishment and development are very similar

regarding the distribution of primary metabolites and cell wall dynamics. The interpretative devices created allowed a bridge between the knowledge produced in the academy and teacher training, giving to the post-graduation an active role in the formation of critical citizenship education, forming citizens capable of understanding the importance of science and its application in everyday life. Moreover, scientific dissemination outside academic environments links the academy-society gap with which we hope to achieve the ultimate goal of scientific literacy.

Keywords: anatomy, educational devices, scientific dissemination, phenology, histochemistry, cell wall immunocytochemistry, plant-insect interaction.

SUMÁRIO

INTRODUÇÃO GERAL

As espécies objeto da tese, *Byrsonima coccolobilofia* Kunth e *B. variabilis* A. Juss., pertencem às Malpighiaceae Juss., família botânica com cerca de 77 gêneros e 1300 espécies de distribuição pantropical, que ocorrem especialmente na América do Sul (Davis e Anderson 2010). Para o Brasil, são citadas 561 espécies distribuídas em 44 gêneros e, para Minas Gerais, 192 espécies distribuídas em 28 gêneros (Mamede et al. 2015). No cerrado, ocorrem cerca de 16 gêneros e 126 espécies ((Reflora 2023). Entre os gêneros que constituem a família, *Byrsonima* Rich. é considerado um dos maiores com 97 espécies (Mamede e Francener 2015). Seu valor comercial está associado a apicultura, utilização da madeira como lenha (Melo e Barbosa 2007) e os seus frutos usados na alimentação. *Byrsonima coccolobifolia*, popularmente conhecida como murici rosa, é uma espécie arbórea que alcança 3-4 m de altura quando adulta (Barbosa et al. 2005) com distribuição nas savanas neotropicais e nos campos cerrado (Medeiros, 2011). As folhas são glabras e têm nervuras rosadas quando jovens e verdes quando maduras, suas flores são rosa pálido com presença de elaióforos dispostos aos pares na base das sépalas (Davis e Anderson 2010). Os frutos semelhantes a drupas têm mesocarpo carnoso e são amarelos quando maduros, com aproximadamente 1,5 cm de diâmetro (Giulietti 1971). A espécie é uma super hospedeira que pode abrigar até cinco morfotipos de galhas em diferentes órgãos – dois morfotipos na folha, um no caule e dois nas gemas.

Esta tese está dividida em duas seções: a seção I é constituída por três capítulos que tratam dos procedimentos, resultados e implicações da pesquisa científica sobre *Byrsonima* spp. e suas galhas e a seção II, constituída por dois capítulos e faz a ponte entre a pesquisa científica, a divulgação científica e o ensino do Botânica com foco na formação inicial de professores da Educação Básica.

O primeiro capítulo da seção I apresenta a fenologia de 10 indivíduos de *B. coccolobifolia* com análise da época de ocorrência e duração das fenofases vegetativa e reprodutiva e, visa elucidar a dinâmica de ocupação temporal do galhador associado à galha foliar cônica induzida por uma espécie de Diptera, Cecidomyiidae. Neste capítulo, testamos as hipóteses de que (1) a planta hospedeira pode ter estratégias fenológicas que permeiem janelas de oportunidade para escapar da indução das galhas, e (2) o ciclo de vida do Cecidomyiidae galhador pode se ajustar às melhores condições de água e nutrição durante a fenologia de *B. coccolobifolia* sem impactar a floração e a frutificação.

Os dois capítulos seguintes abordam aspectos estruturais das galhas e suas peculiaridades frente aos organismos galhadores: um inseto no caso de *B. coccolobifolia* e um fungo no caso de *B. variabilis*. As galhas, tanto em *Byrsonima* quanto nas demais espécies de

plantas hospedeiras, são estruturas que se desenvolvem em resposta a organismos parasitas que podem ser vírus, bactérias, fungos, nematódeos, ácaros ou insetos. A capacidade de induzir galhas é uma estratégia eficiente de utilização dos vegetais pelos herbívoros (Roskam 1992) e os herbívoros galhadores encontram nessas estruturas um microambiente adequado ao seu estabelecimento e nutrição, assim como proteção contra seus inimigos naturais e intempéries ambientais (Price et al. 1986).

No sítio de formação das galhas em suas respectivas plantas hospedeiras, podem ocorrer alterações nos aspectos morfológicos e anatômicos acompanhadas por mudanças nos padrões de desenvolvimento e no metabolismo da planta (Isaias et al. 2015; Oliveira et al. 2017). Tais alterações permitem distinguir as galhas como novos órgãos formados devido aos estímulos oriundos dos galhadores (Mani 1964). A nova organogênese nos locais de desenvolvimento da galha ocorre por meio da diferenciação ou rediferenciação celular (Lev-Yadun, 2003), com especialização tecidual que permite evidenciar compartimentos estruturais e funcionais. A especialização tecidual permite a identificação de compartimentos teciduais funcionalmente distintos. O compartimento externo compartilha funções nutritivas e protetoras, acumulando moléculas energéticas (metabólitos primários) e defensivas, acumulando metabólitos secundários (Bronner 1992; Hartley 1998; Nyman e Julkunen-Tiitto 2000). O compartimento tecidual interno armazena metabólitos primários usados na alimentação direta do galhador e é típico de cada táxon indutor e relacionado às suas demandas nutritivas (Bronner 1992; Ferreira et al. 2017; Bragança et al. 2017). A armazenagem ocorre de forma peculiar no protoplasma e nas paredes celulares, sendo dependentes do perfil químico da planta hospedeira que responde às interações ambientais.

As paredes celulares são elementos essenciais subjacentes ao crescimento, desenvolvimento e resistência a tensões bióticas e abióticas. Sua matriz pode ser modificada, reorganizada e degradada durante o crescimento e desenvolvimento da planta (Barnes e Anderson 2018). Os organismos galhadores controlam estas propriedades, o que resulta na remodelagem e em mudanças nas propriedades mecânicas da parede celular, permitindo o incremento ou inibição de processos celulares tais como divisão, flexibilidade, porosidade e aderência ao longo do desenvolvimento da galha (Park e Cosgrove 2012; Bragança et al. 2020; Costa et al. 2021) e que podem variar em função do estado hídrico da planta hospedeira (Konno et al. 2008; Tenhaken 2015).

A biossíntese da parede celular é um importante dreno de carbono na natureza (Field et al. 1998; Geider et al. 2001; Verbančič et al. 2018) e as galhas, como drenos fisiológicos de nutrientes e de carbono de seu microambiente, ou seja, a planta hospedeira, são modelos sofisticados para estudar alterações nos componentes da parede celular. As alterações na parede

celular e na estrutura das folhas hospedeiras podem ser mapeadas por meio de estudos anatômicos, histométricos, histoquímicos e imunocitoquímicos. Estes estudos em galhas induzidas em espécies congenéricas (*Byrsonima coccolobifolia* e *Byrsonima variabilis*) permitem mapear as alterações que o tecido vegetal sofre devido ao desenvolvimento das galhas.

O segundo capítulo da seção I parte da premissa que haverá um equilíbrio do investimento de *B. coccolobifolia* na formação de tecidos especializados, onde moléculas energéticas e defensivas se acumularão igualmente tanto nas paredes celulares quanto no protoplasma nos compartimentos teciduais da galha. O desenvolvimento das galhas é influenciado e coordenado pelo organismo galhador por meio de fatores ainda não elucidados que redirecionam as células para uma morfogênese característica. Este desenvolvimento se divide em quatro fases: indução, crescimento e desenvolvimento, maturação e deiscência/senescência (Mani, 1964; Rohfritsch, 1992). Neste capítulo, buscamos mapear a dinâmica das células e tecidos envolvidos na manutenção do metabolismo do indutor e da estrutura da galha.

O terceiro capítulo da seção I aborda o desenvolvimento de galhas induzidas por fungos da ferrugem da ordem Pucciniales em *B. variabilis. Byrsonima variabilis* é uma espécie arbustiva e endêmica de campos rupestres muito comum em Minas Gerais, possui pequenas folhas com pilosidade escura. As pétalas originalmente brancas se tornam rosas na maturidade (Anderson, 1992). As análises destas galhas visaram responder as seguintes perguntas: quais modificações estruturais e químicas são induzidas pela galha de fungo ferrugem nas folhas de *B. variabilis*? E quais as respostas da planta hospedeira frente ao ataque desses galhadores?

A seção I desta tese testa a hipótese central de que espécies congenéricas de *Byrsonima* podem apresentar alterações semelhantes nos padrões de desenvolvimento e metabolismo da planta hospedeira em resposta ao desenvolvimento das galhas associadas. Os caracteres das duas galhas estudadas e peculiaridades de sua história de vida serviram, ainda, de inspiração para o desenvolvimento de dispositivos de interpretação ambiental como forma de estabelecer uma ponte entre a pesquisa e a formação inicial de professores.

O estabelecimento de ferramentas para divulgar o conhecimento produzido na pesquisa científica visou ampliar metodologias capazes de tornar o conhecimento botânico atualizado e acessível aos futuros professores da educação básica e ao grande público. O grupo de pesquisa "Estrutura, Fisiologia e Química de Galhas Neotropicais", assim registrado no CNPq em 2008, tem atuado na produção de conhecimento sobre galhas e suas hospedeiras com alcance mundial, enfrentando, atualmente, o desafio de investir na produção de estratégias de ensino e extensão para tornar este conhecimento acessível ao grande público. As estratégias de divulgação

científica e ensino das interações planta-galhador desenvolvidas ao longo da tese compõem os dois capítulos da seção II.

O primeiro capítulo da seção II desta tese apresenta o jogo didático ‘Por dentro das galhas’ que simula o ciclo de vida de um galhador hipotético e suas interações com fatores bióticos e abióticos, bem como as formas de aplicação do jogo como ferramenta de ensino-aprendizagem. O referido jogo tem suas peças, um tabuleiro e um conjunto de cartas acessíveis para impressão no site do grupo de pesquisa (www.neotropicalgallgroup.com.br), gratuitamente, e em três idiomas (português, espanhol e inglês). Sendo uma ferramenta capaz de ser explorada em parceria entre o professor de Ciências/Biologia e os de idiomas estrangeiros. O objetivo do jogo é simular o ciclo de vida das galhas, e detalhar o processo de indução e desenvolvimento destas, levando em consideração também fatores bióticos e abióticos enfrentados pelos indutores das galhas durante seu desenvolvimento. Propomos, ainda, que este jogo seja aplicado na perspectiva da abordagem prática antes da teórica, como um estímulo para aproximar os alunos de um conteúdo científico distante do seu dia a dia, mas que pode ser facilmente alinhado aos conteúdos didáticos dos diferentes níveis de ensino, numa perspectiva multidisciplinar.

O segundo capítulo da seção II compila outros três dispositivos educativos produzidos ao longo da pesquisa visando a divulgação científica e a formação de professores. São eles (1) um vídeo de animação, (2) o aplicativo ‘*Gallocation’* para sistema Android e IOS baseado na ciência cidadã, e (3) um livro infantil que conta a história de duas crianças que encontram uma árvore de murici com galhas. Dispositivos educativos são ferramentas tecnológicas ou materiais didáticos utilizados para auxiliar no processo de ensino e aprendizagem. São projetados para ajudar os alunos a aprender de forma mais eficaz e eficiente, tornando o aprendizado mais atraente e interativo, fornecendo novas maneiras de interagir com diversos conteúdos e ajudar os alunos a desenvolver habilidades e conhecimentos em áreas específicas. Eles podem ser usados para fornecer feedback imediato, permitir a prática de habilidades específicas ou apresentar informações de uma forma mais visual ou auditiva. Podem ser usados em todos os níveis educacionais, desde a educação infantil até o ensino superior. Eles podem ser físicos, como um kit de experimentos científicos, ou virtuais, como um software educativo como exemplo de dispositivos educativos, incluímos softwares educacionais, jogos educativos, livros paradidáticos, vídeos curtos em linguagem acessível ao grande público, kits de experimentos científicos. O termo dispositivos (de interpretação ambiental) foi usado para em uma pesquisa no Parque Estadual Serra Verde - MG em referência a instrumentos criados para traduzir o conhecimento científico e favorecer as conexões entre o interesse da audiência e o significado dos recursos naturais sendo, portanto, utilizados em momentos específicos da visitação ao

referido parque (Portugal, 2014).

A criação de cada estratégia de ensino e divulgação teve o objetivo de trazer de volta a *Scientia amabilis* de Carolus Linneus ao riquíssimo campo de novas descobertas. Os dispositivos educativos foram produzidos em parceria com discentes da licenciatura do curso de Ciências Biológicas na Universidade do Estado de Minas Gerais (UEMG), visando sua incorporação como prática didático-pedagógica no ambiente escolar. Ademais, a divulgação fora dos ambientes acadêmicos faz a ponte academia-sociedade com a qual esperamos alcançar a grande meta do letramento científico (Ulhoa et al. 2008).

É necessário formar profissionais comprometidos e capazes de possibilitar aos alunos entender o papel e o significado da Ciência e da Tecnologia na sociedade contemporânea, compreendendo o que se faz em ciência, por que se faz e como se faz (Krasilchick 2008). Nesse sentido, o campus da UEMG/Ibirité, é rico em áreas verdes com potencial para desenvolvimento de estudos geradores de conhecimento científico sobre botânica e interações ecológicas. Além de abrigar uma escola de educação básica, o campus da UEMG/Ibirité sedia um programa de escola integrada que recebe alunos das escolas municipais de Ibirité, permitindo conciliar pesquisa científica, formação inicial e continuada de professores e educação ambiental.

Os artigos componentes da seção I da tese permitem testar a hipótese de que embora as galhas sejam diferentes do ponto de vista estrutural em função da diferença entre os táxons de organismos galhadores, haverá similaridades nas respostas das espécies congenéricas de *Byrsonima* spp. devido ao seu potencial morfogenético similar.

Seção I:
Pesquisa Científica

Capítulo 1:

Phenological strategies of *Byrsonima coccolobifolia* (Malpighiaceae) and its associated galling Cecidomyiidae

Phenological strategies of *Byrsonima coccolobifolia* (Malpighiaceae) and its associated galling Cecidomyiidae

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Abstract

Gall induction is a sophisticated adaptive strategy in ecological dynamics, which in the case of galling insects, demands the adjustment of their life cycles to the best host plant water and nutrient conditions. Such a hypothesis is herein tested in a *Byrsonima coccolobifolia*-Cecidomyiidae system, which was observed along 18 months. Ten individuals of *B. coccolobifolia* were marked and monitored in an area of Brazilian Savanna at Limeira farm in the municipality of Conceição do Pará, Minas Gerais state, Brazil. The synchronism between host plant phenophases and gall development and their responses to climatic parameters were estimated. *Byrsonima cocolobifolia* is deciduous, and the associated galling Cecidomyiidae has a univoltine life cycle, and the phase of gall induction synchronized with the first leaf flushing. Variations in plant phenology in response to environmental parameters evidenced that vegetative and reproductive phases of *B. coccolobifolia* asynchronous with gall development may be a strategy to escape the competition with the Cecidomyiidae. Nevertheless, gall development synchronized with the *B. coccolobifolia* reproductive phase may have deviated the total allocation of photoassimilates to reproductive shoots but did not impair fruit formation, besides being able to follow the delay at the beginning of leaf flushing as a function of environmental variations.

Keywords: gall life cycle, insect–plant interaction, leaf dynamics, phenological synchrony, water availability.

Introduction

Ecological dynamics may be interdependent and related to numerous biotic and abiotic factors (Franco et al. 2005; Doak et al. 2008; Garcia et al. 2017; García-Núñez et al. 2019). Among the fascinating diversity of ecological dynamics, gall induction is highlighted as a sophisticated adaptive strategy of many insects to obtain shelter, food, and protection from their natural enemies and environmental conditions (Mani 1992; Stone and Schonrögge 2003; Miller and Raman 2018). Galls are new plant organs developed in response to the stimuli imposed by specific gall-inducing organisms, which are mainly insects (Mani 1964). Such stimuli promote cell redifferentiation, leading to the formation of tissues with typical features and functions (Oliveira and Isaias 2009; Ferreira et al. 2019). After induction, gall development follows successive phases named growth and development, maturation, and senescence (Arduin and Kraus 1995) or dehiscence (Rohfritsch 1992), which relate to the temporal co-occurrence of the infesting forms of insects and the reactive plant tissue sites susceptible to manipulations (Oliveira and Isaias 2010, Carneiro et al. 2013). In general, the galling herbivore prefers young plant tissues, due to their ability to react to the stimuli of gall induction (Rohfritsch and Anthony 1992), and to the metabolic sinks established on host leaves and stems along their early developmental phases (Abrahamson and Weis 1987).

The time of appearance of most galling herbivores is synchronized with the time of the active growth of the host plant module in which the galls develop, evidencing ecological relationships to be explored (Mani 1964; Yukawa 2000; Carneiro et al. 2013; Oliveira et al. 2016; Guedes et al. 2018; Costa et al. 2021). For the life cycle of the galling insects, that is, for their developmental phases to be successfully completed, the synchronism with leaf flushing is fundamental for the temporal allocation of resources (Yukawa 2000; Fagundes 2014). These resources are also related to environmental variations, climatic seasonality, and especially to the availability of water in the soil (Borchet 1994; Oliveira et al. 2013; Guedes et al. 2018; Costa et al. 2021). Moreover, the

phenological synchronization of different species can be disrupted if alterations in temperature or in precipitation occur (Franco et al. 2005; Garcia et al. 2017). Such alterations peculiarly affect the phenology of different species (Weis et al. 1988; Yukawa 2000; Asch et al. 2007), with changes in the lifespan and the voltinism of the galling herbivores (Bale et al. 2002).

Species of the genus *Byrsonima* Rich. Ex. Kunth. (Malpighiaceae) has a wide occurrence in Brazilian Savanna areas, different phenological strategies (Mamede 2013), a richness of associated galls (Silva et al. 2018; Campos et al. 2021), and are elegant models for studies on phenological synchronism between host plants and galling insects. One of its species, *Byrsonima coccolobifolia* Kunth, commonly known as "pink murici" is a tropical, deciduous species present in the "cerrado" and "campo cerrado" (Barbosa et al. 2005; CNC Flora 2023), where the individuals are superhosts of gall inducers (Gonçalves-Alvin and Wilson 2001; Araújo et al. 2015), including a Diptera, Cecidomyiidae that induces a conical leaf gall (Araújo et al. 2014). The specificity of the plant-insect interactions and the expected synchronism between *B. coccolobifolia* and this galling Cecidomyiidae were addressed through the investigation along 18 months. Our hypotheses are that (1) the host plant may have subtle phenological strategies toward windows of opportunity to escape gall induction, and (2) the galling Cecidomyiidae life cycle may adjust to the best water and nutritional conditions during the phenology of *B. coccolobifolia* with a negative impact on flowering and fruiting.

Material and Methods

Study area

The phenological events of *B. coccolobifolia* (voucher deposited in herbarium IBIUEMG 167) were analyzed in a forest fragment of Brazilian Savanna on the Limeira farm in the municipality of Conceição do Pará (19°.45' 25.3" S, 44° 50' 01.2" W), state

of Minas Gerais, Southeast Brazil (Figure 1) between September 2018 and March 2020. The area has 704 m of altitude with typical Savanna vegetation and the individuals of *B. coccolobifolia* are arranged at the edge of the forest fragment, a region with sparse vegetation and rocky soil. According to the Köppen system, the climate of the region is classified as Cwa (Humid subtropical with dry winter, and hot summer) (Alvares et al. 2013), with marked seasonality. Meteorological data were collected at the Instituto Nacional de Meteorologia (INMET) in Estação Meteorológica Automática de Divinópolis, Minas Gerais, Brazil, and defined the dry and rainy seasons. The rainy season began in September 2018 and extends to April 2019, with the highest accumulated rainfall (380 mm) in February 2019. The dry season began in May 2019, with no rainfall between July and August 2019. The highest values of maximum temperature were recorded in January 2019 (24.2°C) and the lowest values of minimum temperature were recorded in July 2019 (16.9°C) (Figure 2).

Data sampling of host plant phenology

The phenological observations were conducted at a 15-day interval from September 2018 to February 2020 at the individuals (n=10) of *B. coccolobifloia* with a minimum circumference at breast height greater than 10 cm and good canopy visibility. Ten individuals of *B. coccolobifolia* were marked and monitored in a population of 42 individuals, located in an area of Brazilian Savanna at Limeira farm in the municipality of Conceição do Pará, Minas Gerais state, Brazil. The vegetative phenophases were characterized by the presence of leaf flushing (leaf up to 2 cm long and reddish with green veins (Figure 3A), young leaves (green leaf with pinkish veins) (Fig. 3B), mature leaves (green leaf with light green veins) (Figure 3C), senescence (yellowish leaf with the presence of necrotic areas stained in brown) (Figure 3D), and leaf abscission (Figure 3E). For the reproductive phenophases, the inflorescence bud (terminal raceme-type inflorescence with pinkish flowers) (Figure 3F) and inflorescence anthesis (pale pink

hermaphrodite flowers) (Figure 3G), immature fruits (globose, green) (Figure 3H) and mature fruits (yellow) (Figure 3I).

The percentage of phenophase intensity, i.e., the Fournier index (Fournier 1974), was evaluated using a five-category scale from 0-4 with 25% intervals (0 = absence of the phenophase; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% intensity of the phenophases) in each *B. coccolobifolia* individual. The presence and absence of vegetative and reproductive phenological phases, as well as phenological synchrony, were evaluated for each plant by applying the activity index (Bencke and Morellato 2002). This method estimates the percentage of individuals in the population that present a particular phenological event and indicates the synchrony of this phenological event throughout the year.

Data sampling of the gall developmental phases

The gall developmental phases, induction, growth and development, maturation and senescent phases were rated on five marked branches in each of ten individuals (n = 50 branches). The canopy was radially divided into five parts for branch marking. On each branch, the presence of each gall development phase was counted every 15-day interval from September 2018 to February 2020. The induction phase (hard to be visualized) was defined by the presence of distinct light green spots on the adaxial surface of the young leaf (Figure 4A), and the growth and development phase was indicated by the gall pinkish color on the adaxial surface and green on the abaxial surface (Figure 4B); the maturation phase was indicated by the gall brownish color in the tapered apex on the adaxial surface (Figure 4C); and the senescent phase was indicated by the change in gall to pallid green color (Figure 4D).

Data analysis

The synchrony between the plant phenophases, between individuals in the same phenophase, and of the phenophases with gall development, and the climatic parameters were estimated by the non-parametric Kendall correlation considering the measurements of all the 18 months.

To evaluate the relationship between two quantitative variables (rainfall and temperature), the non-parametric Kendall's correlation was calculated for each pair. Illustrative graphs were constructed for significant relationships. A Principal Components Analysis was performed with the correlation matrix, and prior to the analysis, the data were previously transformed into ranks. This methodology corresponds to evaluation relationships among the vegetative and reproductive phenology, gall development, and climatic variables from a non-parametric perspective (Conover 2012). All statistical analyzes were performed using R software version 3.6.1 (R Core Team 2019).

Results

The phenological behavior of the host plant

Byrsonima coccolobifolia is a deciduous species, which lost all its leaves in the dry season, i.e., between May and September 2019 (Figure 5A-B). Leaf flushing occurred at two periods of the year, with an intensity peak in September 2018. In 2019, there was a peak in February and another one with a higher intensity in October. In 2020, the leaf flushing peak occurred in February. Young leaves occurred from September to November of 2018, but in 2019, this phenophase was less intense and extended until January 2020. Mature leaves had maximum intensity from December to February in both years, with a decrease in April 2019, when the precipitation index decreased. The occurrence of senescent leaves began in April 2019, with a peak of intensity in June 2019. Leaf falling began in June 2019, at the beginning of the dry season. The tree was totally leafless between July and August 2019, when the lowest precipitation was registered along the

dry season (Figure 5A). The floral buds appeared soon after leaf flushing between September and October in both years (2018 e 2019), and full inflorescence anthesis occurred in November; immature fruits had maximum intensity in January 2019 e 2020 and fruit ripening occurred in February 2019 e 2020. A leaf flushing occurred in February (2019 e 2020), and a new reproductive cycle occurred from March to June 2019, concomitant with the end of the rainy season and the beginning of the dry season, and the occurrence of senescent leaves (Figure 5B). Most of the individuals sampled showed synchrony in the vegetative and reproductive phenophases (Figure S1).

The gall phenological behavior

We monitored two gall life cycles, one recorded from September 2018 to May 2019, and another one recorded from October 2019 to February 2020. The maximum peak of induction activity was recorded in October 2018 and November 2019 in synchrony with the occurrence of young leaves, the appearance of floral buds, and the onset of the rainy season. Galls entered the growth and development phase in October 2018 and 2019, with a peak in November of both years. The peaks of the maturation phase occurred on December 2018 and January 2019, coinciding with the peak of mature leaves and the development of immature fruits. The galls entered the senescent phase from February to May 2019 at the onset of the dry season and low temperatures. The second event of gall senescence occurred from January to February 2020 in the middle of the rainy season (Figure 5C). The synchronism between gall phases was observed in most host plants (Figure Sa), and the species of Cecidomyiidae associated with *B. coccolobifolia* has a univoltine life cycle.

Correlations between the phenological phases of the host plant and the gall

Leaf flushing and young leaves were strongly correlated and happened concomitantly ($R = 0.77$; $p<0.001$) (Figure S2 e Figure S3). Leaf flushing ($R = 0,53$, $p =$

0,008), floral buds (R = 0,47, p = 0,017), and galls in the growth and development phase (R=0,59, p=0,006) were moderately correlated and occurred at the same time of the years when the highest water availability occurred (R = 0,44; p = 0,012). The temperature was positive and moderately correlated with the mature leaf (R = 0,45, p = 0,013 and immature fruit (R = 0,39; p = 0,045) phenophases. Precipitation was positive and moderately correlated with the young leaf (R = 0,38, p = 0,38), mature leaf (R = 0,51, p = 0,005), and mature fruit (R = 0,38, p = 0,045) phenophases. Temperature (R = 0,38, p = 0,04) and precipitation (R = 0,51, p = 0,005) were negatively correlated with the leaf abscission phenophase and occurred when water availability was minimal, and the temperature was falling.

Principal Component Analysis (PCA)

The first two axes of the PCA explained 46.5% of the total variability of the matrix. Evaluating by axis 1, we observed the formation of a group of variables positively associated with each other (left quadrants), encompassing the variables: temperature, precipitation, mature leaf, ripe fruit, maturation, and senescence of the galls. This group is negatively associated with the variable leaf abscission, located in the lower right quadrant. A second group is formed by variables that are positively associated with each other, which are leaf flushing, young leaves, inflorescence floral buds, inflorescences, induction, and growth and development, and was negatively associated with senescent leaves.

Two groupings of variables, one being the vegetative period associated with the beginning of flowering and the other the reproductive/dispersive period with the presence of fruits. This last period is associated with the hottest and most humid period of the year (rainy summer). Internal or physiological factors and external or edaphoclimatic factors are positively or negatively associated with the vegetative and reproductive phenophases of the host plant and with the life cycle of the galling insect. The vegetative and

reproductive phenophases overlapped with the growth and development phase of the gall and were positively correlated with temperature and precipitation (Table 1) (Figure 6).

Discussion

From the host plant perspective

The individuals of *B. coccolobifolia* exhibited a deciduous habit characterized by a total leaf falling for three months (June to August) in the dry season. This seasonal behavior has been similarly reported for *B. coccolobifolia* in some Brazilian Savanna areas (Morais et al. 1995), but the semi-deciduous (Barbosa et al. 2005; Pereira 2018) or evergreen habit (Figueiredo 2008) have also been observed. The variation in the leaf dynamics of *B. coccolobifolia* can be related to phenological adjustments at the individual level and may explain the species success in different phytogeographic domains (Barbosa et al 2005).

Variations in water availability and temperature in different habitats can influence the intensity and duration of the vegetative phases of plant populations (Boas et al. 2013; Silveira et al. 2013). In species of the Brazilian Savanna and other seasonal environments, leaf falling synchronizes with lower water availability, as a mechanism to reduce water loss and maximize the acquisition of nutrients (Pedroni et al. 2002; Silvério and Lenza, 2010). In this perspective, the variation in rainfall volume at the beginning of the rainy season (about 1 month later in 2019 than in 2018) interfered with the phenophases of *B. coccolobifolia*. Hence, it is plausible to admit a phenological adjustment of *B. coccolobifolia* individuals to the condition of low water availability of the Brazilian Savanna.

Regarding temperature and rainfall, the ranges between 23-25°C and 80-1020 mm were not directly correlated to leaf flushing, which occurred before the onset of rainfall, and is a strategy reported for other Neotropical savanna species (Borchert 1994; Rivera

et al 2002; Franco et al 2005; Lenza 2005). The occurrence of leaf flushing just before the rains can optimize the photosynthetic rate in deciduous species, as it allows the complete regeneration of the canopy at the onset of the rainy season (Franco et al. 2005) and prevents the prolonged exposure of young leaves to water stress, mitigating the damage imposed by herbivorous insects (Rivera et al 2002), including the gall inducers. In the tropics, herbivorous insect populations decrease in the dry season, and new life cycles start at the beginning of the rainy season (Coley and Barone 1996; Bale et al. 2002). Accordingly, the new leaves produced during the rainy season will suffer less insect damage than leaves produced during the dry season, the time of highest leaf production (Marquis et al 2001).

The low correlation between leaf flushing, floral bud emergence, and gall induction in *B. coccolobifolia*-Cecidomyiidae system with the abiotic parameters suggests that the host plant accumulated nutrient resources that allowed leaf flushing even before the onset of rains as proposed for other phenological studies (Garcia et al. 2017; García-Nuñez et al. 2019). The vegetative and reproductive cycles observed at two different times of the year, at the end of the rainy season, in the individuals of *B. coccolobifolia* may constitute a strategy to escape the attacks by the galling herbivores. This strategy was also evidenced in the early leaf flushing of *Copaifera langsdorffii* before the rainy season and resulted in a higher event of herbivory on plants that emerged later (Fagundes et al. 2016). As the galling Cecidomyiidae associated with *B. coccolobifolia* has a univoltine life cycle, during the second cycle of the year, there are no adult individuals to oviposit, despite the availability of host plant-responsive tissues.

The positive and moderate correlation of young leaf emergence with precipitation and temperature increasing, which coincided with the phases of gall induction and growth and development, indicated an allocation of resources shared by the vegetative, the reproductive phenophases, and the gall development. However, the presence of the galls,

as a third sink of photoassimilates, does not seem to negatively influence the phenological events of *B. coccolobifolia* since fruiting occurred abundantly in both reproductive cycles.

Senescent leaves and leaf abscission had a significant positive correlation with precipitation and temperature. As well, the production of immature and ripe fruit correlated to the lowest precipitation and the highest temperature variations.

From the galling insect perspective

Gall establishment and development require plant-responsive tissues (Carneiro et al. 2013), which are the main components of young leaves, the preferred oviposition sites of the galling Cecidomyiidae associated with *B. coccolobifolia*. The young leaves as preferred sites of oviposition have also been reported for *Piptadenia gonoacantha* (Arduin and Kraus 1995), *Ficus microcarpa* (Souza et al. 2000), *Baccharis concina* and *B. dracunculifolia* (Arduin and Kraus 2001) and *Mimosa gemmulata* (Costa et al. 2021) in different areas of the Brazilian Savanna, as well as in other biomes, as reported for *M. tenuiflora* in Caatinga (Nogueira et al. 2022) and *Schinus polygama* in the Mediterranean climate of southern Chile (Guedes et al. 2018). This preference for young leaves can be interpreted as a strategy from the structural and nutritional points of view, which favor the development of the gall and, consequently, the life cycle of the gall inducer (Miller and Raman 2018). From the structural point of view, young leaves are composed of tissues that are more reactive to gall induction stimuli and respond by hyperplasia and cell hypertrophy, common plant responses observed in galls (Rohfritsch and Anthony 1992). From the nutritional point of view, young leaves are richer in water and nitrogen than mature leaves (Coyle et al. 2010; Liu et al. 2010) due to metabolic sinks, which young leaves establish in their early phases of development (Abrahamson and Weiss 1987).

The peak of gall induction on *B. coccolobifolia* varied about one month from the first to the second year of sampling, indicating that the associated galling Cecidomyiidae

has strategies to exploit the host plant species even with changes in its phenology in response to climatic events. This refined synchronism of the gall inducer was demonstrated in the *Lopesia mimosae* galls on *Mimosa tenuiflora* (Nogueira et al. 2022), as well as, for other groups of gall-inducing insects (Castro et al. 2012; Magalhães et al. 2014).

In the gall growth and development phase, the feeding activity of Cecidomyiidae larvae in plant tissues is intense and alters photoassimilate allocation by acting as an additional sink (Mothes et al. 1961; Stone et al. 2002; Schultz et al. 2013; Ekholm et al. 2019). The gall maturation phase corresponds to the main trophic phase of the gal inducer, and, consequently, the sink of photoassimilates toward the gall is even stronger than in gall induction (Rohfritsch 1992). This new sink can disturb the homeostasis of plant tissues, reducing the availability of plant resources for growth and reproduction (Isaias et al., 2015; Oliveira et al., 2017), which in the case of *B. coccolobifolia*, did not negatively influence the fruiting phase, and suggest the occurrence of a redistribution of photoassimilates.

The synchronism with the host plant phenophases is fundamental to the success of the life cycle of the galling insects (Weis et al., 1988; Yukawa 2000; Oliveira et al., 2016). In *B. coccolobifolia*, the associated Cecidomyiidae has a univoltine life cycle in which gall induction was synchronized with the first event of leaf flushing observed in 2018. The second leaf flushing observed in the population of *B. coccolobifolia* in 2019 occurred when there were no adults of Cecidomyiidae able to oviposit. This asynchrony with the life cycle of the Cecidomyiidae configures a window of opportunity for the host plant escape gall induction, which corroborates our first hypothesis. The galling Cecidomyiidae life cycle adjusts to the environmental variations, but does not have a negative impact on the flowering and fruiting of *B. coccolobifolia*, which does not corroborate our second hypothesis.

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CRediT authorship contribution statement

RSMM: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, review & editing. JNM: Conceptualization, Validation, Formal analysis, Writing – original draft. ECC: Writing – review & editing. GPPB: Writing – review & editing. RMSI: Conceptualization, Resources, Writing – original draft, review & editing, Supervision, Project administration.

Declaration of Competing Interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data Availability

Data will be made available on request.

Declaration of Funding

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578

579 **Legends**

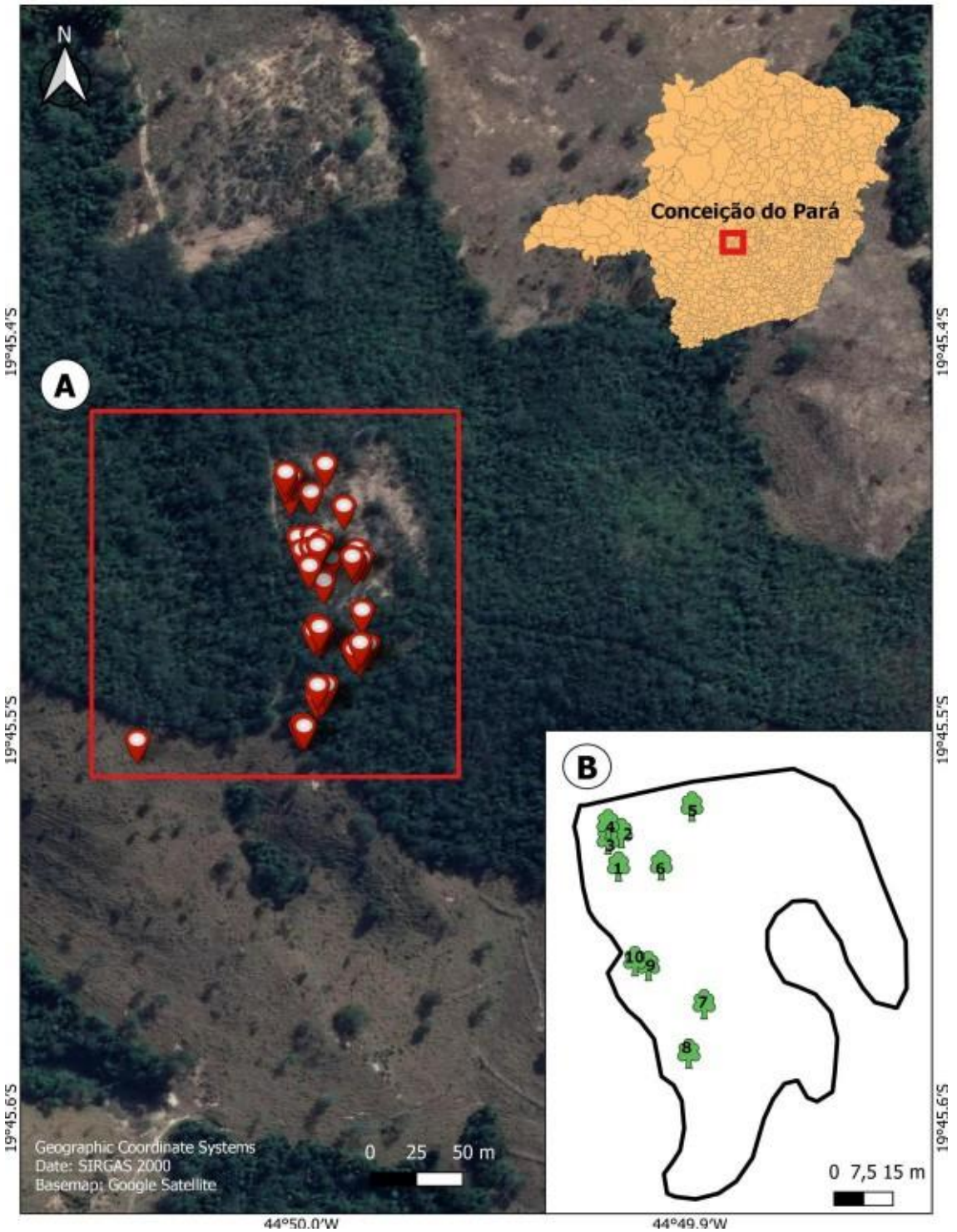


Figure 1 - Forest fragment of Brazilian Savanna in the Limeira farm in the municipality of Conceição do Pará, state of Minas Gerais, Southeast Brazil. A) Aerial view of the region and location of the 42 individuals of *Byrsonima coccolobifolia* Kunth (Malpighiaceae) (dotted rectangle). B) Contour of the study area and location of the 10 individuals of *Byrsomina coccolobifolia* Kunth (Malpighiaceae) monitored for 18 months.

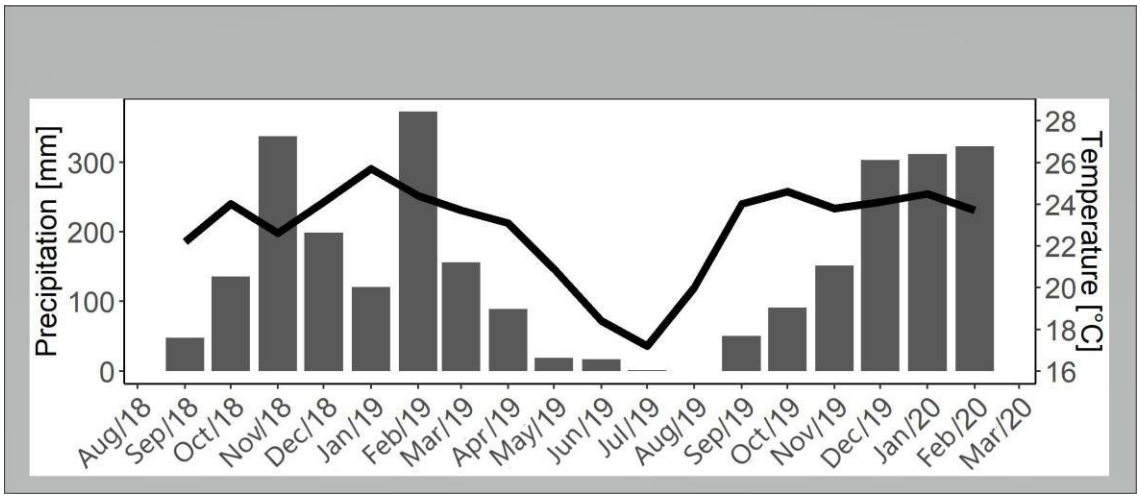


Figure 2 - Climatic diagram with mean annual rainfall (mm) and temperature (°C) during the 18 months of study from September 2018 to February 2020.

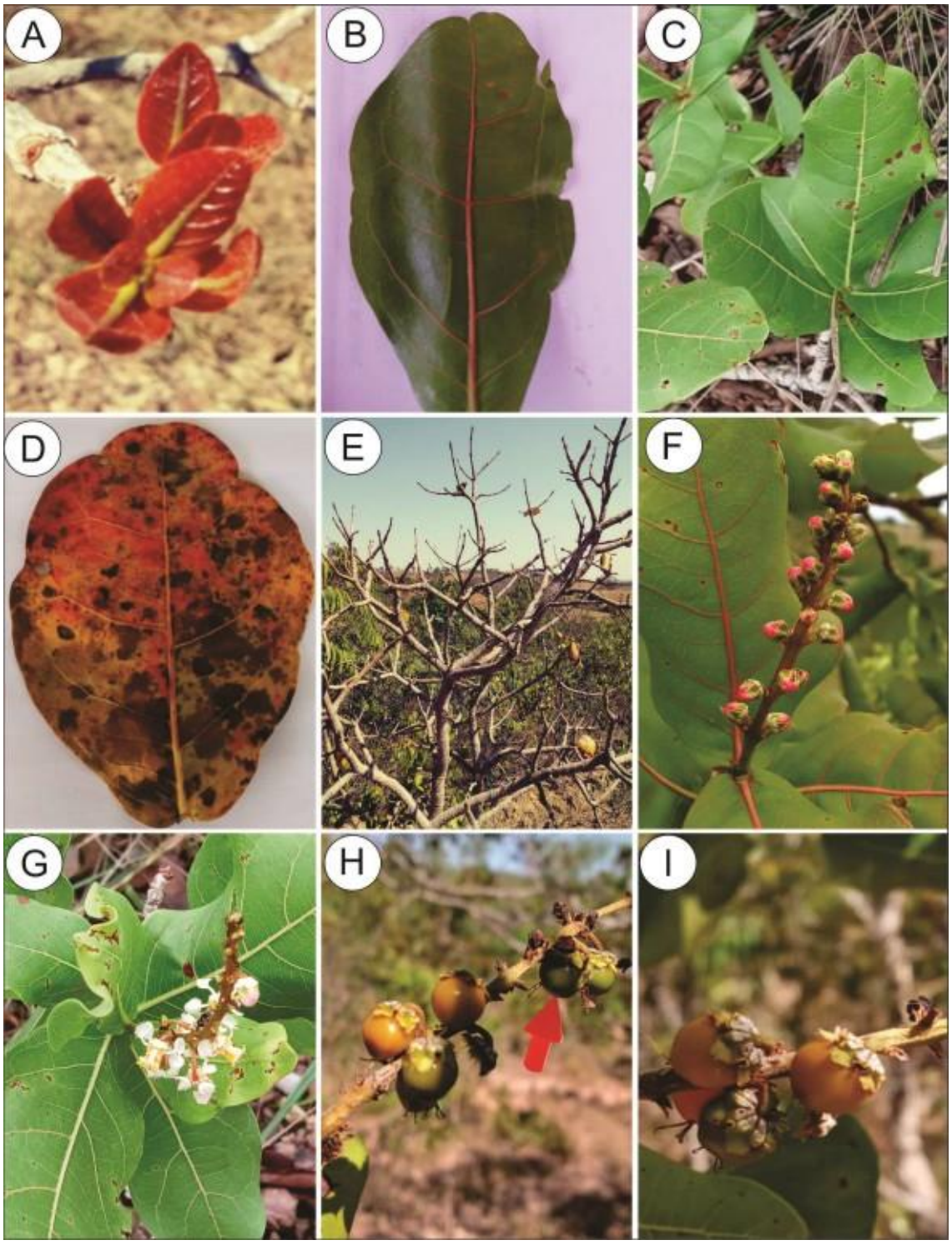


Figure 3 - Vegetative and reproductive phenophases *of Byrsonima coccolobifolia* Kunth (Malpighiaceae). A) Leaf flushing. B) Young leaves. C) Mature leaves. D) Senescent leaves. E) Leaf abscission. F) Inflorescence buds. G) Inflorescence anthesis. H) Immature fruit (red arrow). I) Ripe fruit.

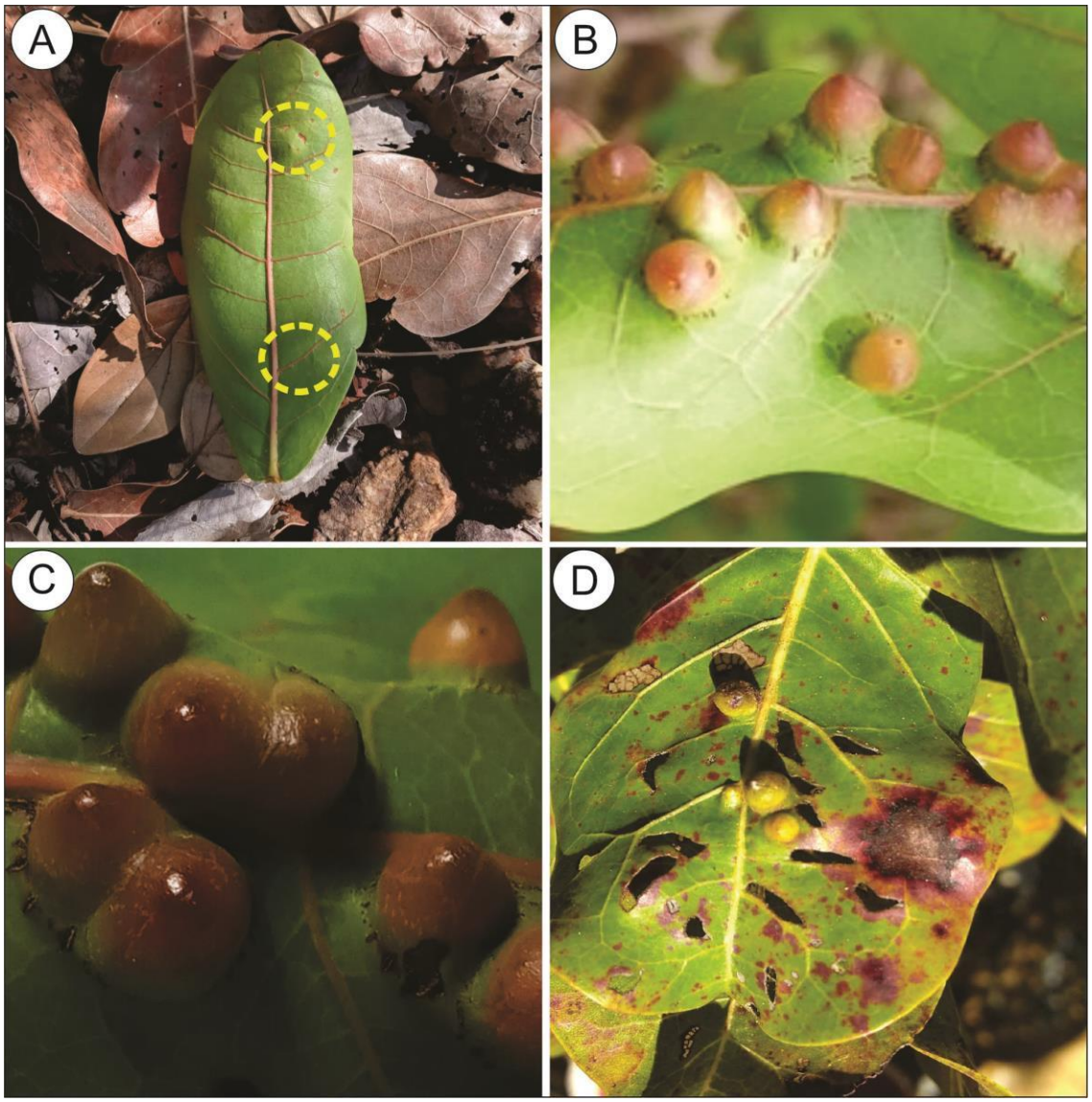


Figure 4 - Developmental phases of the leaf conical galls induced by a Cecidomyiidae (Diptera) on *Byrsonima coccolobifolia* Kunth (Malpighiaceae). A) Induction (yellow dotted). B) Growth and development. C) Maturation. D) Senescence.

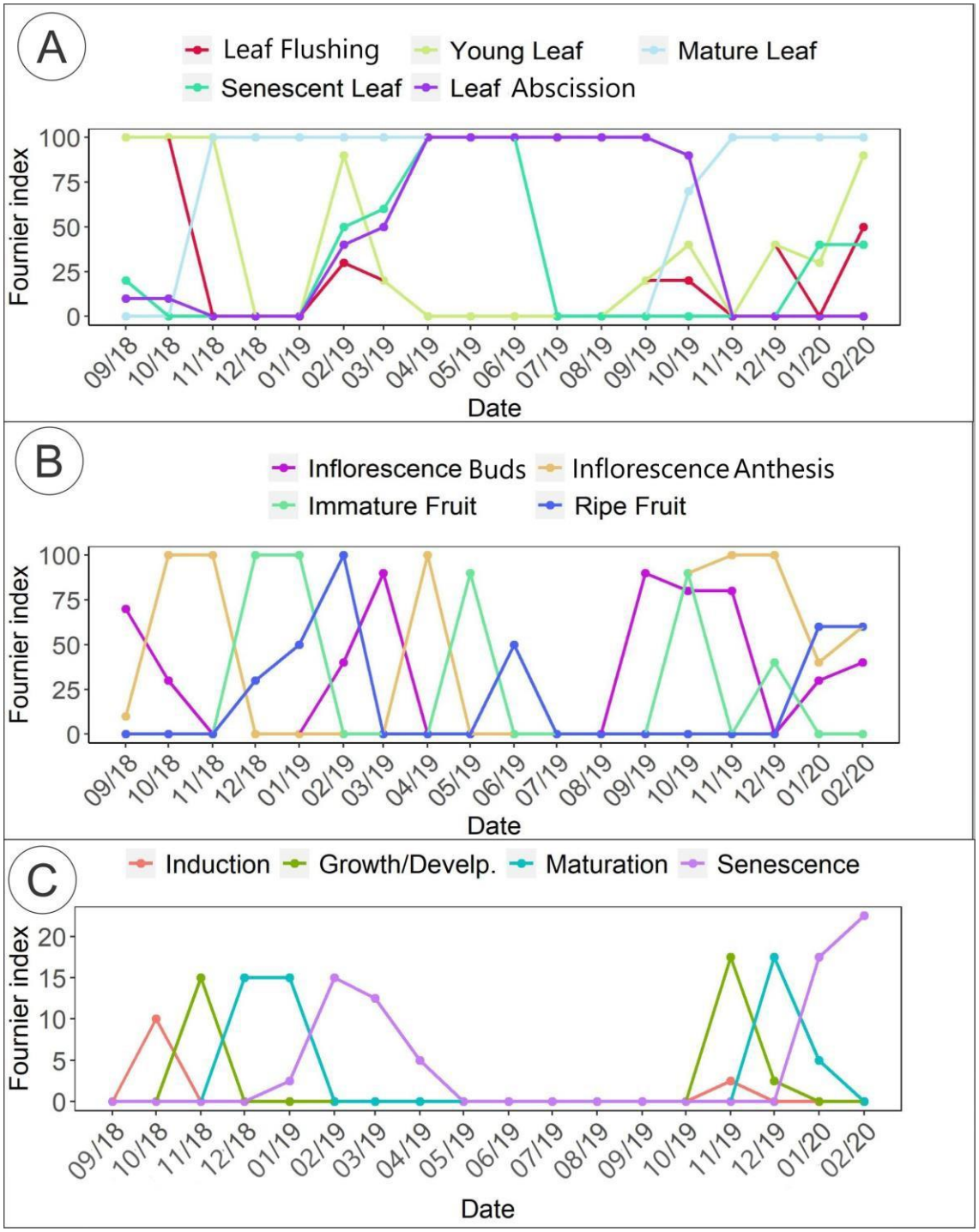


Figure 5 - Temporal variation of phenological indices during the phenology of the *Byrsonima coccolobifolia* Kunth (Malpighiaceae) and of the gall life cycles of Cecidomyiidae (Diptera). A) Synchrony between the vegetative phenophases (Fournier index) of the *B. coccolobifolia*. B) Synchrony between the reproductive phenophases of the *B. coccolobifolia* (Fournier index). C) Synchrony between the phenophases (Fournier index) of the gall.

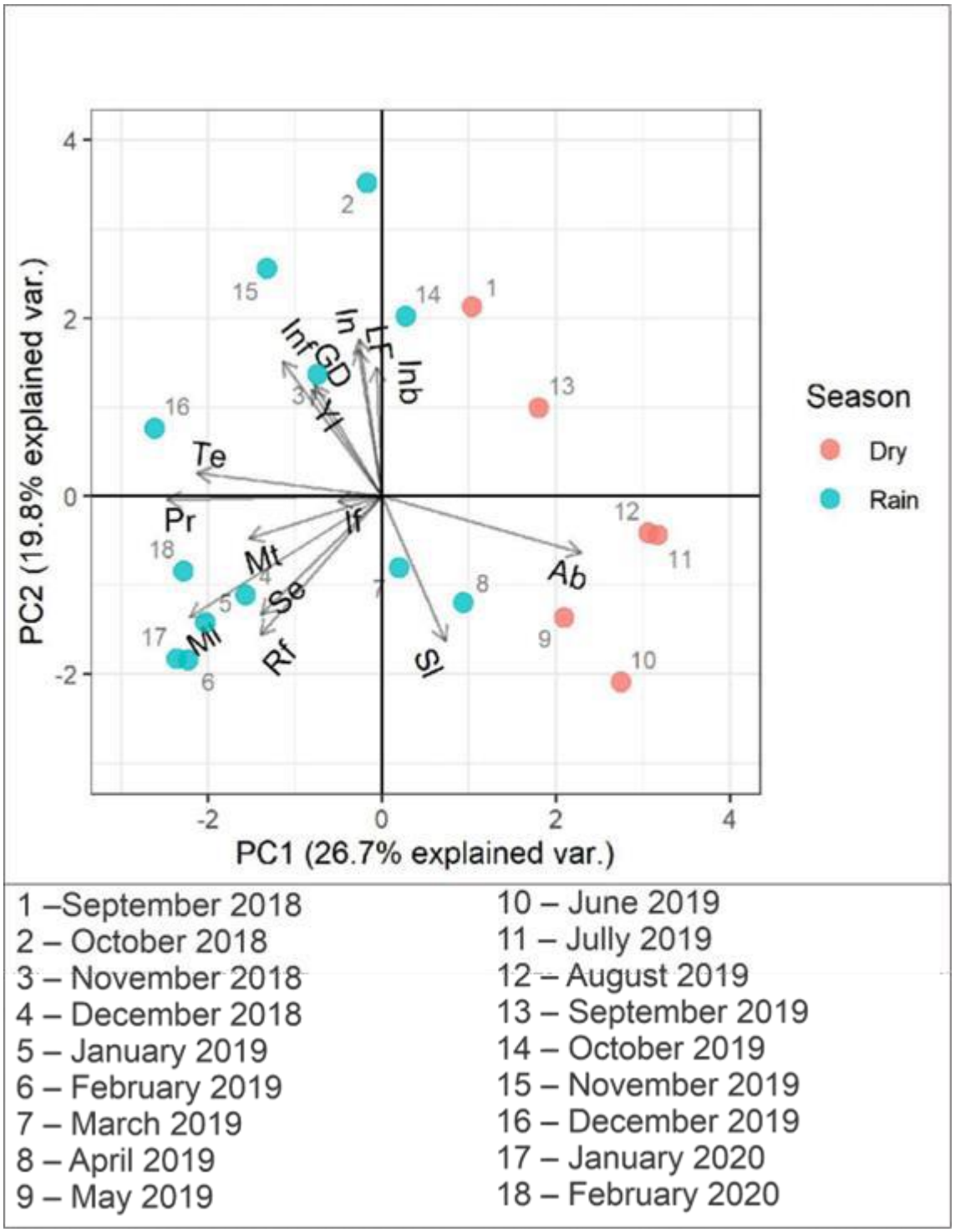
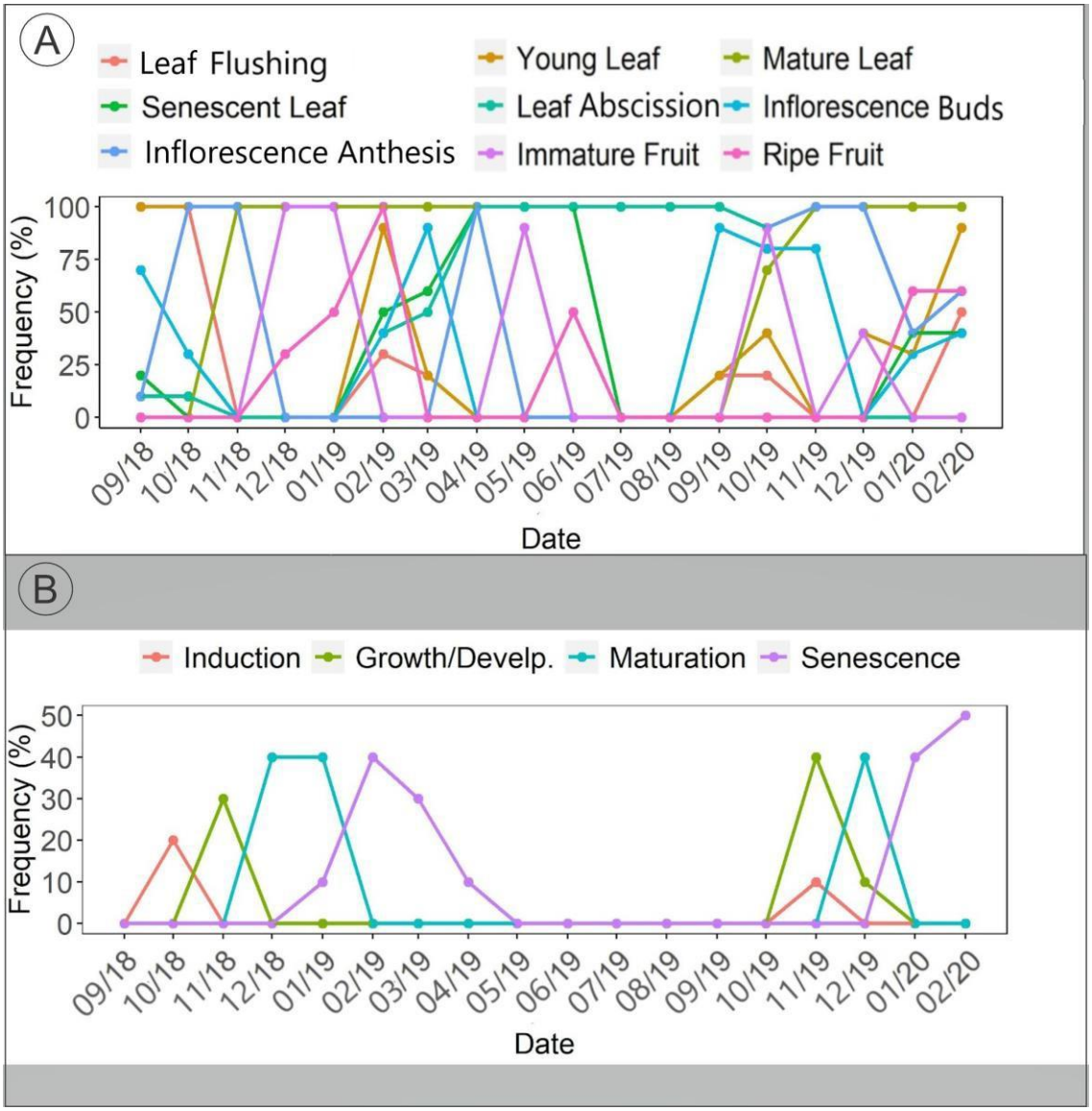
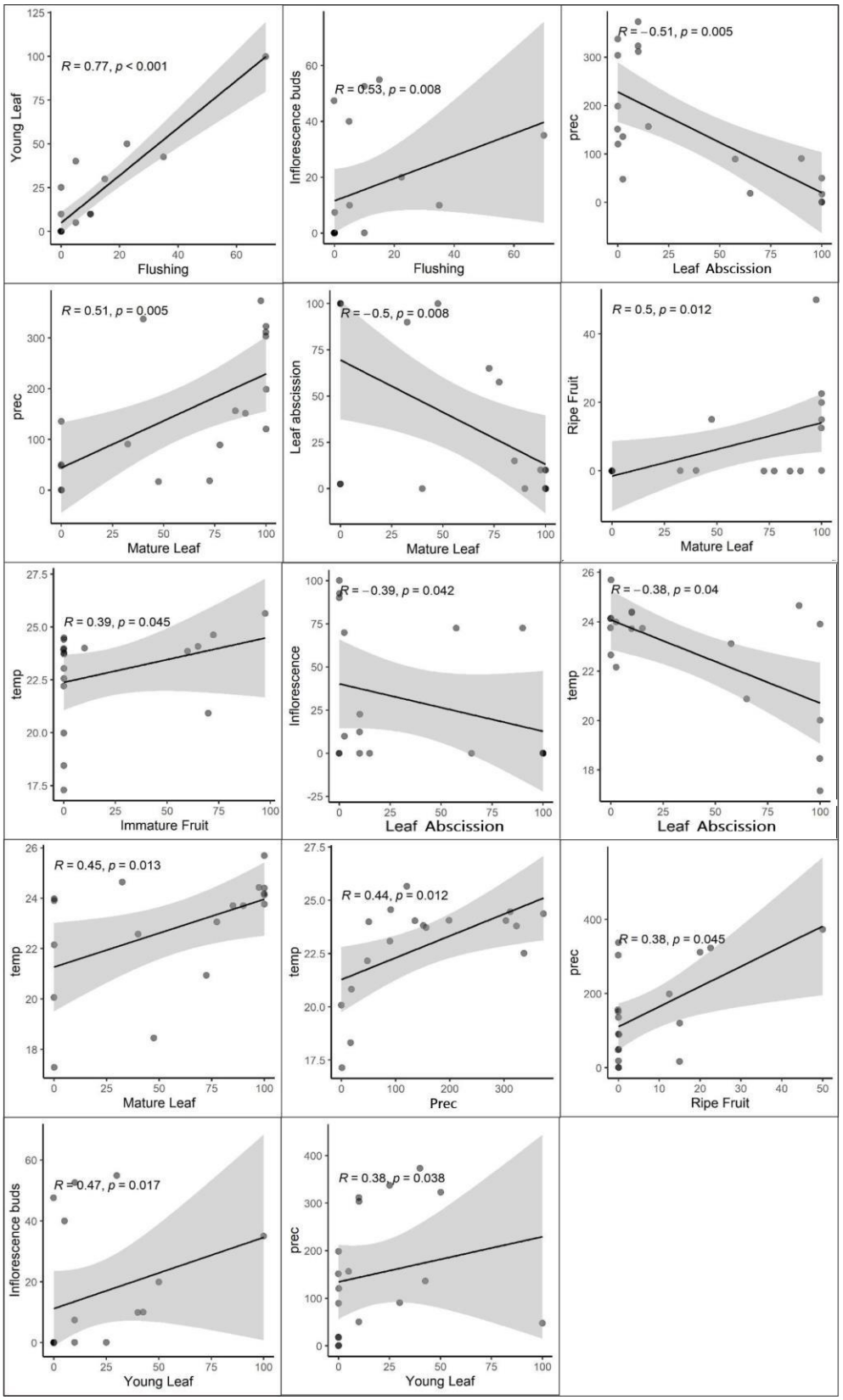


Figure 6 - Principal component analysis (PCA) of the phenophases of *Byrsonima coccolobifolia* Kunth (Malpighiaceae) and the gall induced by an unidentified species of Cecidomyiidae (Diptera). Vectors represent explanatory variables derived from host phenology: leaf flushing (LF), young leaf (YL), mature leaf (ML), senescence leaf (SL), leaf abscission (Ab), inflorescence buds (InB), inflorescence anthesis (Inf), immature (IF) and ripe fruits (RF). Gall phases: induction (In), growth and development (GD), maturation (Mt), and senescence (Se).



Supplementary a - Temporal variation of the variables measured during the eighteen months of the study. A - Synchronism of vegetative and reproductive phenophases among the ten individuals of *Byrsonima coccolobifolia* Kunth (Malpighiaceae) monitored between September 2018 to February 2020 evaluated at relative frequency index. B - Synchronism of gall phenophases among the ten individuals of *Byrsonima coccolobifolia* monitored between September 2018 to February 2020 evaluated at relative frequency index.



Supplementary b - Illustrative plots of the host plant phenophases for the significant non-parametric Kendall's correlations followed by their respective p-values. Strong correlations are above 0.7 and correlations between 0.4 and 0.69 are considered moderate. Correlations near 1, indicate a high synchrony, values near -1.

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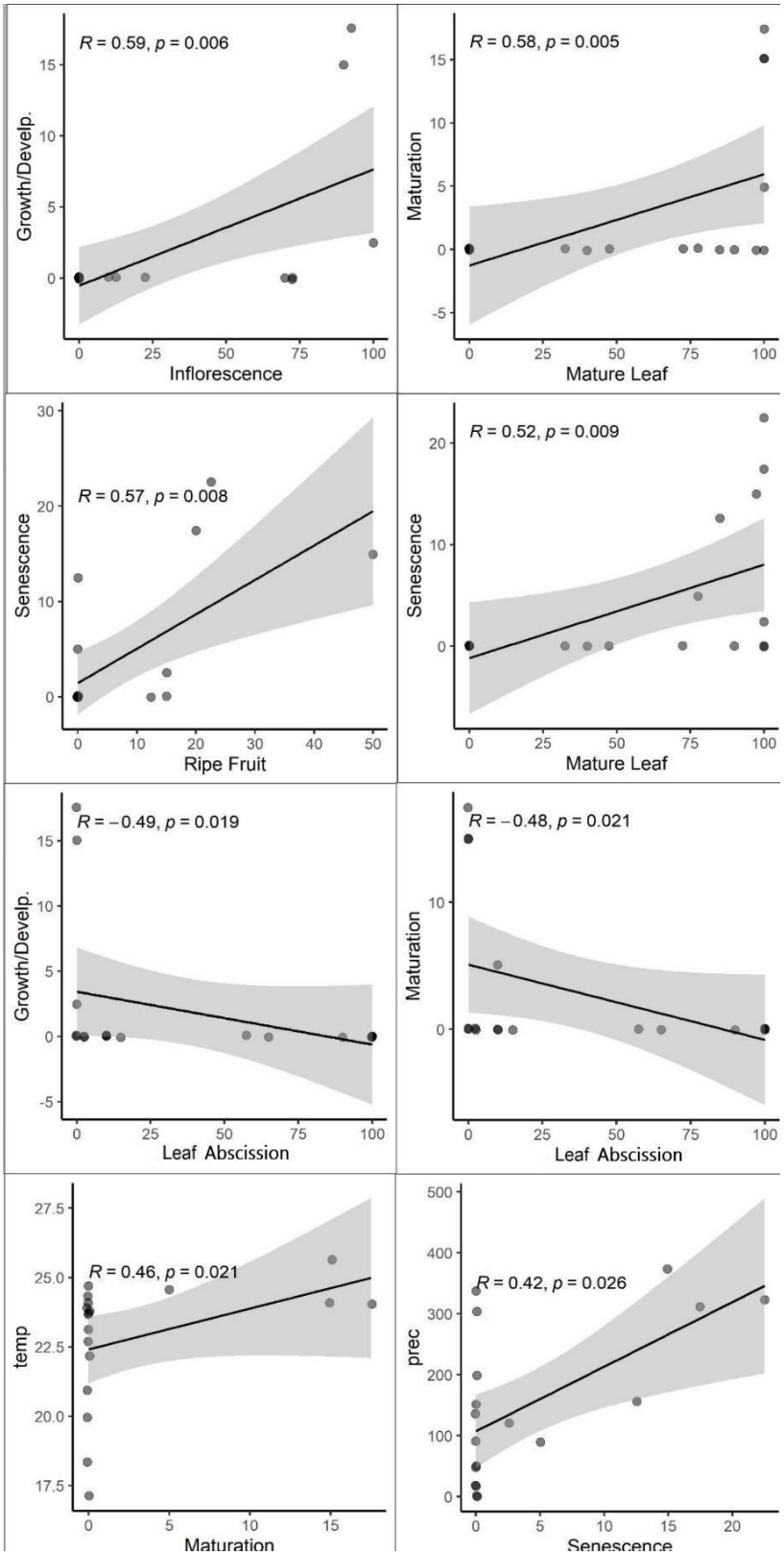

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642 **Supplementary c** - Illustrative plots of the gall phenophases for the significant non-
643 parametric Kendall's correlations followed by their respective p-values. Strong
644 correlations are above 0.7 and correlations between 0.4 and 0.69 are considered moderate.
645 Correlations near 1, indicate a high synchrony, values near -1.

Table 1 - Principal components 1 (PC 1) and 2 (PC 2) obtained from the correlation matrix of the phenological phases of the *Byrsonima coccolobifolia* Kunth (Malpighiaceae) and life cycle phases of the galls.

Variables	Axes	
	PC1	PC2
Flushing	-0.05	0.35
Young Leaf	-0.15	0.25
Mature Leaf	-0.40	-0.29
Senescent Leaf	0.13	-0.34
Leaf abscission	0.41	-0.13
Inflorescence buds	-0.01	0.30
Inflorescence anthesis	-0.21	0.32
Immature Fruit	-0.09	-0.01
Ripe Fruit	-0.25	-0.33
Induction	-0.05	0.37
Growth/Develp.	-0.14	0.26
Maturation	-0.28	-0.10
Senescence	-0.25	-0.28
Precipitation	-0.45	-0.01
Temperature	-0.39	0.05
Explained variance (%)	26.7	19.8

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Capítulo 2:

Toward nutrition and protection: anatomical, histochemical and immunocytochemical alterations induced by Cecidomyiidae on *Byrsonima coccolobifolia* leaves

.

Toward nutrition and protection: anatomical, histochemical and immunocytochemical alterations induced by Cecidomyiidae on *Byrsonima coccolobifolia* leaves

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Abstract

Tissue specialization resulting from gall development allows the identification of structural and functional compartments. Host plant leaf tissues are altered in the number of cell layers, the cell elongation axis, and the accumulation of metabolites. The outer tissue compartment accumulates metabolites with nutritive and protective functions, and the inner tissue compartment accumulates energetic molecules involved in the nutrition of the inducer. Using anatomical, histometric, histochemical, and immunocytochemical techniques, we mapped tissue origin and destiny, and evaluated the level of investment in each original tissue layer toward the mature gall. Our results revealed a 92-fold higher investment in the development of gall storage and nutritive tissues,and an approximately 5-fold higher investment in protective tissues compared to the non-galled tissues. By the immunocytochemical techniques, we mapped xyloglucans accumulated as reserve carbohydrates in cell walls. The histochemical techniques allowed the detection of starch

grains accumulated in the protoplasm of the CST cells, and lipids and reducing sugars accumulated in the protoplasm TNT cells. The dynamics of the cell wall components in gall tissues revealed changes involved in the development of gall structure, and in the nutrition and defense of the inducer. We concluded that there is a balance of structural and chemical investment of *B. coccolobifolia* in the formation of specialized tissues for nutrition and protection of the galling Cecidomyiidae, both by the increase in the number of cell layers compared to the non-galled tissues and by the distribution of defensive molecules along the two gall tissue compartments.

Keywords: cell walls, gall anatomy, histochemistry, immunocytochemistry

Introduction

The new organogenesis in gall developmental sites occurs through cell differentiation or re-differentiation (*sensu* Lev-Yadun, 2003), with peculiar changes in tissue specialization, in the axis of cell elongation, and in the accumulation of metabolites both in cell walls and protoplasm (Formiga et al. 2013; Ferreira et al. 2017; Bragança et al. 2020). Tissue specialization allows the identification of structural and functional compartments, with the gall outer tissue compartment commonly composed of the epidermis, the common storage tissue, and the mechanical zone. This outer compartment shares nutritive and protective functions, accumulating both energetic and defensive molecules. The inner tissue compartment is formed by the typical nutritive tissue and accumulates energetic molecules involved in the nutrition of the inducer (Bragança et al. 2017). In comparison to the original non-galled tissues, such compartments go through alterations in the number of cell layers, the axis of cell elongation, and the accumulation of metabolites, whose features indicate the level of investment of each original tissue layer toward the mature gall, and its new functional traits.

Histochemical and immunocytochemical techniques allow the evaluation of the functional traits of the gall tissue compartments, specifically at the level of protoplasm in specific tissue sites of accumulation, and the peculiarities of cell-to-cell traffic of the molecules are determinants for the support of gall structure and of the inducer. The peculiarities of the structural-functional traits at the protoplasm level are indicated by the production and storage of primary and secondary metabolites (Bronner 1992, Hartley 1998, Nyman and Julkunen-Tiitto 2000). While the accumulation of primary metabolites is typical of each gall-inducing taxon and related to its nutritive demands (Bronner 1992; Ferreira et al. 2017; Bragança et al. 2017), the accumulation of secondary metabolites occurs in response to a cascade of metabolic events and is usually related to the protection of the galling herbivore (Abrahamson et al. 1991; Bragança et al. 2017; Kuster et al. 2019) but may have other metabolic involvements. The phenolic compounds, for instance, may minimize the effects of the oxidative burst caused by the ROS in mature galls (Oliveira et al. 2011; Isaias et al. 2015 Czerniewicz et al. 2017; Kmieć et al. 2018). In addition, the phenolic compounds are related to the modulation of the level of auxins causing cell hypertrophy in gall developmental sites (Suzuki et al. 2015; Bedetti et al. 2017; Bragança et al. 2020).

The modification, reorganization, and degradation of cell walls are concomitant to the biosynthesis of their components, creating a physiological carbon sink (Field et al. 1998; Geider et al. 2001), which is as strong as the wider the cell wall remodeling is. Cell wall remodeling in gall tissues is a sophisticated model of study for elucidating structural-functional traits at the apoplast level, which are under the control of the gall inducers but vary in terms of cell division, flexibility, porosity, and adhesion, due to the hydric status of the plant (Konno et al. 2008; Tenhaken 2015). In the cell walls, the elaborate networks of polysaccharides (hemicelluloses and pectins) and glycoproteins are responsible for regulating cell growth and development, communication, and defensive responses, and

imply different levels of cell adhesion, depending on the gall tissue functional specialization (Harris 2005; Bragança et al. 2017). Accordingly, cell wall matrix can be modified, reorganized, and degraded during growth and development (Barnes and Anderson 2018).

The anatomical, histometric, histochemical, and immunocytochemical study of the Cecidomyiidae galls induced on *B. coccolobifolia* aims to map the alterations plant tissue suffers due to gall development. The anatomical plus histometric evaluation of gall cells and tissues will add knowledge to the axis of cell elongation and investment in specialized tissue compartments. The histochemical and immunocytochemical techniques will localize the metabolites in protoplasm and cell walls. All the analyses will follow the purpose of mapping the dynamics of cells and tissues involved in support of the inducer and of the gall structure, as well. As the induction and early development of Cecidomyiidae-induced galls are synchronized with the leaf flushing on *Byrsonima coccolobifolia* and concomitant to the rainy season (Migliorini-Mendes et al. 2023 - submitted), we assume that the host plant does not face the dilemma of growing or defending (Herms and Mattson 1992) from the attack of the galling herbivore. Instead, there will be a balance of *B. coccolobifolia* investment in the formation of specialized tissues, where energetic and defensive molecules will equally accumulate both in the cell walls and protoplasm in gall tissue compartments.

Material and methods

Sampling and collection

Samples of non-galled mature leaves (NGL) (n = 20) and mature galls (MG) (n = 50) were collected from individuals (n = 10) of *B. coccolobifolia* (Fig. 1a) in a Savanna environment on the Limeira farm, Conceição do Pará (19°.45’ 25.3” S, 44° 50’ 01.2” W), state of Minas Gerais, Brazil, between September 2018 and March 2020. Mature galls

(Fig. 1a-b) with the larvae of the galling Cecidomyiidae (Fig. 1c) alive in the larval chamber were selected. This is the main trophic phase of the inducer, and is usually related to the gall final stage of development (Rohfritsch 1992), in which tissue compartments have been established. The conical mature galls are brown, intralaminar, convex on the adaxial surface with the cone-shaped projection on the abaxial surface (Fig. 1b). The samples were stored in plastic bags, transported in a cooler containing ice to the laboratory, where a set of the fresh samples were submitted to histochemical analyses, and a another set of samples were fixed in Karnovsky solution (2.5% glutaraldehyde, 4.5% formaldehyde in 0.1 $mol.l^{-1}$ phosphate buffer, pH 7.2) (Karnovsky 1965) for 48 h at room temperature for structural and immunocytochemical analyses.

Anatomical analyzes

Fixed fragments of the NGL (n = 10) and MG (n = 10) were dehydrated in an n-butyl series (Johansen 1940), embedded in Paraplast (Sigma®) (Kraus and Arduin 1997), and sectioned in a rotatory microtome (12-14 µm) (Leica® Biocut 2035). The sections were deparaffinized in butyl acetate and hydrated in an ethanol series (Kraus and Arduin 1997). After staining with Astra blue-safranin solution (9:1, v/v) (Bukatsch 1972, modified to 0.5%), the sections were dehydrated in an ethanol series and mounted with Acrilex® colorless varnish (Paiva et al. 2006). The Astra blue-safranin combination distinguishes between lignified (stained in red) and non-lignified cellulosic cell walls (stained in blue) (Vazquez-Cooz 2002). The sections were photographed under a light microscope (LEICA® DM500; Leica, Wetzlar, Germany) with a coupled digital camera (Leica ICC50 HD®).

Histometrical analyzes

In the NGL, cuticle thickness on adaxial and abaxial surface, the thickness and cell areas of the adaxial and abaxial epidermis, and the mesophyll thickness were measured (n = 5 leaves; 1 cross section per leaf; 4 sites/cells per tissue in each section, totaling 20 cells per tissue) with AxioVision Release 4.8® software. In the MG, the thickness of the cuticle on the adaxial and abaxial surfaces, the thickness and cell areas of the adaxial and abaxial epidermis, and the thickness of the mechanical zone (MZ), typical nutritive tissue (TNT), and common storage tissue (CST) were measured (n = 5 galls; 1 cross section per gall; 4 sites/cells per tissue in each section, totaling 20 cells per tissue). The histometric data of the NGL and the MG were compared by fitting generalized estimating equation models for each response variable (tissue measurements). For all variables, an exchangeable autocorrelation framework was used. After fitting the models, the mean values and respective 95% confidence intervals were calculated for each group (NGL and MG). Tukey's correction was applied to each test. All statistical analyses were performed using R software version 3.6.1 (R Core Team 2019) using packages geepack (Højsgaard et al. 2006) and emmeans (Lenth 2021).

Histochemical analyses

Handmade sections of fresh samples of NGL (n = 5) and MG (n = 5) were submitted to histochemical tests for the detection and histolocalization of primary and secondary metabolites (Table 1). For all the histochemical tests, blank sections were analyzed as controls. All sections were mounted according to each protocol, and immediately observed and photographed under a light microscope (LEICA® DM500; Leica, Wetzlar, Germany) with a coupled digital camera (Leica ICC50 HD®).

Immunocytochemical analyses

Fixed fragments of the NGL and the MG (n = 3 for each sample) were dehydrated in an ethanol series (Johansen 1940), embedded in Paraplast (Sigma®) (Kraus and Arduin 1997), deparaffinized, and incubated with the monoclonal antibodies (MAbs): LM1, LM19, LM20, LM5, LM6, LM10, and LM15 (Centre of Plant studies, University of Leeds) (Table 2).

For the detection of the epitopes of hemicelluloses, the sections were pre-incubated in pectate lyase at 10 μg/mL, diluted in 50 mM N-cyclohexyl-3-aminopropane sulfonic acid (CAPS) and 2 mM CaCl2 buffer, pH 10, for 2 h at room temperature (Marcus et al. 2008). For the detection of hemicelluloses, pectins, and glycoproteins, the whole set of slides was incubated in 5% powder milk in phosphate-buffered saline (PBS) 0.1 mol L−1, pH 7.2 (w/v), for 30 min, to prevent cross-labeling. Afterward, the slides were incubated in the MAbs diluted in 5% powder milk/PBS (1:10), for 90 min for hemicelluloses, or 120 min for pectins and glycoproteins, in the darkness. The slides were washed in PBS and incubated in the secondary antibody anti-rat IgG linked to FitC, diluted in 5% powder milk/PBS (1:100, v/v), for 90 min, in the darkness. After washing in PBS, all sections were mounted in 50% glycerin kept in darkness, analyzed, and photographed under a fluorescence microscope (Leica® DM 2500 LED), with blue excitation light (450–490 nm) and green emission light (515 nm), coupled to a digital camera (Leica® DFC 7000T). For the control, a set of sections to which the primary antibodies were suppressed was used to manually adjust the microscope and eliminate eventual autofluorescence. The positive labeling was acquired in the sections submitted to the MAbs in strictly the same fluorescence conditions.

The images of immunocytochemical analysis were submitted to intensity measurement using Image J version 1.51k (http://rsb.info.nih.gov/ij). The fluorescence intensities of the epitopes of hemicelluloses, pectins, and glycoproteins were evaluated by grayscale methodology (Gy = Gray value) with triplicate analysis for each tissue.

After the measurements, we categorized the intensities as: (-) negative (= 0 Gy values), (+) weak (<12 Gy values), (++) moderate (12-24 Gy values), and (+++) intense (> 24 Gy values) (Table 3).

Results

Anatomical features of the non-galled mature leaves and mature galls

In the mature leaves, the epidermal cells of the adaxial surface are elongated in the periclinal axis, whereas those of the abaxial surface are anticlinally elongated. Both epidermal surfaces are lined by thin cuticle (0,08 µm). The leaves are hipostomatic with paracytic stomata. The mesophyll is isobilateral with palisade parenchyma (PP) composed of 1-3 anticlinally elongated cell layers on the adaxial surface, and 1-2 anticlinally elongated cell layers on the abaxial surface. The spongy parenchyma (SP) has 4-6 layers isodiametric cells and reduced intercellular spaces (Figure 2a). The vascular system of the midrib vein has collateral arrangement, with the convex region facing the abaxial face (Figure 2b). The minor veins are also collateral and surrounded by a sclerenchymatic sheath (Figure 2a - yellow dotted circle). The mature galls are intralaminar and characterized by hyperplasia and cell hypertrophy of both dermal and ground systems. The outer tissue compartment (OTC) comprises the epidermis, the common storage tissue (CST), and the mechanical zone (MZ), while the inner tissue compartment (ITC) comprises the typical nutritive tissue (TNT) (Figure 2c). Epidermal cells are small cells and surround the gall. The CST and MZ are originated from parenchymatic cells of the NGL. The CST is composed of 15-18 layers of cells, and the MZ has 2-3 layers of lignified cells. Two collateral vascular bundles are immersed in the CST and located one on each side of the larval chamber (Figure 2c). The epidermal cells of the abaxial surface originated 6-7 cell layers of the TNT around of the larval chamber

(Figure 2d-e). The gall escape channel is lined and obliterated by trichomes in the ostiole region (Figure 2f), which is directed toward the leaf abaxial surface.

Histometrical features of the non-galled mature leaves and mature galls

Quantitative changes in the dermal and ground systems occur from the NGL towards the MG. Epidermal cells from adaxial surface in the MG is 7-fold thinner (Figure 3a; $p < 0.001$) than in the NGL. There is no significant difference in cuticle thickness on the epidermis from adaxial surface between NGL and MG (Figure 3b; $p = 0.0442$). The cell area of the epidermis from adaxial surface of the NGL is more than 9-fold larger than in the MG (Figure 3c; $p < 0.001$). Differently, the abaxial epidermal cell area in the NGL is more than 3-fold larger than MG (Figure 3d; $p < 0.001$), the cuticle thickness is 23-fold larger than in the MG than in the NGL (Figure 3e; $p < 0.001$), and the cell area of the epidermis from abaxial surface in the MG is more than 6-fold larger than in the NGL (Figure 3f; $p < 0.001$).

Investment in cell differentiation to form new tissues in the MG revealed that the abaxial epidermis thickness increased by 68-fold to form the TNT compared to epidermis of the NGL (Figure 3g; $p < 0.001$). To form the CST the increase in mesophyll NGL thickness is 24-fold (Figure 3h; $p < 0.001$). The increase in mesophyll thickness for MZ formation is 5-fold greater than the thickness of the NGL mesophyll (Figure 3i; $p < 0.001$).

Histochemical features of the non-galled mature leaves and mature galls

In the NGL, reducing sugars and starch grains are detected in the cells of the PP and SP, as well in the cortical parenchyma of the midrib vein (Figures 4a-b). The cuticle has positive reaction to Sudan red B on cuticles of both surfaces and in the stomata guard cells, and lipid droplets occur in the cytoplasm of ordinary epidermal cells (Figure 4c).

Proteins are detected in the protoplasm of the PP and SP cells, and in the nucleus of the epidermal cells (Figure 4d). Phenolic compounds are detected in the protoplasm of the epidermal cells, PP and SP cells (Figure 4e). Lignins are detected in the cell walls of the tracheal elements and of the pericyclic fibers (Figure 4f).

In the MG, reducing sugars are detected, forming a centripetal gradient of accumulation from the CST cells toward the TNT cells (Figure 4g). Starch grains are accumulated in the CST cells (Figure 4h), and in the TNT cells. Lipids are detected in the cuticle, and as droplets in the cytoplasm of the CST (Figure 4i), and TNT cells. Proteins are detected in the cytoplasm of the CST, sclerenchyma, and TNT cells, as well as in the phloem cells, forming a centripetal gradient (Figure 4j). Phenolic compounds are detected in the cytoplasm of the epidermal, CST, sclerenchyma and TNT cells (Figure 4k). Lignins are detected in the walls of the tracheal elements and of the sclerenchyma cells (Figure 4l).

Immunocytochemical features of the non-galled leaves and galls

In NGL, the epitopes of extensins are not labeled by LM1 in the cell walls of any tissues. The epitopes of (1→4) β-D-galactans are weakly labeled by LM5 in the cell walls of the PP and SP, and of the vessel elements (Figure 5a). The epitopes of the (1→5) α-L-arabinans are weakly labeled by LM6 in the cell walls of the epidermis and of the PP and SP (Figure 5b). The epitopes of homogalacturonans (HGs) methyl esterified are weakly labeled by LM20 in the cell walls of the PP and SP (Figure 5c). The epitopes of unesterified HGs are weakly labeled by LM19 in the cell walls of the epidermis (Figure 5d), PP and SP (Figure 5e), as well as in the homogeneous parenchyma cells in the midrib (Figure 5f). The epitopes of xylans are moderately labeled by LM10 in the walls of the vessel elements in the midrib and in the secondary veins (Figures 5g-h). The epitopes of

xyloglucans are moderately labeled by LM15 in the cell walls of the PP and SP (Figure 5i).

In the MG, the epitopes of extensins labeled by LM1 are weakly labeled in the cell walls of the epidermis, CST, and sclereids in the MZ (Figure 6a). The epitopes of (1→4) β-D-galactans labeled by LM5 are weakly labeled in the medium lamella of xylem cells (Figure 6b). The epitopes of the (1→5) α-L-arabinans are moderately labeled by LM6 in the walls of the sclereids in the MZ (Figure 6c). The epitopes of methyl esterified HGs are moderately labeled by LM20 in the cell walls of the epidermis, CST (Figure 6d), and sclereids in the MZ (Figure 6e). The epitopes of unesterified HGs are moderately labeled by LM19 in the cell walls of the epidermis (Figure 6f), weakly labeled in the cell walls of the CST, and sclereids in the MZ, and intensely labeled in the cell walls of the TNT (Figure 6g). The epitopes of xylans are moderately labeled by LM10 in the walls of sclereids in the MZ, and vessel elements (Figure 6h). The epitopes of xyloglucans are moderately labeled by LM15 in the cell walls of the CST and TNT (Figure 6i).

Discussion

The dynamics of cells and tissues reorganization toward the support of the inducer and of gall structure

The Cecidomyiidae-induced galls on *B. coccolobifolia* have a high investment in the development of the CST and TNT, which accumulate energy resources for supporting the gall life cycle, and the nutrition of the inducer.

In the OTC, the diminutive size of epidermal cells on both surfaces indicates an intense site of hyperplasia, which is confirmed by the 9-fold reduction in cell area and a change from the anticlinal elongation axis in the NGL to the periclinal elongation axis in the galls. The high structural investment in the CST is evidenced by the storage of water and nutrients, which particularly occurs during the rainy season, when the host plant is not under stressful conditions (Patra et al. 2009; Oliveira et al.2013; Costa and Araújo 2019).

The CST is separated from the larval chamber by the mechanical zone, a region of cells with lignified walls, which in the *B. coccolobifolia* galls is constituted of sclereids. The MZ neoformation in gall developmental site evidences an investment of 5-fold more cell differentiation in protective tissues compared to NGL tissues. This relative lower investment in protection may be balanced by the accumulation of phenolic compounds in the protoplasm of the CST and TNT cells. In addition, several layers of small cells form a robust barrier to the penetration of the ovipositor apparatus of possible parasitoids (Gahra et al. 2011; Broski 2013), complementing the protection of the lignified cell layers of the MZ, separating the larval chamber from the OTC.

In ITC, the high investment in the neoformation of tissues involved in the direct nutrition of the galling Cecidomyiidae indicates that the gall is in the maturation phase, and the galling herbivore has a high feeding demand. This characteristic is confirmed by the metabolites stored in the TNT, as well as by the dynamics of HGs, which together support the development both of the inducer and of the gall structure, and will be discussed next.

The detection of metabolites in the protoplasm and cell walls determines gall functional traits

Regarding primary metabolites, the starch grains accumulated in the CST demand the breakdown smaller sugars by the enzymatic activity of invertases and sucrose synthase (Oliveira et al. 2010; Oliveira et al. 2016). Once broken, these sugars may be translocated through the symplast from the CST to the TNT cells, and eventually take part in the construction of the pectins and hemicelluloses of the cell walls of the CST on *B. coccolobifolia* galls.

The cell-to-cell transport system of sugars and other compounds is energy-efficient and can have specific adjustments depending on communication and nutrition

needs, as well as cell type, stage of development, and environmental conditions (Miras et al. 2022). In *B. coccolobifolia*, reducing sugars can be translocated from the CST to the TNT cells by crossing the MZ sclereids through pits in the secondary cell walls and act in the nutrition of the galling Cecidomyiidae. Similar structural and histochemical analyses were reported for *Mimosa gemmulata* galls (Costa et al. 2022), *Lantana camara* (Moura et al. 2008), *Aspidosperma spruceanum* Oliveira et al. 2010), *Marcetia taxifolia* (Ferreira and Isaias, 2014), *Mimosa tenuiflora* (Nogueira et al. 2018) indicating a probable general pattern for cell-to-cell communication and the nutritional processes in galls.

Regarding secondary metabolites, the phenolic compounds are commonly histolocalized in the outer tissue compartment of Cecidomyiidae galls (Carneiro et al. 2014, Isaias et al. 2014), and are often associated with chemical defenses against predatory herbivores. However, in *B. coccolobifolia*, the phenolic compounds accumulated in the protoplasm of the TNT cells may impair the nutrition of the galling Cecidomyiidae, which does not seem to occur and may be related to peculiarities of the insect saliva gut fluid (Martin et al. 1987). In addition, the accumulation of phenolic compounds together with proteins in the TNT cells of mature galls on *B. coccolobifolia* leaves may be related to the dissipation of oxidative stress, minimizing the effects of reactive oxygen species (ROS) burst (Oliveira et al. 2011, Isaias et al. 2015, Czerniewicz et al. 2017), and the production of cell wall modifying enzymes (Cantu et al.2008; Malinovsky et al. 2014; Hansen and Nielsen, 2018), respectively. Accordingly, the lignification of cell walls in *B. coccolobifolia* galls is favored by the accumulation/availability of phenolic compounds in the MZ, and may be associated with the stress generated by the dynamics of HGs in the adjacent TNT cells.

Even though Cecidomyiidae galls usually accumulate carbohydrates in the TNT cells (Bronner 1992, Oliveira et al. 2010, Oliveira et al. 2011), the detection not only of

carbohydrates but also of proteins and lipids in the inner tissue compartment of *B. coccolobifolia* galls indicates a high demand for energetic resources, as pointed out for other insect galls (Carneiro et al. 2017; Costa et al. 2021). The histochemical and immunocytochemical profiles of the Cecidomyiidae galls on *B. coccolobifolia* galls evidences that the protoplasm of all tissue compartments contribute both to defense, due to phenolic accumulation, and to nutrition, due to the accumulation of carbohydrates, proteins and lipids.

Cell wall dynamics determines the functional characteristics of the outer and inner tissue compartments

In the *B. coccolobifolia* galls, the epitopes of (1 → 4) β-D-galactans and (1 → 5) α-L-arabinans labeled in the cell walls of the MZ sclereids confer porosity to the cell walls and favor the transport of nutrients and water from the CST toward the TNT. In addition to the cell-to-cell trafficking of molecules, both the galactans and the arabinans can contribute to the maintenance of mechanical stability (Silva et al. 2021), flexibility (McCartney et al. 2000), and cellular adhesion (O'Donoghue and Sutherland 2012). The moderate labeling of xylans in the walls of the MZ sclereids and of the vessel elements in the vascular bundles confirms their expected association with the lignification of the cell walls (McCartney et al. 2005, Evert 2006; Rennie & Scheller 2014), providing rigidity (Ferreira et al 2019; Bragança et al. 2021; Nogueira et al. 2022). Once de MZ sclereids are live cells, their position lining the inner tissue compartment and the composition of their cell walls do not prevent but, otherwise, favor the translocation of water and nutrients toward the TNT.

The dynamics of HGs reveals the cell wall as a monitoring system to detect threats caused by various invaders (Ridley et al. 2001; Ochoa-Villarreal et al. 2012), that is, the cell walls are not merely a passive defensive barrier but a dynamic and highly controlled

cell component. As the pectins are one of the main components of the cell walls, they are multifunctional and their ability to form gels confers porosity to cell walls (Chanliaud and Gidley 1999; Ridley et al. 2001), also contributing directly to the movement and access of metabolites through the cell wall matrix. In the *B. coccolobifolia* galls, the moderate labeling of HGs in the methyl-esterified state in cell walls of the parenchyma cells of the CST may indicate that the storage cells have reached maturity and low cell division capacity (Willats et al. 2001a, 2001b) as the gall tissues are established, and the inducer has reached its final trophic phase. However, the intense labeling of HGs in the unesterified state in the cell walls of the TNT suggests cell juvenility maintained due to the continuous stimuli of the galling herbivore (Cosgrove 2000; Formiga et al. 2013; Carneiro et al. 2014, 2015).

The weak labeling of the epitopes of extensins in the cell walls of the epidermis and of the CST seems to confirm the capacity of using cell wall in detecting threats caused by various invaders. In the cell walls of the Cecidomyiidae galls on *B. coccolobifolia*, this weak labeling may indicate the inhibition of the host plant ability to deposit more extensins under pathogenic attack (Guo et al. 2010; Castilleux et al. 2020), and may configure a subtle protective resource of the OTC cells. This protective trait, even low, is a mechanism conferred by the extensins that has been similarly evidenced in the rust fungi galls (Basidiomycota: Pucciniales) on *Byrsonima variabilis* leaves (Migliorini Mendes et al. 2023), and here in the insect galls on *B. coccolobifolia* leaves, which may indicate a feature yet to be explored in other *Byrsonima* spp. associated to galling herbivores.

The xyloglucans labeled in cell walls of the TNT in the Cecidomyiidae galls on *B. coccolobifolia* have been previously associated with carbohydrate reserves in other Cecidomyiidae galls induced on *Inga ingoides* (Bragança et al. 2021) and *Mimosa gemmulata* (Costa et al. 2022), for instance. To be accessed by the gall inducers, the xyloglucans must be converted into smaller sugars by the activity of the endoglucanase

(Park et al., 2003) and transglycanases (Franková and Fry 2013; Bragança et al. 2020), and may represent and additional nutritive resource acting in the metabolic support of the gall inducer and gall structure.

Conclusion

The formation of two tissue compartments with distinct specialized functions, and an increase in the number of cell layers is expected for Cecidomyiidae galls. The apparently higher structural investment in the formation of tissues specialized for the nutrition of the inducer compared to the formation of protective specialized tissues is compensated by the accumulation of defensive molecules mainly in the gall inner tissue compartment. Therefore, our hypothesis is corroborated, i.e., there is a balance in the formation of specialized tissues and the accumulation of energetic and defensive molecules. The energetic molecules mapped in this study are xyloglucans accumulated as reserve carbohydrates in cell walls, as starch grains accumulated in the CST cells, and as lipids and reducing sugars accumulated in TNT cells. The transport of reducing sugars and proteins from the CST to the TNT through the MZ is ensured by the dynamics of arabinans and galactans in the cell walls of the sclereids. The energetic molecules accumulated in the protoplasm of the CST and TNT cells, indicating that the favorable water supply of the rainy season positively influences gall development.

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Data availability.

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Conflicts of interest.

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Histochemical tests applied for the detection and histolocalization of primary and secondary metabolites in non-galled leaves and mature galls induced by Cecidomyiidae (Diptera) on *Byrsonima coccolobifolia* A.Juss. (Malpighiaceae).

	Reagents	Metabolites	Positive colors	References
Primary metabolites	Fehling's test	Reduce sugars	Brown precipitates	Sass (1951)
	Sudan red B	Lipids	Red oils	Brundett et al. (1991)
	Lugol's reagent	Starch grains	Purple	Johansen (1940)
	Mercuric bromophenol blue	Total proteins	Blue precipitates	Baker (1958)
Secondary metabolites	Ferric chloride	Polyphenols	Black reaction	Johansen (1940)
	Wiesner's reagent	Lignins	Pink walls	Johansen (1940)

Table 2. Monoclonal antibodies (MAbs) used for the immunocytochemical recognition of the epitopes of hemicelluloses, pectins and proteins.

Class	MAbs	Epitope	References
Glycoproteins	LM1	Extensins	Smallwood et al. (1996), Cassab (1998) Sabba and Lulai (2005) Leroux et al. (2011)
Pectins	LM5	(1→4) β-D-galactans	Jones et al. (1997)
	LM6	(1→5) -α-L-arabinans	Willats et al. (1998)
	LM19	Unesterifed homogalacturanans (HGs)	Vander-bosch et al. (1989), Knox et al. (1990), Willats et al. (2000), Clausen et al. (2004)
	LM20	Esterifed HGs	Knox et al. (1990), Willats et al. (2000), Clausen et al. (2004),
Heminelluloses	LM10	Xylans	McCartney et al. (2005)
	LM15	Xyloglucans	Marcus et al. (2008)

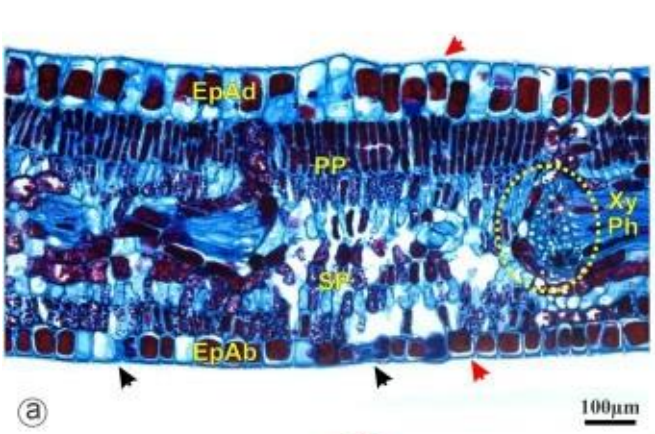
Table 3. Average of gray values and intensity of reactions of the epitopes of hemicelluloses, pectins, and proteins to the monoclonal antibodies for in the tissues of the mature galls on *Byrsonima coccolobifolia* leaves (Malpighiaceae).

	Non-galled leaves				**Galls**				
	Adaxial epidermis cells	**Abaxial epidermis cells**	**Mesophile cells**	**Xylem cells**	**Epidermis cells**	**Common store cells**	**Mechanical zone cells**	**Nutritive cells**	**Xylem cells**
LM1	0 (-)	0 (-)	0 (-)	0 (-)	9,01 (+)	9,85 (+)	11,09 (+)	0 (-)	0 (-)
LM5	0 (-)	0 (-)	11,82 (+)	8,63 (+)	0 (-)	0 (-)	0 (-)	0 (-)	11,1 (+)
LM6	9,28 (+)	0 (-)	8,38 (+)	0 (-)	0 (-)	0 (-)	15,04 (++)	0 (-)	0 (-)
LM19	0 (-)	8,83 (+)	8,24 (+)	7,75 (+)	12,28 (++)	9,04 (+)	7,97 (+)	25,96 (+++)	0 (-)
LM20	0 (-)	0 (-)	8,02 (+)	0 (-)	12,43 (++)	15,55 (++)	8,96 (+)	0 (-)	0 (-)
LM10	0 (-)	0 (-)	0 (-)	19,29 (++)	0 (-)	0 (-)	20,24 (++)	0 (-)	19,63 (++)
LM15	0 (-)	0 (-)	18,78 (++)	0 (-)	0 (-)	19,10 (++)	0 (-)	17,10 (++)	0 (-)

Intensity of reaction: (-) negative (= 0 Gy values), (+) weak (<12 Gy values), (++) moderate (12-24 Gy values), and (+++) intense (>24 Gy values)

Legends

Figure 1 – Conical gall in *Byrsonima coccolobifolia* Kunth. (Malpighiacceae). a- Mature galls on leaves of *B. coccolobifolia*; b – Mature gall in detail; c – larvae of inducer Cecidomyiidae. EpAb: abaxial epidermis, EpAd: adaxial epidermis.



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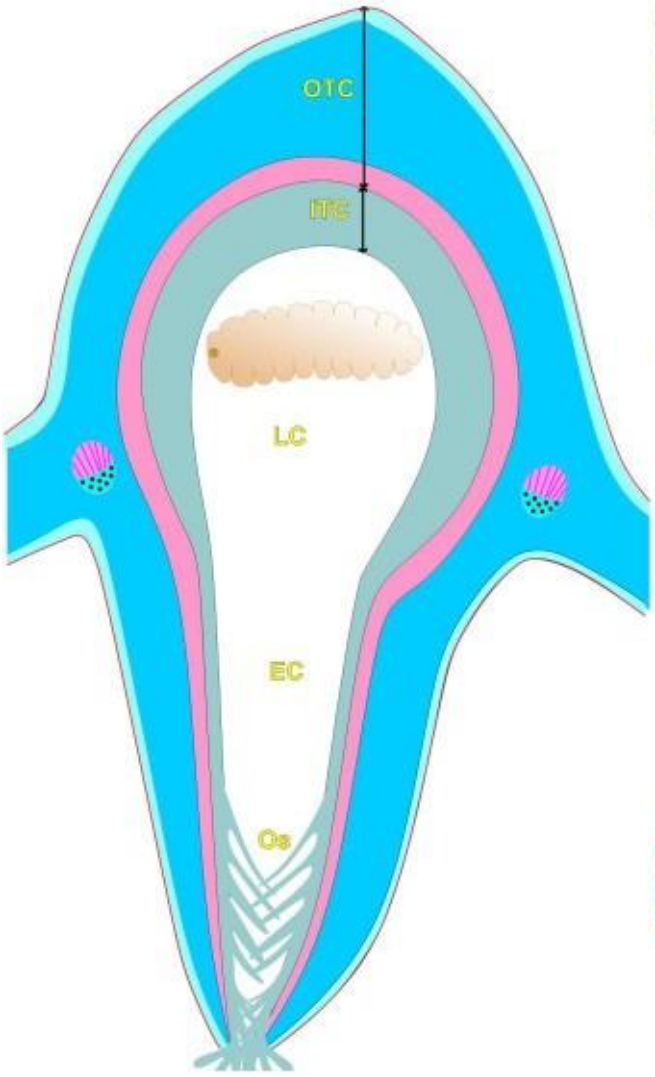

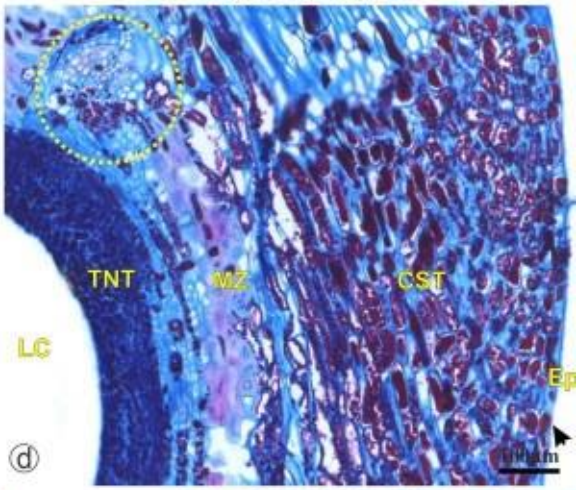

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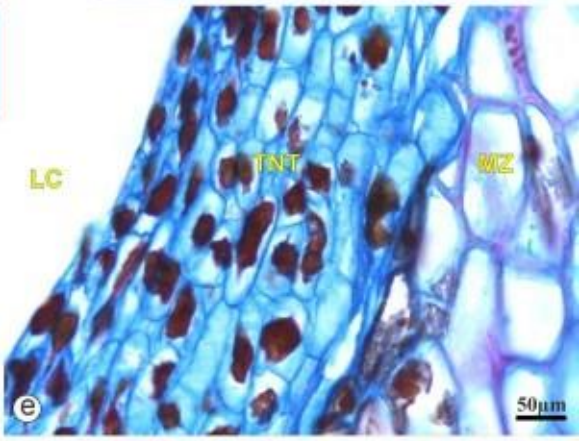

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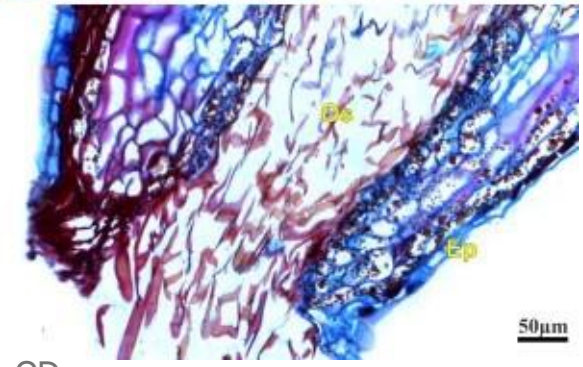


0Epidermis
Dcommon storage tissue
DMechanical zone
0Typical nutritive tissue
cuticle
CiilXylem
Phloem

©

CD

Figure 2 - Structural profiles of leaf and gall associated. a-b: Anatomical profile of *Byrsonima coccolobifolia* non-galled leaf in transverse section. a – non-galled leaf lamina in cross section; b - Collateral arrangement of the vascular system of the midrib. c-f: Anatomical profile of gall associated in transverse section. c - diagram of gall evidencing outer tissue compartment (blue), and mechanical zone (pink), inner tissue compartments (Gray), larval chamber with larva, exit channel (EC) and ostiole (Os) obliterated by trichomes (gray); d- gall in cross section evidencing common storage tissue (CST), mechanical zone (MZ), collateral vascular bundles (yellow dotted circle), and typical nutritive cells (TNT); e – detail of typical nutritive tissue (TNT) and mechanical zone (MZ); f - exit orifice obliterated by trichomes (Oe). EpAb: abaxial epidermis, EpAd: adaxial epidermis, HP: homogenous parenchyma, EC: exit channel, ITC: inner compartment tissue, Os: Ostiole, OTC: outer compartment tissue, LC: larval chamber, PP: palisade parenchyma, Ph: phloem, SP: spongy parenchyma, Xy: xylem. Bars: (a, b, d) 100 µm; (e, f) 50 µm.

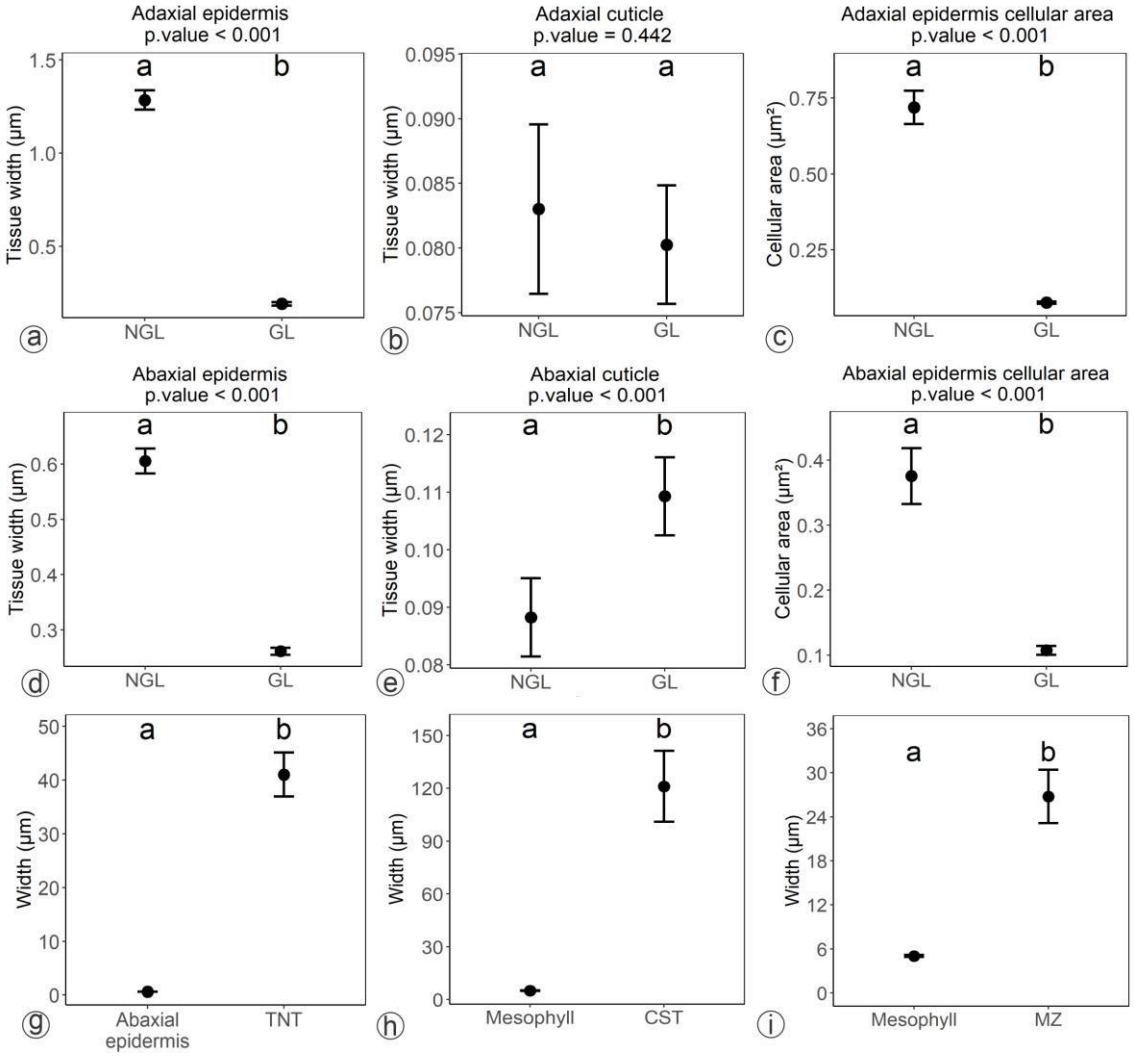
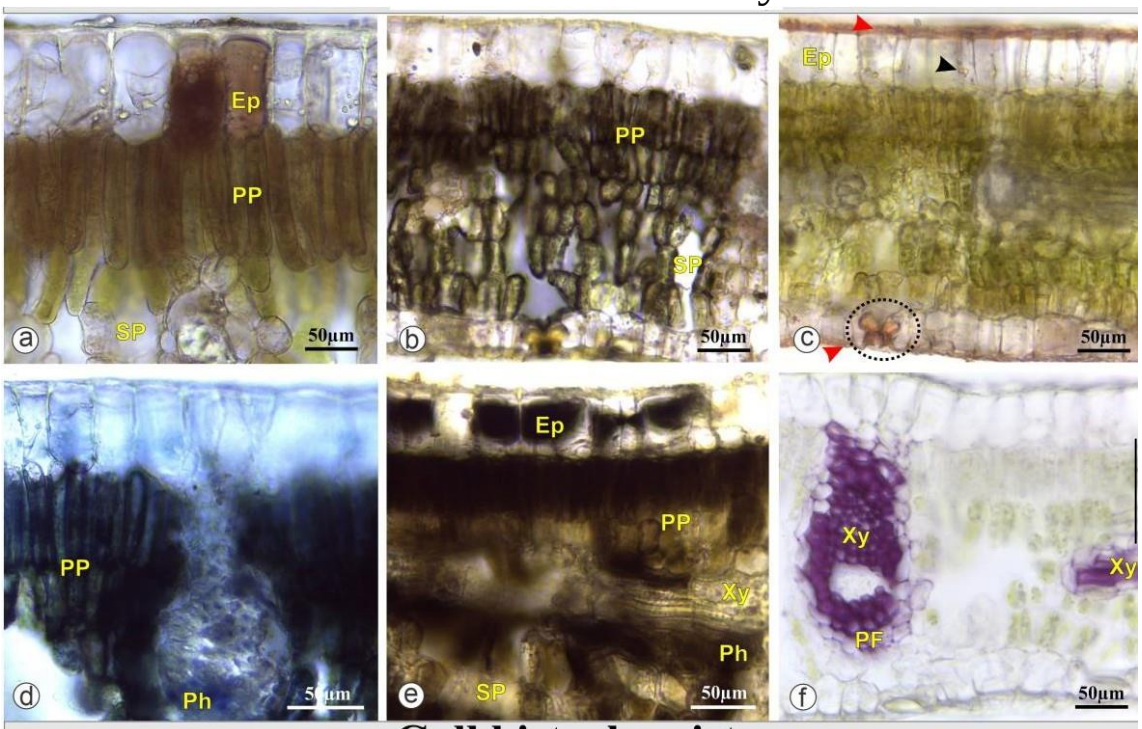


Figure 3 - Cytometry and histometry of non-galled leaves (NGL) and galls (GL): a- Adaxial epidermal thickness in NGL and GL; b - Adaxial cuticle thickness in NGL and GL; c- Cell area of the adaxial epidermis in NGL and GL; d - Abaxial epidermal thickness in NGL and GL; e - Abaxial cuticle thickness in NGL and GL; f- Cell area of the abaxial epidermis in NGL and GL; g-i: comparative investment between NGL and GL tissues. g – abaxial epidermis and typical nutritive tissue (TNT); h – Mesophyll and common storage tissue (CST); i – mesophyll and mechanical zone (MZ) Same letters indicate statistically equal values for the same variable, and different letters indicate different values.

Leaf histochemistry



Gall histochemistry

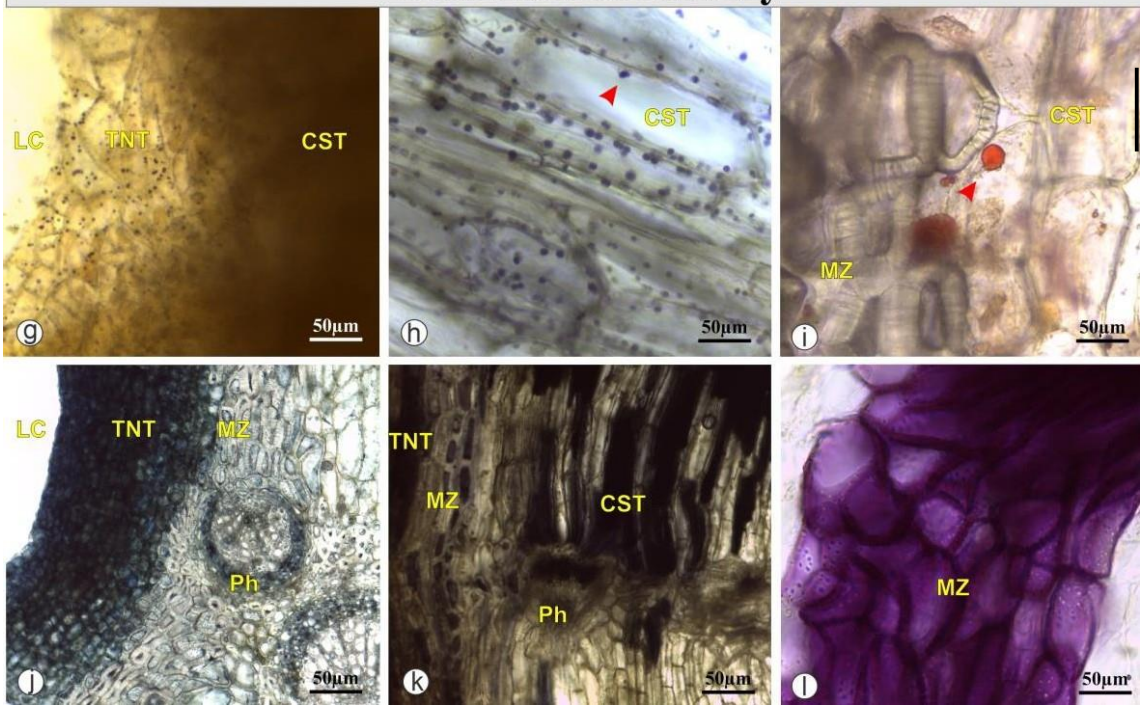


Figure 4 - Histochemical profiles of the non-galled leaf and conical gall on *Byrsonima coccolobifolia* Kunth (Malpighiaceae) leaf in transverse sections. a-f: non-galled leaf. a - reducing sugars detected in the cells of the palisade parenchyma (PP) and spongy parenchyma (SP); b - starch grains detected in the cells of the palisade parenchyma (PP) and spongy parenchyma (SP); c - lipophilic substances detected in the cuticle on the adaxial and abaxial epidermis (red arrow head), in the stomata guard cells (black dotted circle), and as droplets in ordinary cells of the epidermis (black arrow head); d - Proteins detected in the protoplasm of the palisade parenchyma (PP) and spongy parenchyma (SP) cells; e - Phenolic compounds detected in the protoplasm of the epidermal cells (Ep), palisade parenchyma (PP) and spongy parenchyma (SP) cells, and of the perivascular fibers; f - Lignins detected in the cell walls of the tracheal elements and of the pericyclic fibers; g-l: Gall. g - reducing sugars detected in the common storage tissue (CST) and typical nutritive tissue (TNT) cells; h - Starch grains detected in the common storage tissue (CST) cells; i - Lipophilic substances detected in the cuticle, and as droplets (red arrowhead) in the protoplasm of the common storage tissue (CST); j - proteins detected in the protoplasm of the common storage tissue (CST), sclerenchyma, typical nutritive tissue (TNT) cells, and in the phloem (Ph) cells; k - phenolic compounds detected in the common storage tissue (CST), sclerenchyma, and typical nutritive tissue (TNT); l - lignins detected in the walls of the tracheal elements and of the sclerenchyma cells in the mechanical zone (MZ). EpAd: adaxial epidermis, LC: larval chamber, PP: palisade parenchyma, PF: perivascular fibers, Ph: phloem, SP: spongy parenchyma, Xy: xylem. Bars: (a - l) 50 µm.

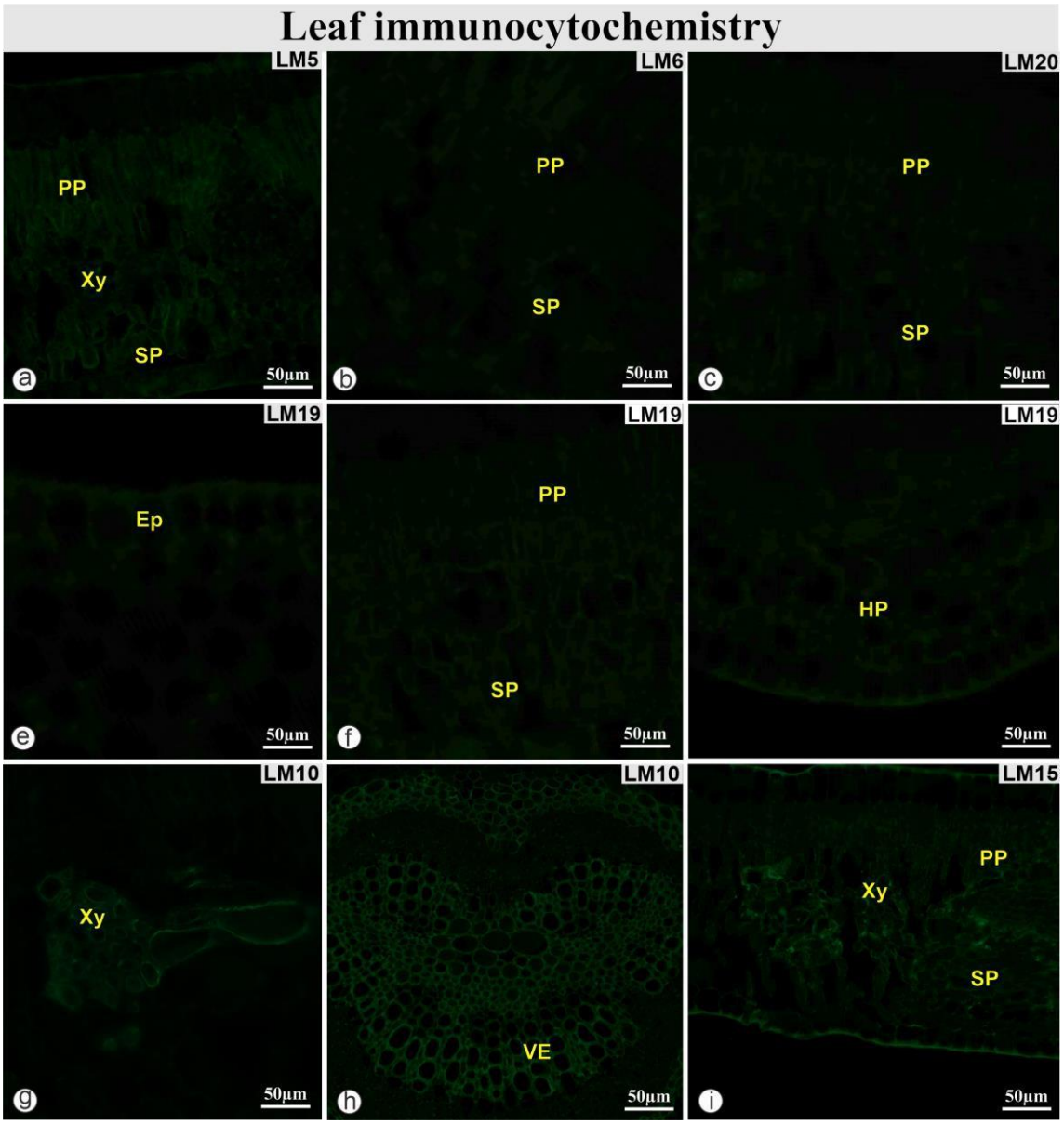


Figure 5 - Immunocytochemical profiles of the non-galled leaf of the *Byrsonima coccolobifolia* Kunth (Malpighiaceae) leaf in transverse section. a- (1→4) β-D-galactans labeled by LM5 in the cell walls of the palisade parenchyma (PP) and spongy parenchyma (SP), and of the vessel elements; b - (1→5) α-L-arabinans labeled by LM6 in the cell walls of the epidermis and palisade parenchyma (PP) and spongy parenchyma (SP); c - methyl esterified HGAs labeled by LM20 in the cell walls of the palisade parenchyma (PP) and spongy parenchyma (SP); d - unesterified HGAs labeled by LM19 in the cell walls of the epidermis (Ep); e - unesterified HGAs labeled by LM19 in the cell walls of the palisade parenchyma (PP) and spongy parenchyma (SP); f - unesterified HGAs labeled by LM19 in the homogeneous parenchyma (HP) cells in the midrib; g - xylans labeled by LM10 in the walls of the vessel elements in the midrib; h - xylans labeled by LM10 in the walls of the secondary veins; i - xyloglucans labeled by LM15 in the cell walls of the palisade parenchyma (PP) and spongy parenchyma (SP). EpAd: adaxial epidermis, PP: palisade parenchyma, Ph: phloem, SP: spongy parenchyma, VE: vessel elements, Xy: xylem. Bars: (a - i) 50 µm.

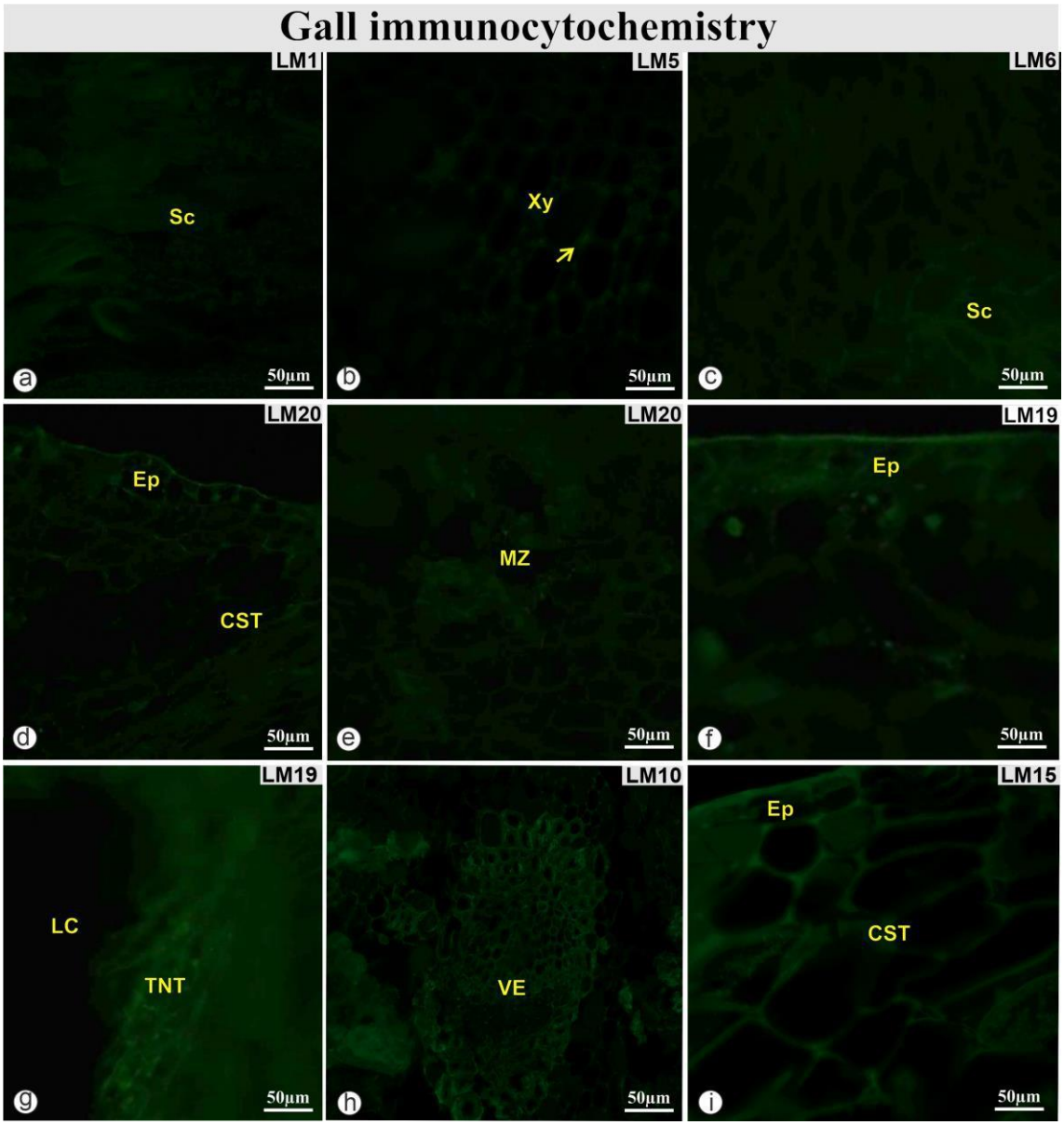


Figure 6 - Immunocytochemical profiles of the conical gall on *Byrsonima coccolobifolia* Kunth (Malpighiaceae) leaf in transverse section. a - extensins labeled by LM1 in the cell walls of the epidermis (EpAd), common storage tissue (CST), and sclereids in the mechanical zone (MZ); b - (1→4) β-D-galactans labeled by LM5 in the medium lamella of the xylem (yellow arrow); c - (1→5) α-L-arabinans labeled by LM6 in the walls of the sclereids (Sc) in the mechanical zone (MZ); d - methyl esterified HGAs labeled by LM20 in the cell walls of the epidermis, common storage tissue (CST); e - methyl esterified HGAs labeled by LM20 sclereids in the mechanical zone (MZ); f - unesterified HGAs labeled by LM19 in the cell walls of the epidermis and of the common storage tissue (CST); g - unesterified HGAs labeled by LM19 in the cell walls of the TNT; h - xylans labeled by LM10 in the walls vessel elements; i - xyloglucans labeled by LM15 in the cell walls of the common storage tissue (CST) and typical nutritive tissue (TNT). EpAd: adaxial epidermis, LC: larval chamber, MZ: mechanical zone, Sc: sclereids, VE: vessel elements, Xy: xylem. Bars: (a - i) 50 µm.

Capítulo 3:

Dynamics of cell wall components and histochemical profile of a rust fungi gall (Basidiomycota: Pucciniales) on *Byrsonima variabilis* A.Juss. (Malpighiaceae)

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Dynamics of cell wall components and histochemical profile of a rust fungi gall (Basidiomycota: Pucciniales) on *Byrsonima variabilis* A.Juss. (Malpighiaceae)

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ABSTRACT

Context. An obligate biotrophic parasitism with a rust fungus led to gall formation on *Byrsonima variabilis*. **Aims.** The hypothesis that the host leaf–rust fungi interaction alters the dynamics of plant cell walls and the histochemical profile toward favouring the plant cell-to-fungi cell translocation of metabolites is tested. **Methods.** Gall samples were sectioned and submitted to anatomical, histometric, histochemical, and immunocytochemical techniques to evaluate structural alterations and the detection of primary and secondary metabolites, as well as the epitopes of glycoproteins, pectins, and hemicelluloses. **Key results.** Fungi gall development results in the hypertrophy of the stomatal chamber and the hyperplasia of epidermis and spongy parenchyma. The cell-to-cell translocation of metabolites from plant mesophyll cells toward the rust fungi gall is favoured by the epitopes of homogalacturonans (HGs) and (1 → 5) α-L-arabinans detected in the hyphae passage sites in the pycnial and aecial stages. The arabinogalactan-proteins (AGPs) may favour mycelial nutrition and differentiation, and cell wall adhesion. HGs and arabinans confer porosity to mesophyll cell walls, which favours the traffic of molecules toward the rust fungi gall. **Conclusions.** The unexpected labelling of AGPs, HGs, and arabinans in fungi cell walls is a novelty regarding the plant–fungi interaction. The primary metabolites detected in rust fungi support hyphae growth and spore maturation. **Implications.** The immunolabelling of host plant cell wall components on fungi cell walls indicates the integrative role of some plant cell wall components in the biological process of pathogen colonisation in leaf tissues.

Keywords: AGPs, gall anatomy, histochemistry, histometry, immunocytochemistry, pectins, plant–fungi interaction.

Introduction

The rust fungi (Basidiomycota: Pucciniales) represent the most diverse, environmentally and economically important, group of plant pathogenic fungi (Toome-Heller 2016; Aime *et al.* 2018). They are obligate biotrophic pathogens that do not produce fruit bodies but erupt like sores on the surface of their host plants (Kendrick 2001; Mendgen and Hahn 2002). The rust fungi may have up to five spore stages, which may alternate between haploid and dikaryotic nuclear conditions on two unrelated host plants (Singh 1969; Petersen 1974; Meyer 1987) or may have complex life cycles, and be specialised to specific hosts (Kolmer *et al.* 2009; Lorrain *et al.* 2019). The rust fungi must feed, grow, and reproduce inside the tissues of living host plants, where they differentiate specific infection structures called haustoria. The haustoria are necessary to establish intimate interactions with the infected host tissues (Chen *et al.* 2014; Polonio *et al.* 2020), and in the case of the rust fungi associated with *Byrsonima variabilis* A.Juss. (Malpighiaceae) (Coelho *et al.* 2013; Araújo *et al.* 2014; Guimarães *et al.* 2014) they intersperse with leaf cells through the adaxial leaf surface toward mesophyll. Rust fungi of the genera *Crossopsora*, *Aecidium* (Carvalho Júnior *et al.* 2008), and *Crossopsorella* (Souza *et al.* 2018)

induce galls on other species of the genus *Byrsonima*, namely *B. coccolobifolia* (Carvalho Júnior *et al.* 2008; Souza *et al.* 2018), *B. verbascifolia*, *B. laxiflora*, *B. crassa*, *B. pachyphylla* (Souza *et al.* 2018), *B. intermedia*, and *B. crassifolia* (Carvalho Júnior *et al.* 2008). The rust fungi galls are induced by the germination of basidiospores, which produce small, hemi- spherical sori called pycnia with pycnospores. These pycnospores germinate and originate dikaryotic hyphae, which form a mycelium responsible for the formation of a cup-shaped sorus, the aecium, with aeciospores (Health 1997; Kendrick 2001; Quilliam and Shattock 2003; Kolmer *et al.* 2009; Lorrain *et al.* 2019). The aeciospores are responsible for the infection of other susceptible sites in the same or in an alternative host plant (Nilsson 1983), from where they are dispersed by wind and germinate (Kolmer *et al.* 2009; Lorrain *et al.* 2019). The aecium is surrounded by the peridium, a tissue that limits the mass of aeciospores (Berndt and Uhlmann 2006). All of the rust fungi life cycle stages described above are peculiar to rust fungi galls (Health 1997; Kendrick 2001; Mendgen and Hahn 2002; Quilliam and Shattock 2003; Berndt and Uhlmann 2006; Kolmer *et al.* 2009; Cooper *et al.* 2016; Lorrain *et al.* 2019), and were also observed in *Byrsonima variabilis*, the current study model. Fungi cell walls differ from plant cell walls by the presence of chitin rather than cellulose, although both substances play the same role in preserving the overall cell wall integrity and osmotic stability (Bowman and Free 2006). In common with plants, the fungi cell wall is composed of homo- and heteropolymers of polysaccharides and proteins that are often associated with one or more of the polysac- charide constituents of the host plant cell wall (Ullah *et al.* 2021), such as hemicelluloses, pectins, and glycoproteins.

The hemicelluloses (Bragança *et al.* 2020; Costa *et al.* 2021), pectins, and some glycoproteins have been related to cell wall remodelling processes in insect (Formiga *et al.* 2013), nematode, and acari galls (Ferreira *et al.* 2020; Silva *et al.* 2021). On fungus cell walls, glucans are the main structural polysaccharide reported (Bowman and Free 2006; Ullah *et al.* 2021). However, as far as we are concerned, the structural and functional implications of the interactions of the fungi–host plant cell wall polysaccharides are not deeply explored.

Fungi-induced galls are more frequent on eudicots, where the site of induction is preferably the leaf (Mani 1964). In these neoplasms, the tissues may differ from those of the host leaf, and cell hypertrophy, hyperplasia, histological deviations, such as the disappearance of intercellular spaces, absence of sclerenchyma due to non-lignification of the cell walls, and differentiation of collenchyma cells may occur (Mani 1964). The developmental alterations are particularly dependent on the formation of the haustoria that plays a key role in fungi establishment, nutrient uptake, and effector delivery (Polonio *et al.* 2020), crucial for the maintenance of a close physiological relationship of the fungus with the host plant cells and tissues (see Voegele and Mendgen 2003).

We assume that alterations in plant cell wall composition in areas adjacent to the fungi gall establishment may favour the traffic of molecules toward the gall developmental site, which may also result in peculiarities in the histochemical profile of the host leaf tissues and rust fungi structures. To test this hypothesis, the dynamics of cell wall components and the histolocalisation of some primary and secondary metabolites in host leaf tissues and in the structures of rust fungi galls induced by Pucciniales on *B. variabilis* were analysed. We assessed (1) how the rust fungi install in *B. variabilis* leaf tissues, (2) the main anatomical responses of the host leaf tissues to fungi establishment, (3) the cell wall dynamics in the light of the translocation of metabolites toward the rust fungi gall, and (4) the histochemical profiles of the rust fungi gall and of the adjacent host leaf tissues.

Material and methods

Sampling and fixation

Mature non-galled leaves (NG) ($n \geq 18$) and rust fungi galls (RG) ($n \geq 18$) were sampled from vegetative branches of *B. variabilis* ($n = 5$) (Fig. 1*a*) at Parque Estadual do Itacolomi (20°26′04.3″S 43°30′37.4″W), Ouro Preto municipality, Minas Gerais state, Brazil. The voucher specimens of *B. variabilis* were deposited in the Herbarium of the Universidade Federal de Minas Gerais Collection, under the registration number BHCB-144184.

Fragments (2 mm^2) of NG and RG were fixed in 2.5% glutaraldehyde and 4.5% formaldehyde in 0.1 mol L^{-1} (Karnovsky 1965, modified to pH 7.2 phosphate buffer) for 48 h at room temperature, and were used for anatomical and immunocytochemical analyses.

Anatomical and histometric analyses

A first set of fixed NG ($n = 10$) and RG ($n = 10$) fragments were dehydrated in an *n*-butyl series (Johansen 1940), embedded in Paraplast (Sigma®) (Kraus and Arduin 1997), and sectioned in a rotatory microtome (12–14 μm) (Leica® Biocut 2035). The sections were deparaffinised in butyl acetate and hydrated in an ethanol series (Kraus and Arduin 1997). After staining with Astra blue–safranin solution (9:1, v/v) (Bukatsch 1972, modified to 0.5%), the sections were dehydrated in an ethanol series and mounted with Acrilex® colourless varnish (Paiva *et al.* 2006). The Astra blue– safranin combination distinguishes between lignified (stained in red) and non-lignified cellulosic cell walls (stained in blue) (Vazquez-Cooz and Meyer 2002).

A second set of RG fragments ($n = 5$) were dehydrated in an ethanol series, embedded in glycolmethacrylate Leica® Historesin (Kraus and Arduin 1997), and sectioned in the rotatory microtome (5–7 μm). The sections were stained

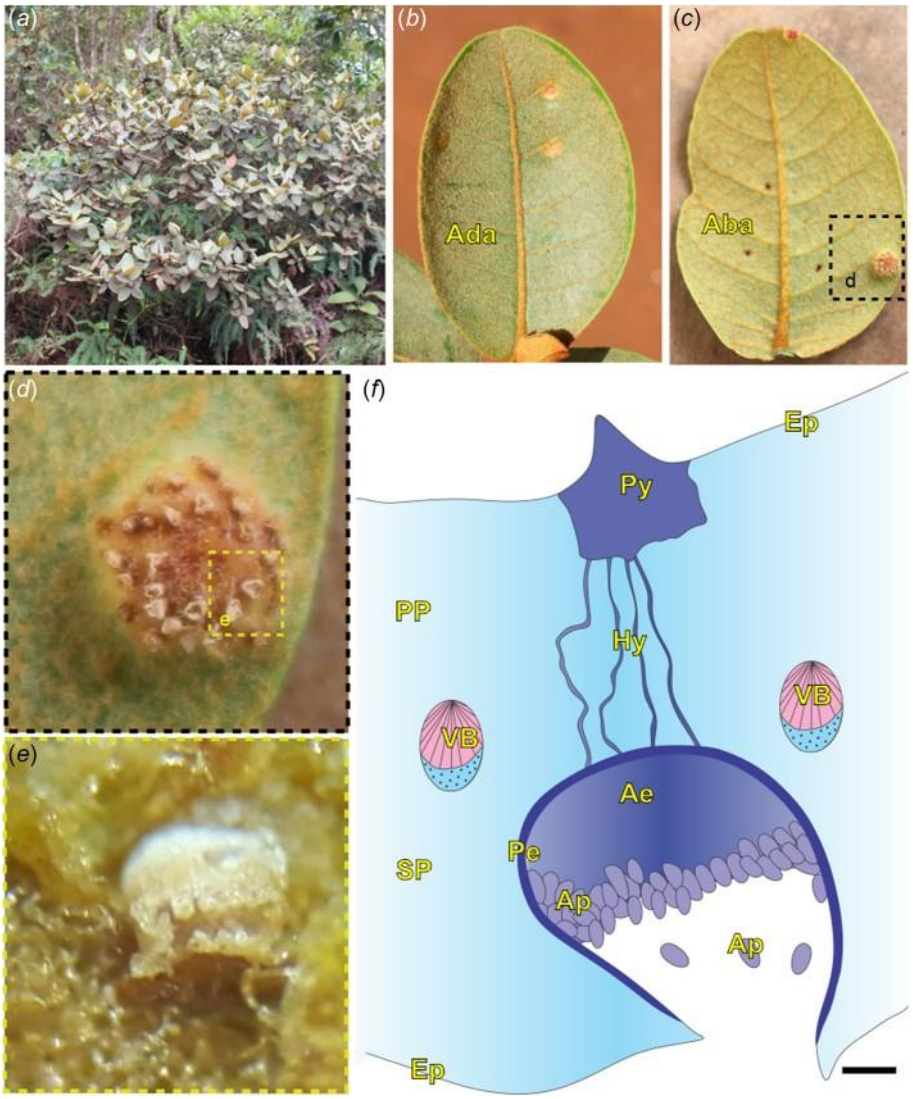

Fig. 1. *Byrsonima variabilis* (Malpighiaceae) with rust fungi galls induced by Pucciniales (Basidiomycota): (*a*) Individual of *B. variabilis*; (*b*, *c*) galled leaves. (*b*) Rust fungi galls projected to the adaxial leaf surface (Ada); (*c*) Rust fungi galls projected to the abaxial leaf surface (Aba). (*d*) Detail of the lenticular gall on the abaxial surface, evidencing several aecium. (e) Detail of the aecium. (*f*) Diagram of a rust fungi gall in transverse section. The pycnium (Py) install in the adaxial epidermis (Ep) and the hyphae (Hy) grow interspersed to mesophyll (palisade PP and spongy SP parenchyma). The vascular bundles (VB) have non-altered xylem and phloem. The peridium (Pe) limits the aecium (Ae) with their aeciospores (Ap) in the level of spongy parenchyma cells. Bar: 200 μm.

with 5% cotton blue in lactophenol (phenol/glycerol/lactic acid/distilled water) for 20 min, followed by safranin for 5 min, and washed in distilled water three times (1 min each) (Marques *et al*. 2013). The hyphae stained in blue, and the plant cell walls stained in red. The histological sections were analysed and photographed under a light microscope (LEICA® DM500; Leica, Wetzlar, Germany) with a coupled digital camera (LEICA® ICC50 HD; Leica, Wetzlar, Germany). The thickness of the cuticle, on the adaxial and abaxial leaf surfaces and of the mesophyll; the areas of the epidermal cells on the adaxial and abaxial leaf surfaces, and of the palisade parenchyma were measured (n = 10 galls; 1 transverse section per gall; 4 cells per tissue in each section, totalling 40 cells per tissue) with the AxioVision Release 4.8® software. The histometric data of the NG and the RG were compared by adjusting generalised estimation equation models for each response variable (cell and tissue measurements). This type of model has the flexibility to handle distinct probability distributions and it is suitable for the design of repeated measurements, where the same subject was measured at

different points in time (Guimarães and Hirakata 2012). For all variables, an exchangeable autocorrelation structure was used. After adjusting the models, the mean values and the respective 95% confidence intervals were calculated for each group (NG and RG). Tukey's correction was applied to each test. All statistical analyses were performed using software R version 3.6.1 (R Core Team 2019) using packages geepack (Højsgaard *et al*. 2006) and emmeans (Lenth 2021).

Immunocytochemical analysis

A third set of fixed fragments of the NG and the RG (n = 3 different leaves and three different galls) were dehydrated in an ethanol series (Johansen 1940), embedded in Paraplast (Sigma®) (Kraus and Arduin 1997), deparaffinised, and incubated with the monoclonal antibodies (MAbs): LM1, LM2, LM5, LM6, LM19, LM20, LM10, LM15, and LM21 (Centre of Plant Studies, University of Leeds) (Table 1).

For the detection of the epitopes of hemicelluloses, the slides were pre-incubated in pectate lyase at 10 µg mL^{-1}, diluted in 50 mM N-cyclohexyl-3-aminopropane sulfonic acid (CAPS) and 2 mM $CaCl_2$ buffer, pH 10, for 2 h at room temperature (Marcus *et al*. 2008). For the detection of hemicelluloses, pectins, and glycoproteins, the whole set of slides was incubated in 5% powdered milk in phosphate-buffered saline (PBS) 0.1 mol L^{-1}, pH 7.2 (w/v), for 30 min, to prevent cross-labelling. Afterward, the slides were incubated in the MAbs diluted in 5% powdered milk/PBS (1:10), for 90 min for hemicelluloses, or 120 min for pectins and glycoproteins, in the darkness. The slides were washed in PBS and incubated in the secondary antibody anti-rat IgG linked to FITC (fluorescein isothiocyanate), diluted in 5% powdered milk/PBS (1:100, v/v), for 90 min, in the darkness. After washing in PBS, all sections were mounted in 50% glycerin kept in darkness, analysed, and photographed under a fluorescence microscope (Leica® DM 2500 LED), with blue excitation light (450–490 nm) and green emission light (515 nm), coupled to a digital camera (Leica® DFC 7000T). For the control, a set of sections to which the primary antibodies were suppressed were used to manually adjust the microscope, and eliminate eventual autofluorescence. The positive labelling was acquired in the sections submitted to the MAbs in strictly the same fluorescence conditions.

The images of immunocytochemical analysis were submitted to intensity measurement using Image J version 1.51k (http://rsb.info.nih.gov/ij). The fluorescence intensities of the epitopes of hemicelluloses, pectins, and glycoproteins were evaluated by greyscale methodology (Gy = Grey value) with triplicate analysis for each tissue. After the measure- ments, we categorised the intensities as: (−) negative (=0 Gy values), (+) weak (<10 Gy values), (++) moderate (10– 18 Gy values), and (+++) intense (≥19 Gy values).

Table 1. Monoclonal antibodies (MAbs) used for the immunocytochemical recognition of the epitopes of hemicelluloses, pectins and glycoproteins.

Classes	MAbs	Epitope	References
Glycoproteins	LM1	Extensins	Smallwood *et al.* (1996); Cassab (1998); Sabba and Lulai (2005), Leroux *et al.* (2011)
	LM2	Arabinogalactan-proteins	Yates *et al.* (1996); Smallwood *et al.* (1996)
Pectins	LM5	(1 → 4) β-D-galactans	Jones *et al.* (1997)
	LM6	(1 → 5) – α-L-arabinans	Willats *et al.* (1998)
	LM19	Unesterifed homogalacturonans (HGs)	Vanden-Bosch *et al.* (1989); Knox *et al.* (1990); Willats *et al.* (2000); Clausen *et al.* (2004)
	LM20	Esterifed HGs	Knox *et al.* (1990); Willats *et al.* (2000); Clausen *et al.* (2004)
Hemicelluloses	LM10	Xylans	McCartney *et al.* (2005)
	LM15	Xyloglucans	Marcus *et al.* (2008)
	LM21	Heteromannans	Marcus *et al.* (2010)

Histochemical analyses

Handmade sections of fresh samples of the NG (n = 5) and the RG (n = 5) were submitted to histochemical tests for the detection and histolocalisation of primary and secondary metabolites (Table 2). For all the histochemical tests, blank sections were analysed as controls. All sections were mounted in water, observed, and immediately photographed under a light microscope (LEICA® DM500; Leica, Wetzlar, Germany) with a coupled digital camera (Leica ICC50 HD®).

Results

Gall structural aspects and fungi installation in leaf tissues

The RG on *B. variabilis* are intralaminar, lenticular, and protrude to the adaxial leaf surface (Ada) as an orange spot (Fig. 1*b*), and as a brown spot on the abaxial leaf surface (Aba) (Fig. 1*c*, *d*). The RG are induced in internervural regions (Fig. 1*c*), and fungi installation occurs through the adaxial leaf surface. The germination of the pycniospores goes toward the mesophyll cells, where the hyphae grow among the cells of the palisade and the spongy parenchyma, forming the aecium with aeciospores (Fig. 1*d*–*f*).

Table 2. Histochemical tests applied for the detection and histolocalisation of primary and secondary metabolites in non-galled leaves and rust galls induced by Pucciniales (*Basidiomycota*) on *Byrsonima variabilis* (Malpighiaceae).

	Reagents	Metabolites	Positive colours	References
Primary metabolites	Fehling's reagent	Reducing sugars	Brown	Sass (1951)
	Sudan red B	Lipids	Red	Brundrett *et al.* (1991)
	Lugol's reagent	Starch	Purple	Johansen (1940)
	Best Carmine	Glycogen	Pink	Mallory (1961)
	Bromophenol blue	Total proteins	Blue	Baker (1958)
Secondary metabolites	Ferric chloride	Polyphenols	Black	Johansen (1940)
	Wiesner's reagent	Lignins	Pink	Johansen (1940)

Responses of the host plant tissues to RG installation

The epidermal ordinary cells of *B. variabilis* are anticlinally elongated on the adaxial surface, and periclinally elongated on the abaxial surface. The stomata (St) occur exclusively on the abaxial epidermis and have external ridges emerging from the cuticular thickening. The mesophyll of the NG has a 2–3-layered palisade parenchyma, and an 8–10-layered spongy parenchyma (Fig. 2*a*). Crystals occur in mesophyll cells. The midrib has two arches of xylem and phloem in collateral arrangement organised around a parenchymatic pith. The vascular bundles (VB) of the minor veins also have collateral arrangement, and are immersed in the mesophyll (Fig. 2*b*).

The RG is originated by the germination of basidiospores on the adaxial leaf surface leading to the degradation of the epidermal (Ep) cells in the region of pycnium (Py) installation, and separation of the palisade parenchyma (PP) cells (Fig. 2*c*); the vascular system remains unaltered. The mesophyll (Me) of the RG remains with 2–3-layered palisade parenchyma, and the spongy parenchyma (SP) increases to 16–19 layers of hypotrophic cells with sinuous walls. The pycnium is a sorus that shelters pycniospores (Fig. 2*c*). The pycniospores germinate originating the hyphae (Hy) that grow within the apoplastic pathways of mesophyll cells (Fig. 2*d*). The hyphae grow toward the stomatal chambers on the abaxial surface, where the aecium (Ae) with the aeciospores (Ap) installs (Fig. 2*e–f*). The stomatal chambers are visibly larger due to the formation of the aecia inside them (Fig. 2*e*), and the 1–2 layers of the peridium (Pe) are anticlinally elongated and limit the aecium, whose aeciospores are organised in chains (Fig. 2*f–g*). The basal aeciospores of these chains have evident nuclei and intense divisions (Fig. 2*h*), while the distal aeciospores are differ- entiated with verrucous ornamentation (Fig. 2*i*), and ready for dispersion.

Quantitative alterations in the dermal and ground tissue systems from the NG to the RG condition occur. The cuticle is thinner in the RG than in the NG, both on the adaxial (Fig. 3*a*) and the abaxial (Fig. 3*b*) surfaces. The epidermal cell area reduces 70% from NG to RG on the adaxial surface (Fig. 3*c*); and on the abaxial surface, the reduction in epidermal cell is of 50% from NG to RG (Fig. 3*d*). The mesophyll has an increase of 12% in the thickness from NG to RG (Fig. 3*e*), but there are no significant differences between the palisade parenchyma cell area in NG and RG (Fig. 3*f*).

Immunocytochemical profiles

In the NG (Table 3; Fig. 4), the epitopes of glycoproteins, pectins, and hemicelluloses were immunolabelled in all tissues, but with different intensities (Fig. 4*a*). The epitopes of extensins are moderately labelled by LM1 in the cell walls of the palisade parenchyma (Fig. 4*b*), while the epitopes of arabinogalactan-proteins (AGPs) are weakly labelled by LM2 in the cell walls of the palisade and spongy parenchyma (Fig. 4*c*). The epitopes of (1 → 4) β-D-galactans are moderately labelled by LM5 (Fig. 4*d*) in the cell walls of the palisade and spongy parenchyma, and the epitopes of (1 → 5) α-L-arabinans are moderately labelled by LM6 (Fig. 4*e*) in cell walls of the palisade and spongy parenchyma and vascular bundle cells. The epitopes of unesterified homogalacturonans (HGs), and the epitopes of esterified HGs are moderately labelled by LM19 (Fig. 4*f*) and LM20 (Fig. 4*g*), respectively, in the cell walls of the palisade parenchyma. The epitopes of xylans are moderately labelled by LM10 in the cell walls of the xylem (Fig. 4*h*), while the epitopes of xyloglucans are strongly labelled by LM15 in the cell walls of the parenchyma and xylem (Fig. 4*i*). The epitopes of heteromannans are not labelled by LM21.

Unexpectedly, the epitopes of plant cell wall components were labelled by the MAbs in fungi cell walls (AGPs, HGs and (1 → 5) α-L-arabinans) (Fig. 5*a*). In the RG (Table 3; Fig. 5), the epitopes of extensins are weakly labelled by LM1 only in plant cell walls at the region of pycnia installation (Fig. 5*b*), where the epitopes of the AGPs are moderately labelled by LM2 (Fig. 5*c*). The epitopes of AGPs are strongly labelled in the cell walls at the installation sites of the peridium (Fig. 5*d*), the aecium, and the aeciospores (Fig. 5*e*, *d*). The epitopes of (1 → 4) β-D-galactans are moderately labelled by LM5 in the cell walls of the pycnia

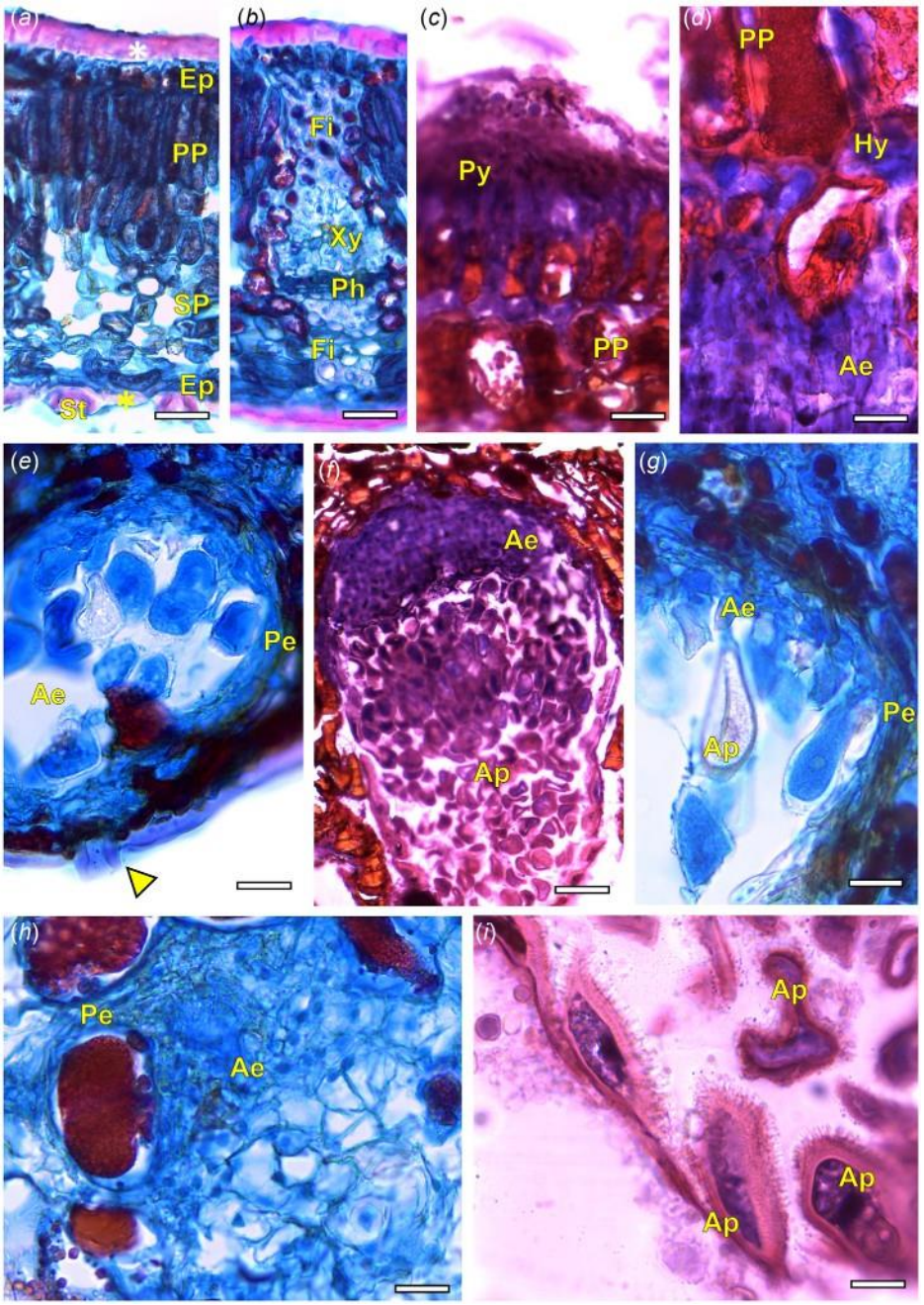

Fig. 2. Anatomical profile of non-galled leaves and rust fungi galls induced by Pucciniales (Basidiomycota) on *Byrsonima variabilis* (Malpighiaceae). (*a*, *b*) Non-galled leaf; (*a*) Mesophyll of non-galled leaves evidencing epidermis (Ep) with thick cuticle (asterisk), palisade (PP) and spongy parenchyma (SP), and stomata (St) on the abaxial surface; (*b*) Secondary vein evidencing xylem (Xy), phloem (Ph), and perivascular fibres (Fi); (*c*–*i*) Rust galls; (*c*) The pycnium (Py) install in the adaxial epidermis; (*d*) Detail of hyphae (Hy) growing in apoplastic pathways of parenchyma cells and formed aecium (Ae); (e) Detail of the aecium in hypertrophied stomatal chamber (yellow arrowhead in stomata) surrounded by the peridium (Pe); (*f*) Aecium with aeciospores (Ap); (*g*) Detail of peridium (Pe) lining the aecium with aeciospores; (*h*) Detail of intense divisions in aecium with aeciospores in development; (*i*) Distal aeciospores differentiated and verrucous ornamentation. (*a*–*b*, e, *g*–*h*) staining with Astra blue–safranin (9:1, v/v); (*c*–*i*) staining with cotton blue and safranin. Bars: 200 μm (*a*-*b*); 50 μm (*c*–*g*); 20 μm (*c*–*d*, *f*, *i*).

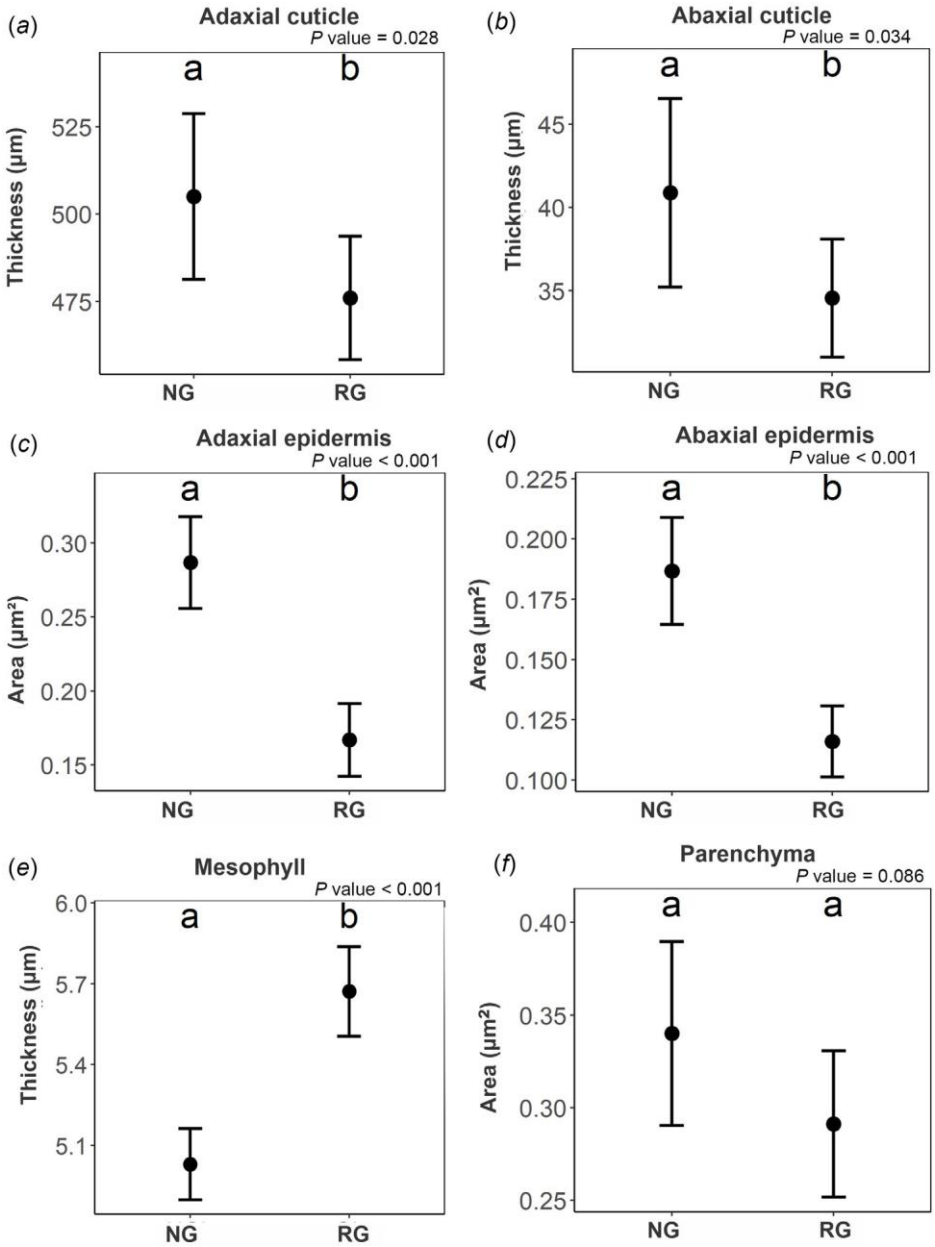

Fig. 3. Cytometry and histometry of mature non-galled leaves and rust fungi galls induced by Pucciniales (Basidiomycota) on *Byrsonima variabilis* (Malpighiaceae). (*a*) Thickness of adaxial cuticle on non-galled leaves (NG) and rust galls (RG). (*b*) Thickness of abaxial cuticle on NG and RG. (*c*) Cell area of adaxial epidermis on NG and RG. (*d*) Cell area of abaxial epidermis on NG and RG. (*e*) Mesophyll thickness on NG and RG. (*f*) Cell area of palisade parenchyma on NG and RG; $P < 0.05$. Same letters indicate statistically equal values for the same variable, and different letters indicate different values.

(Fig. 5*f*), of the peridium (Fig. 5*g*), and of the aecia (Fig. 5*h*). The epitopes of (1 → 5) α-L-arabinans are weakly labelled by LM6 in the pycnia cell walls (Fig. 5*i*), and strongly labelled in the cell walls of the peridium (Fig. 5*j*), and of the aecia (Fig. 5*k*). The epitopes of unesterified HGs are weakly labelled by LM19 in the cell walls of the pycnia (Fig. 5*l*), and strongly labelled in the cell walls of the peridium (Fig. 5*m*). The epitopes of esterified HGs are weakly labelled by LM20 in the cell walls of the pycnia (Fig. 5*n*) and moderately labelled in the aeciospores (Fig. 5*o*). The epitopes of xyloglucans are moderately labelled by LM15 in the peridium cell walls, and weakly labelled in the cell walls of the aecia (Fig. 5*p*). The epitopes of heteromannans and xylans are not labelled by LM10 and LM21, respectively.

Table 3. Average of grey values and intensity of reactions of the epitopes of hemicelluloses, pectins, and glycoproteins to the monoclonal antibodies, in the tissues of the rust galls on *Byrsonima variabilis* leaves (Malpighiaceae).

	NG			RG			
	Ep	Me	VB	Ep	Py	Pe	Ae
Extensins	0 (−)	14.88 (++)	0 (−)	0 (−)	7.09 (+)	0 (−)	0 (−)
Arabinogalactan-proteins	0 (−)	4.76 (+)	0 (−)	0 (−)	13.56 (++)	22.76 (+++)	22.64 (+++)
(1 → 4) β-D-galactans	0 (−)	10.93 (++)	0 (−)	0 (−)	11.79 (++)	16.84 (++)	12.17 (++)
(1 → 5) α-L-Arabinans	0 (−)	10.17 (++)	16.67 (++)	0 (−)	9.28 (+)	20.02 (+++)	27.97 (+++)
Unesterified homogalacturonans (HGs)	0 (−)	11.22 (++)	0 (−)	0 (−)	4.1 (+)	19.78 (+++)	0 (−)
Esterified HGs	0 (−)	10.94 (++)	0 (−)	0 (−)	7.47 (+)	0 (−)	11.87 (++)
Xyloglucans	0 (−)	0 (−)	28.58 (+++)	0 (−)	0 (−)	11.17 (++)	6.06 (+)
Xylans	0 (−)	0 (−)	15.98 (++)	0 (−)	0 (−)	0 (−)	0 (−)
Hereromannans	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)

Intensity of reaction: (−) negative (=0 Gy values), (+) weak (<10 Gy values), (++) moderate (10–18 Gy values), and (+++) intense (≥19 Gy values).

Histochemical profiles of NG and RG

In the NG, total proteins are detected in the cells of the palisade and spongy parenchyma (Fig. 6*a*). Lipids constitute the cuticle and occur as oil droplets in the cytoplasm of the parenchyma cells (Fig. 6*b*). Starch grains (Fig. 6*c*) and reducing sugars (Fig. 6*d*) are detected in the cells of the palisade and spongy parenchyma. Phenolic compounds occur in the vacuoles of the epidermal and mesophyll cells (Fig. 6*e*); and lignins (Fig. 6*f*) are detected in the walls of the vessel elements and of the perivascular fibres of the midrib, and secondary veins.

In the RG, total proteins are detected in the cytoplasm of the pycnium and their pycniospores (Fig. 7*a*), in the fungi hyphae installed in the apoplastic pathways (Fig. 7*b*), in the aecium (Fig. 7*c*), and in the nuclei of the aeciospores (Fig. 7*d*, *e*). Lipids constitute the cuticle and are detected as oil droplets in the cytoplasm of the parenchyma cells, of the pycnium and their pycniospores, in the peridium of the aecium, and in the aeciospores (Fig. 7*f*, *g*). Reducing sugars are detected in the mesophyll and peridium cells (Fig. 7*h*). Starch grains are not detected. Glycogen is detected in the peridium, and in the aeciospores (Fig. 7*i*). Phenolic compounds are detected in the vacuoles of the epidermal cells, and in the vacuoles and walls of the parenchyma cells (Fig. 7*j*). The lignins are detected in the walls of the vessel elements, and of the perivascular fibres (Fig. 7*k*).

Discussion

The labelling of plant cell wall components in the structures of the rust fungi was unexpected but favoured the translocation of molecules toward gall developmental sites. The main changes in cell wall components of the host plant and the rust fungi in the infection process were the increased immunolabelling of (1 → 5) α-L-arabinans, and the moderate labelling of epitopes of extensins in cell walls of the palisade parenchyma adjacent to the fungi. Regarding the histochemical profile, the accumulation of starch and reducing sugars was impaired in the gall developmental site, where glycogen and proteins were detected, and related to the metabolic support for gall installation, growth, and development.

Implications of gall installation for plant leaf tissues

The dorsiventral pattern of *B. variabilis* leaf lamina is not altered by the RG installation, but the aecium development involves the passageway of hyphae imposing mechanical force through the apoplast of parenchyma cells, as reported for *Phakopsora pachyrhizi* in soybean leaves (Edwards and Blond 2011), for example. The growth strategy of the rust fungi associated with *B. variabilis* results in the development of large intercellular spaces, in the decreased communication between plant cells, and in the hypertrophy of the stomatal chamber, as well as in the hypotrophy of spongy parenchyma cells located below the aecia installation and around the peridium.

The Pucciniales produce a wide range of effector proteins that support their ability to successfully digest the cuticle and colonise their host tissues toward obtaining nutritional benefits, as observed in rust fungi in leaves of beans and soybeans (Petre *et al*. 2014; Cooper *et al*. 2016). This mechanism of colonisation may occur in the establish- ment of the pycnia with their pycniospores in the RG on *B. variabilis*, as there is a difference in cuticle thickness just in the region where the pycnium is installed. The cuticle chemical properties and thickness constitute the first barrier surpassed by fungal penetration (Kolattukudy *et al*. 1987), and do not result in any other anatomical alteration (Heller 2020). However, the hypotrophy of *B. variabilis* epidermal cells indicates cell divisions at the site of spore germination,

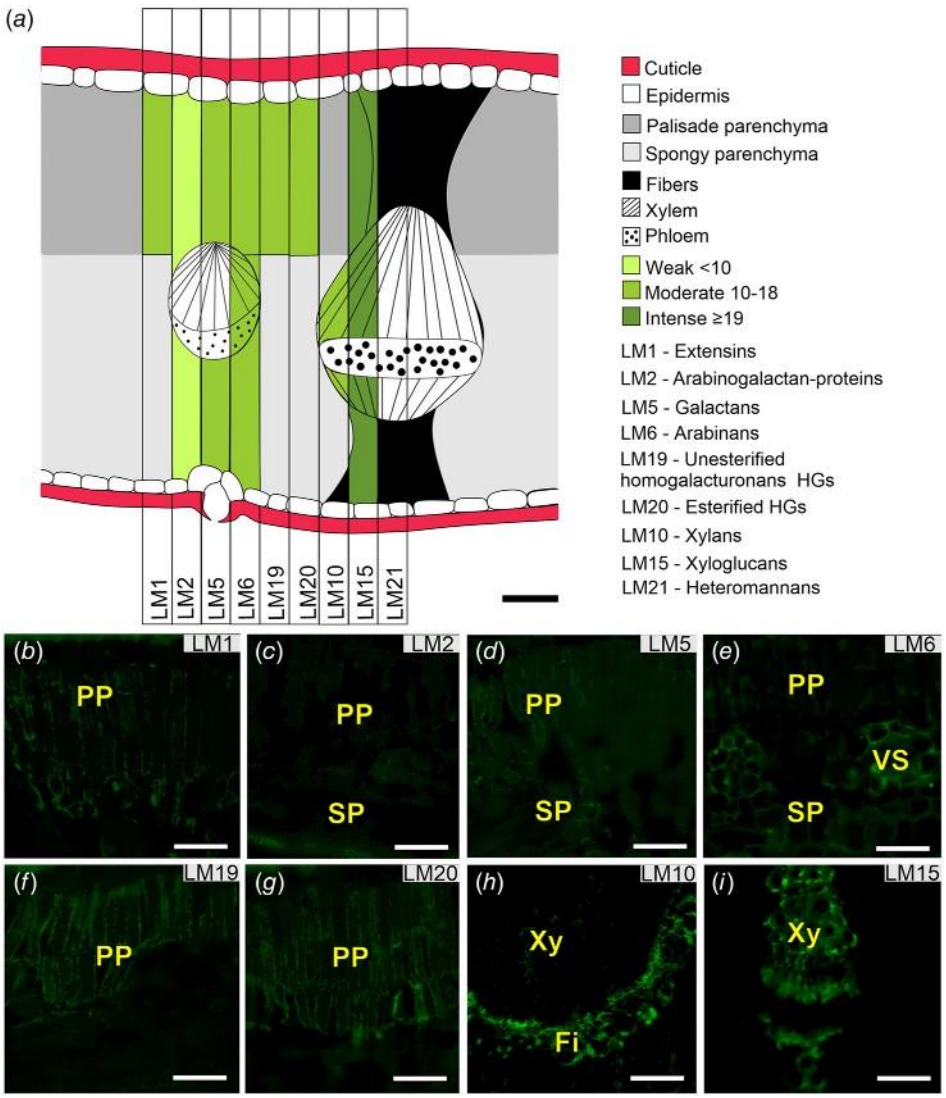

Fig. 4. Immunocytochemical profiles of proteins, pectins and hemicelluloses in the cell walls of non-galled leaves of *Byrsonima variabilis* (Malpighiaceae). (*a*) Schematic depicting the immunolabels of antibodies and their respective epitopes. Grey scale labels indicate palisade (dark grey) and spongy (light grey) parenchyma, vascular bundle sheath (grey), and cuticle (pink). Perpendicular bars indicate positive labelling – dark green: intense, green: moderate and light green: weak. (*b–c*) Glycoproteins. (*b*) Extensins detected by LM1. (*c*) Arabinogalactan-proteins detected by LM2. (*d–g*). Pectins. (*d*) (1 → 4) β-D-galactans detected by LM5. (e) (1 → 5) α-L-arabinans detected by LM6. (*f*) Unesterified homogalacturonans (HGs) detected by LM19. (*g*) Esterified HGs detected by LM20. (*h–i*) Hemicelluloses. (*h*) Xylans detected by LM10. (*i*) Xyloglucans detected by LM15. Heteromannans undetected by LM21. Fi: fibres, PP: Palisade parenchyma, SP: Spongy parenchyma, VS: vascular system, Xy: Xylem. Bars: 200 µm (*a*); 50 µm (*b–i*).

has not been associated with the interaction of rust fungi and host plant before.

The hyphae developed from the pycniospores and their growth through the mesophyll apoplast dissolves the middle lamella (Heller 2020), increases the intercellular spaces, which consequently, decreases cell-to-cell communication, which may prevent the triggering of defence responses in host plant. Even though the palisade parenchyma cells are the second line of contact along the fungus installation, the there is no evidence of defensive responses (structural or histochemical) in these tissue layers, but changes in the chemical composition of cell walls occur, such as increased immunolabelling of (1 → 5) α-L-arabinans, in addition to the maintenance of immunolabelling of extensins, discussed in more detail in the next topic. The formation of the the aecium and the development of the aeciospores imply the

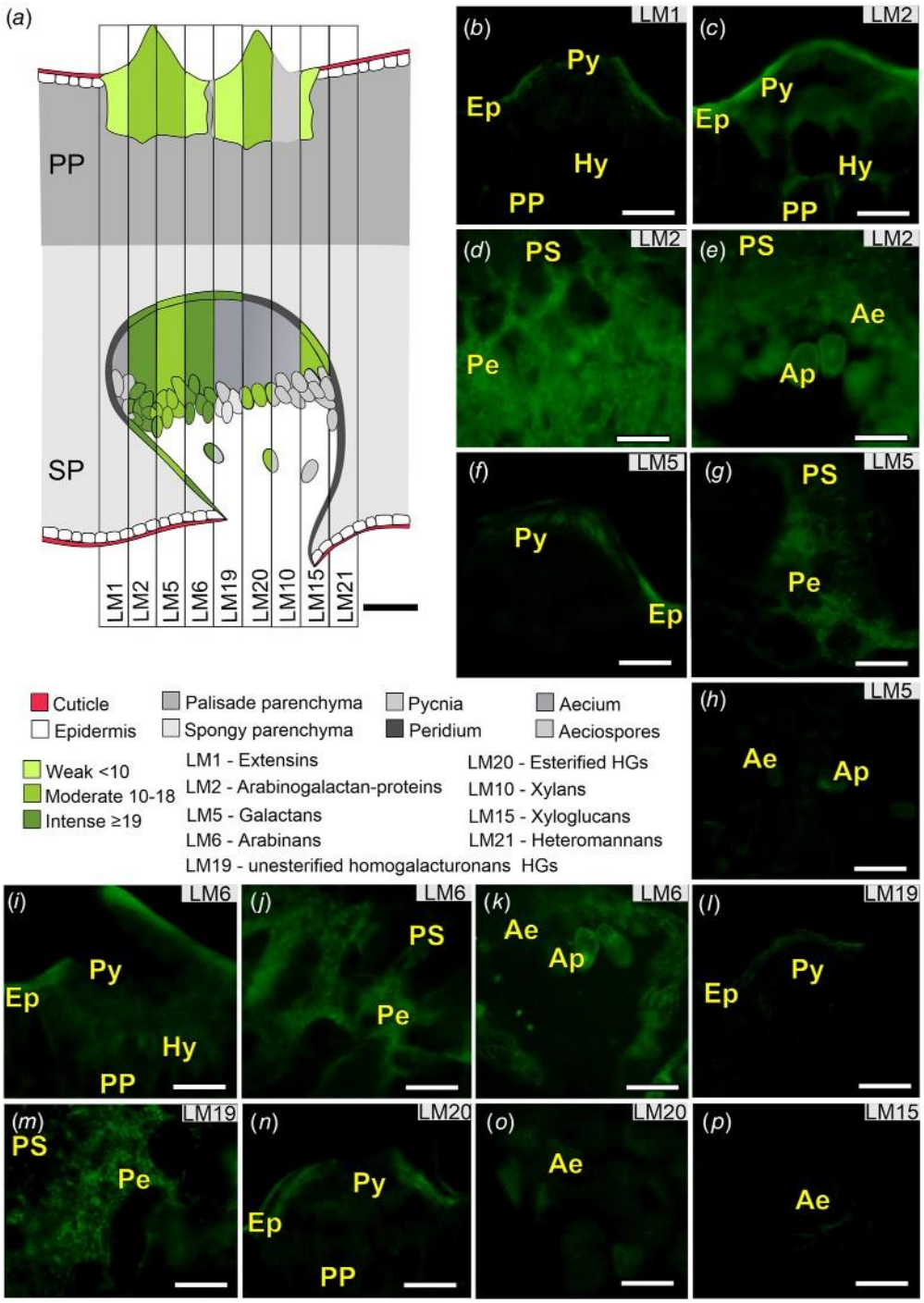

Fig. 5. Immunocytochemical profiles of glycoproteins, pectins, and hemicelluloses in rust fungi galls induced on *Byrsonima variabilis* (Malpighiaceae). (*a*) Schematic depicting the immunolabels of antibodies and their respective epitopes. Greyscale labels indicate palisade (PP) (dark grey) and spongy (SP) (light grey) parenchyma, and cuticle (pink). Perpendicular bars indicate positive labelling – dark green: intense, green: moderate and light green: weak. (*b*–*e*) Glycoproteins. (*b*) Extensins in pycnia (Py) detected by LM1. (*c*–*e*) Arabinogalactan-proteins (AGPs) detected by LM2. (*c*) in pycnia, (*d*) in peridium (Pe), (*e*) in aecium (Ae) and aeciospores (Ap). (*f*–*o*). Pectins. (*f*–*h*) (1 → 4) β-D-galactans detected by LM5. (*f*) in pycnia, (*g*) in peridium, (*h*) in aecium and aeciospores. (*i*–*k*) (1 → 5) α-L-arabinans detected by LM6. (*i*) in pycnia and hypha (Hy), (*j*) in peridium, (*k*) in aecium and aeciospores. (*l*–*m*). Unesterified HGs detected by LM19. (*l*) in pycnia, (*m*) in peridium. (*n*–*o*) Esterified HGs detected by LM20. (*n*) in pycnia, (*o*) in aecium. (*p*) Xyloglucans detected by LM15 in aecium. Xylans detected by LM10. Heteromannans detected by LM21. Bars: 200 μm (*a*); 50 μm (*b*–*p*).

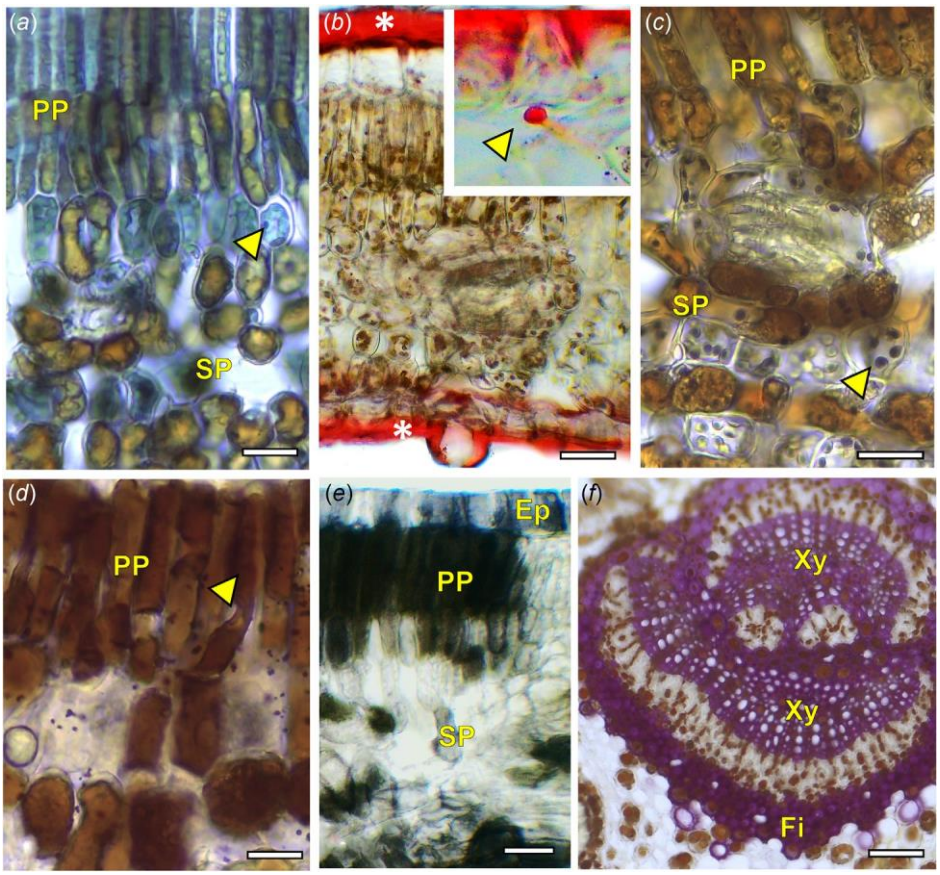

Fig. 6. Histochemical profile of non-galled leaves of *Byrsonima variabilis* (Malpighiaceae). (*a*) Total Proteins (yellow arrowhead) in the cytoplasm of the palisade (PP) and spongy (SP) parenchyma cells; (b) Lipids in the cuticle (asterisk) and parenchyma cells (detail of lipidic droplet – yellow arrowhead); (c) Starch grains (yellow arrowhead) in palisade and spongy parenchyma cells; (*d*) Reducing sugars in palisade parenchyma cells (brownish vacuole content – yellow arrowhead); (*e*) Phenolic compounds in the vacuoles of epidermal (Ep) and parenchyma cells; (*f*) Lignins in xylem (Xy) and perivascular fibres cell walls (Fi). Bars: 200 μm (*b*, *e*); 50 μm (*a*, *c*–*d*, *f*).

hyperplasia of the spongy parenchyma, as well as the hypertrophy of the stomatal chamber, where the aecium is established, as expected for the Pucciniales (Kolmer *et al*. 2009; Heller 2020). The spongy parenchyma cells in the RG are smaller than the spongy parenchyma cells in the NG, and have sinuous walls, which indicate of sites of hyperplasia in galls (Oliveira and Isaias 2010; Oliveira *et al*. 2011).

Cell wall dynamics and RG functional traits

Most of the major cell wall components of fungal pathogens are not represented in plants, and therefore the immune system of plants has evolved to recognise many of the conserved elements of fungal walls (Gow *et al*. 2017). In common with plants, the fungi cell wall is composed of homo- and heteropolymers of polysaccharides and proteins that are often associated with one or more of the polysaccharide constituents of the host plant cell wall (Ullah *et al*. 2021). In most fungal species the inner cell wall consists of a core attached branched β-(1,3) glucan (Latgé 2007). The outer layers of fungi vary much more than the inner skeletal layer (Gow *et al*. 2017).

The unexpected labelling of plant cell wall components (AGPs, HGs and (1 → 5) α-L-arabinans) in rust fungi structures is herein interpreted as a result of the integrative interaction of the fungi with plant cells toward its installation and gall development. The moderate labelling of the epitopes of extensins by LM1 in the palisade parenchyma cell walls indicates a defensive response of plant cell walls to the RG installation. The same results were reported for these epitopes during colonisation of the *Phytophthora parasitica* on *Arabidopsis thaliana* (Castilleux *et al*. 2020). Plant- parasitic fungi modify the morphology of their hyphae depending on the structural and physiological characteristics of the host organ after germination (De Bary 1866). Obligate biotrophic fungi develop their infection structures, exhibit limited secretory activity, especially of lytic enzymes, maintain carbohydrate- and protein-rich interfacial layers

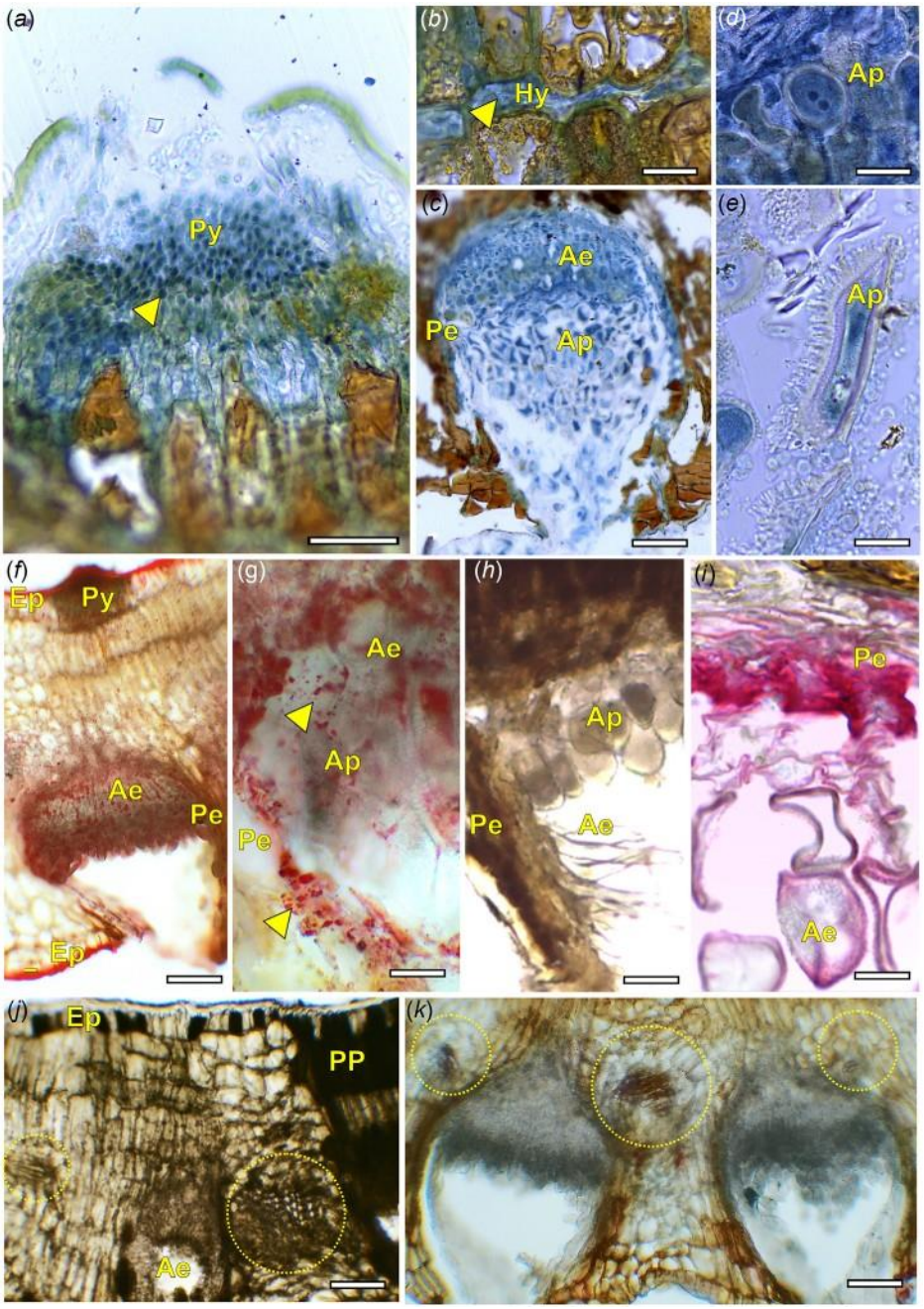

Fig. 7. Histochemical profile of rust fungi galls induced by Pucciniales (Basidiomycota) on leaves of *Byrsonima variabilis* (Malpighiaceae): (*a*–e). Total proteins detected in the cytoplasm of (*a*) pycnium (Py), and pycniospores (yellow arrowhead); (*b*) hyphae (Hy) (yellow arrowhead); (*c*) aecium (Ae); (d) nuclei of the aeciospores (Ap), and (e) differentiated aeciospore; (*f*) Lipids detected in the cuticle, and in the cytoplasm of the parenchyma cells, pycnium, peridium (Pe), and aeciospores; (*g*) Detail of aecium evidencing lipids (yellow arrowhead); (*h*) Reducing sugars detected in the peridium and aeciospores; (*i*) glycogen detected in the peridium, and aeciospores; (*j*) Phenolic compounds detected in the vacuoles of epidermal (Ep) and parenchyma cells, and cell walls of xylem (yellow dotted line). Lignins detected in cell walls of xylem (yellow dotted line). Bars: 200 µm (*c*, *f*, *j*, *k*); 50 µm (*a*, *g*–*h*); 20 µm (*b*, *d*, e, *i*).

that separate fungal and plant plasma membranes, sustain long-term suppression of host defence, and grow haustoria for nutrient uptake and metabolism (Mendgen and Hahn 2002). The weak labelling of the epitopes of extensins is

related to the first phase of RG installation, and suggests the ability of the fungus to overcome the plant's expected defence mechanisms of depositing more extensins under pathogen attacks (Castilleux *et al.* 2020). After the pycnia installation, hyphae development through the host leaf apoplast demands the digestion of the middle lamella, which is composed of pectins. The pectins together with the AGPs reinforce plant cell walls (Tan and Mort 2020) but does not limit fungus colonisation in *B. variabilis* leaves in the present study. The moderate labelling of the epitopes of AGPs by LM2 in the region of the pycnia, and their intense labelling in the region of the peridium and in the cells of the aecium and aeciospores indicate the involvement of AGPs in cell wall adhesion in vascular plants (Cosgrove 1997; Mastroberti and Mariath 2008), but they may also be involved in the mycelium nutrition and differentiation (Pennell and Roberts 1990), and in cell divisions in vascular plants (Willats and Knox 1996; Ma *et al.* 2018; Su and Higashiyama 2018) for formation of aeciospores. Another possible relationship of the intense labelling of AGPs relates to its association with the intense labelling of (1 → 5) α-L-arabinans in RG installation sites, as responsible for maintaining the osmotic balance and the reduction of cell dehydration (Ganie and Ahammed 2021), which are important for the RG develop- ment. It is plausible to assume that in this biotrophic interaction, the AGPs facilitate cell wall adhesion and translocation of metabolites toward the development of the aecia, and the aeciospores in the RG developmental sites.

Regarding the interaction of the fungi hyphae with cell wall pectins, the non-labelling of the epitopes of HGs in unesterified condition by LM19, and the weak labelling of the HGs in esterified condition by LM20 indicate the poor ability of the RG to mobilise the HGs of the host leaf cell walls toward its own cell wall construction. The host leaf cell walls have both the unesterified and esterified HGs labelled by LM19 and LM20, respectively, as indicative that the mesophyll cells maintain both juvenile and mature features, typical of totipotent cells (Willats *et al.* 2000; Albersheim *et al.* 2011). The intensity of cell divisions is evidenced by the histometry, which reveals the hyperplasia and cell hypotrophy of leaf parenchyma near the peridium, where there is an intense labelling both of the AGPs, and of the HGs. The labelling of the HGs in such neo-formed cells indicates they represent sites of great cell wall porosity and cell adhesion (Ferreira *et al.* 2020) and may be involved in the translocation of primary metabolites toward the aecium and the aeciospore formation.

The interaction of the fungi hyphae with (1 → 5) α-L-arabinans and (1 → 4) β-D-galactans indicates the potential investment in cell wall porosity along the hyphae pathway, as the epitopes of (1 → 4) β-D-galactans were labelled by LM5 and the epitopes of (1 → 5) α-L-arabinans were labelled by LM6 in the pycnia region. The strong labelling of (1 → 5) α-L-arabinans in the region of the peridium and the aecia, reinforced the ubiquity of the labelling of the epitopes of (1 → 4) β-D-galactans that have shown relatively uniform distribution in primary cell walls near the plasma membrane in different cell types (Sun *et al.* 2021), and appear to be associated with the maintenance of mechanical stability and increased porosity (Silva *et al.* 2021).

The NG spongy parenchyma offers less cell wall carbohydrates to be manipulated and redirected for RG installation, as only the epitopes of AGPs and (1 → 4) β-D-galactans were labelled by LM2 and LM5, respectively, in the NG. However, in the cell walls adjacent to the peridium, the epitopes of AGPs, unesterified HGs, and (1 → 5) α-L-arabinans are moderately or strongly labelled by LM2, LM19, and LM6, respectively. The labelling of the unesterified HGs in peridium cells indicates their synthesis and mobilisation from the adjacent host plant cells, but the (1 → 5) α-L-arabinans, not conjugated with the (1 → 4) β-D-galactans, may relate to the flexibility (McCartney *et al.* 2000), and adhesion (O'Donoghue and Sutherland 2012) of the RG cell walls. The labelling of the AGPs by LM2 also suggests cell adhesion and proliferation, corroborating the anatomical results of hyperplasia in the peridium region.

The network of hemicelluloses is altered in the site of RG installation, as indicated by the weak labelling of xyloglucans in the peridium region, where they may prevent the cellulose microfibrils from slipping (Bragança *et al.* 2020), as expected for hypotrophic cells. The hemicelluloses interfere with cell wall rigidity (Cavalier *et al.* 2008; Cosgrove and Jarvis 2012) and are also crucial for supporting expansive plant growth. Such functions seem to be attenuated in the RG installation site. Moreover, the starch grains and the reducing sugars detected in the cytoplasm of palisade parenchyma cells in NG (sites of intense labelling of xyloglucans), and the weak labelling in the peridium (RG) may reinforce the ability of the fungus to manipulate the accumulation of carbohydrates and redirect such molecules for its cell wall components. Accordingly, the fungus is able to degrade cell wall xyloglucans (Xu and Mendgen 2007), carbohydrate reserves in plants (Liepman *et al.* 2007; Moreira and Filho 2008; Scheller and Ulvskov 2010), and in galls (Bragança *et al.* 2020). Due to the non-detection of starch grains and the low detection of xyloglucans in peridium (RG), we assume that the rust fungi can mobilise these carbohydrates to compose their own cell walls.

Using the *B. variabilis* host leaf cell walls as substrate, the RG seems to mobilise the chemical components of the plant cell walls toward the formation of their own structures, as previously discussed for rust fungi (Heller 2020). Such supposition is supported by the immunolabelling of the epitopes of AGPs, HGs, (1 → 5) α-L-arabinans, and xyloglucans in the cell walls along the passageway of the hypha and the formation of aeciospores. The xylans labelled in the vascular fibres of the NG are not labelled in the RG cell walls, as they are components of secondary walls not deposited in fungi.

Histochemical profile and RG functional traits

In the RG on *B. variabilis*, the proteins accumulated in the pycnia and aecia may be involved in structural and metabolic support along gall growth, while the main energetic primary metabolites related to gall development are lipids and glycogen detected in the fungi. The abundance of lipid droplets and glycogen grains in aeciospores is a prominent feature that has been related to the metabolic support for fungus nutrition (Hatcher 1995; Moore-Landecker 2002), and the RG growth in *B. variabilis*. The accumulation of lipids is associated with many types of fungi, as reported for the aeciospores of *Puccinia distincta* on *Bellis perennis* leaves (Weber and Davoli 2002) and may represent one of the signals that promote and maintain the filamentous growth of fungi in host plants (Klose *et al.* 2004; Wongsuk *et al.* 2016). The starch grains detected in the spongy parenchyma of the *B. variabilis* NG, are undetected in the RG, which is indicative of the conversion of carbohydrates, starch grains, and reducing sugars, into the glycogen accumulated in the peridium.

The histochemical profiles of the NG and the RG in *B. variabilis* also indicate the involvement of phenolic compounds in gall functional traits, as the phenolics usually take part in plant defence and in the dissipation of oxidative stress (Close and McArthur 2002). In some rust fungi, the phenolics have been related to defensive responses of their host plants (Health 1997). Nevertheless, in *B. variabilis*, the accumulation of the phenolic compound in the palisade parenchyma cells does not impair the germination of the spores, the growth of the hyphae, or the installation of the RG. Moreover, there are no alterations in the sites of accumulation of phenolic compounds, another indication that the phenolics are not directly involved in the chemical mediation of the RG establishment in *B. variabilis*.

Conclusively, the development of the RG results mainly in the hyperplasia of spongy parenchyma, culminating in hypotrophied cells. The histochemical results indicate that proteins support the rust fungi installation, growth, and development; and the reducing sugars are mobilised in the form of glycogen, which together with the lipids constitute the energy reserves of the aeciospores. We emphasise that understanding cell wall modifications during pathogenic interactions is critically important, as the cell wall serves as the first line of defence for plant cells (Castilleux *et al.* 2020). Our results evidence other important roles of cell walls in rust fungi–plant cell interactions, for the unexpected immunolabelling of plant cell wall components in fungi cell walls provided unique evidence of the integrative role of some plant cell wall components in the biological process of pathogen colonisation of leaf tissues. We interpreted such interaction as the ability of the fungus to break down and incorporate plant cell wall molecules so that the epitopes were detectable in the various sites of rust fungi installation. Suitably, the functional traits of the cell wall components may work out in RG successful interaction with *B. variabilis* host leaf cells by increasing the adhesion (AGPs) as well as the porosity (arabinans and HG esterified) of host plant and rust fungus cell walls, thus favouring the traffic of molecules from the plant mesophyll toward the rust fungi gall.

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Seção II:
Divulgação Científica e Ensino de Botânica

Capítulo 1:

Por dentro das galhas: jogos didáticos como ferramenta de ensino e aprendizagem

Publicado no livro Ciências Biológicas: Gênese na formação multidicisplinar

CAPÍTULO 15

POR DENTRO DAS GALHAS: JOGOS DIDÁTICOS COMO FERRAMENTA DE ENSINO E APRENDIZAGEM

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RESUMO: O conhecimento sobre as plantas e o ensino de botânica têm grande relevância para a sociedade, considerando-se tanto a importância das plantas para os ambientes naturais quanto para os processos culturais e sistemas econômicos. No entanto, o ensino de botânica em espaços formais é, por vezes, tratado como matéria árida, entediante e fora do contexto moderno. Tendo como premissa que ferramentas didáticas interativas são eficientes em aproximar os conteúdos didáticos do interesse dos alunos, apresentamos o jogo “Por dentro das Galhas”. O objetivo do jogo é simular o ciclo de vida das galhas, e detalhar o processo de indução e desenvolvimento das mesmas, levando em consideração também fatores bióticos e abióticos vivenciados pelos indutores das galhas durante seu desenvolvimento. Propomos, ainda, que este jogo seja aplicado na perspectiva da abordagem prática antes da teórica, como um estímulo para aproximar os alunos de um conteúdo científico distante do seu dia a dia, mas que pode ser facilmente alinhado aos conteúdos didáticos dos níveis de ensino fundamental, médio e superior, numa perspectiva multidisciplinar.

PALAVRAS-CHAVE: Aprendizagem, ensino de botânica, ensino de ciências, metodologia ativa, Parâmetros Curriculares Nacionais.

GALLS FROM INSIDE OUT: EDUCATIONAL GAMES AS TEACHING AND LEARNING TOOLS

ABSTRACT: The knowledge on plant biology and the teaching of botany has great relevance to society, taking into account the importance of plants both for natural environments and for cultural processes and economic systems. However, when it comes to the teaching of botany in formal spaces, this content has been assumed as arid, tedious, and outside the modern context. We assume that interactive teaching tools may be efficient in solving the distancing of the didactic contents and the interest of the students. As an

interactive tool, the game “ Galls from Inside Out” aims to simulate the life cycle of the galls, and to detail the process of induction and development thereof, also considering the biotic and abiotic stresses that the gall inducers face along their development. We also propose the game as an approach of the practice previous to the theory, as a mean to stimulate and put the students closer to a scientific content that is far from their daily lives. Accordingly, the concepts on the game may be easily aligned with the programmed didactic content of elementary, high school and undergraduate levels in multidisciplinary approaches.
KEYWORDS: Active Methodology, Botany Teaching, Learning, Science Teaching, National Curriculum Parameters.

JOGOS COMO METODOLOGIAS ATIVAS E O ENSINO DE BOTÂNICA

O ensino de Botânica tem valor imensurável nos processos educacionais (Neves et al. 2019), pois as plantas fornecem benefícios ambientais e culturais tangíveis, bem como relevância nos sistemas econômicos (BACHI et al., 2020). No entanto, o ensino de Botânica é frequentemente negligenciado ou ignorado inteiramente nas salas de aula desde à educação básica (WANDERSEE; SCHUSSLER, 1999; SENICIATO; CAVASSAN, 2004; TOWATA et al., 2010) até o ensino superior. Na atualidade, grande parte das pessoas que passam pelos ensinos fundamental e médio, e até pelo ensino superior, veem a botânica como matéria escolar árida, entediante e fora do contexto moderno (SALATINO; BUCKERIDGE, 2016; NEVES et al., 2019).

Um dos desafios da carreira docente é construir continuamente modos de despertar a atenção dos alunos para a contextualização de conteúdos e, por meio do lúdico, tornar estes conteúdos mais atraentes, fugindo da abordagem meramente instrucionista e memorística (GUTIERREZ, 2014). Neste sentido, jogos, livros paradidáticos e vídeos demonstrativos surgem como ferramentas metodológicas capazes de gerar a aproximação do alunado com os conteúdos de botânica. Todas essas ferramentas visam simular situações que exponham o indivíduo a vários tipos de linguagens, em diferentes contextos disciplinares, culminando em diferentes práticas colaborativas (ANDRADE, 2002). Os jogos didáticos são ferramentas metodológicas eminentemente colaborativas, pois implicam na existência de jogadores que cumprem etapas de modo a alcançar os objetivos simulados. Os jogos sobre temas biológicos podem ainda, agregar estratégias multidisciplinares, caso incluam no ambiente simulado seres vivos como personagens, processos celulares e teciduais representando etapas a serem vencidas, bem como interações ecológicas que representem a interdependência dos meios bióticos e abióticos para a manutenção da vida.

Os jogos sempre estiveram presentes na história da humanidade e são uma constante em todas as civilizações, sendo mediadores de vínculos entre os povos e facilitadores da comunicação e interações interpessoais (SANT’ANNA; NASCIMENTO, 2011). O jogo didático, por sua vez, quando utilizado como aliado na prática docente, é uma forma lúdica de mediar o processo de ensino e aprendizagem podendo agir como catalisador de

motivação e autonomia na aprendizagem (FRANKLIN et al., 2003; GUTIERREZ, 2014). Neste contexto, extrapolam uma simples atividade, pois podem estimular a criação de estratégias, de senso crítico, serem desenvolvedores de confiança e de competências formativas em vários quesitos como, liderança e trabalho em equipe, além de atuarem no desenvolvimento motor e cognitivo (GONZAGA et al., 2017).

A utilização de elementos próprios do jogo como objetivos, regras, competição com a finalidade de motivar, despertar o interesse e promover a aprendizagem (gameficação) proporciona aos alunos uma experiência única que influencia na construção do conhecimento (SHI; CRISTEA, 2016; GONÇALVES et al., 2016). Partindo dessa premissa e, com o objetivo de contribuir para um ensino de Botânica mais atrativo e eficiente, criamos o jogo “Por dentro das galhas”. Este jogo tem como tema central uma interação parasita-planta, no caso ora simulado, uma interação inseto-planta, que resulta na formação de galhas. Galhas são curiosas neoformações induzidas em todos os órgãos vegetais não somente por insetos, mas também por ácaros, nematódeos, vírus, bactérias e até mesmo plantas (MANI, 1964; HARRIS e PITZSCHKE, 2020). Durante uma breve caminhada em um parque urbano, um fragmento de mata ou até mesmo no quintal de casa, é possível encontrar galhas e investigá-las (PORTUGAL-SANTANA; ISAIAS, 2014).

O jogo “Por dentro das galhas” simula o ciclo de vida e as intempéries, bióticas e abióticas, enfrentadas por um inseto galhador em sua planta hospedeira, trazendo para o espaço formal uma realidade que demanda, normalmente, de 3 meses a 1 ano (YUKAWA, 2000) para ser acompanhada na natureza. Além disso, o uso deste jogo como ferramenta metodológica permite uma abordagem multidisciplinar, sendo as galhas o tema motivador para a introdução de conteúdos relativos à biologia de insetos, ciclos de vida dos seres vivos, interações ecológicas, metabolismo vegetal, fatores abióticos e bióticos, bem como pragas agrícolas em plantas. Pretendemos que a utilização do jogo aqui apresentado como ferramenta didática possa (1) enriquecer a interação pedagógica através do engajamento dos alunos, (2) estimular o protagonismo do aluno no processo ensino-aprendizagem, (3) auxiliar no processo de ensino e aprendizagem de conceitos básicos sobre as relações entres os seres vivos, focado na história de vida das galhas e, finalmente, possa (4) fomentar a percepção dos alunos sobre a interdependência de fatores abióticos e bióticos que interagem durante o ciclo de vida dos seres vivos.

O JOGO E SUA METODOLOGIA DE APLICAÇÃO

O jogo é composto por tabuleiros (Figura 1) e cartas (Figura 2) e pode ser jogado por dois jogadores, que devem dispor de 2 tabuleiros e 48 cartas ou por quatro jogadores, que devem dispor de 4 tabuleiros e 96 cartas. O jogo “Por dentro das galhas” está disponível em português, e também nas versões em língua inglesa (The galls from inside out) e em língua espanhola (Por dentro de las agallas) e pode ser acessado livremente para impressão no

site do Neotropical Gall Group (https://www.neotropicalgallgroup.com/cool-science).

Regras do jogo:

1. Cada jogador tem seu tabuleiro representando o ciclo de vida de um indutor e de sua planta hospedeira e começa o jogo comprando 5 cartas do baralho, previamente embaralhado, que fica depositado entre os tabuleiros.

2. Para iniciar o jogo, um dos jogadores deve ter a carta 'OVIPOSIÇÃO' ou 'INDUÇÃO DE GALHA' e deve colocá-la na casa correspondente (= OVIPOSIÇÃO ou INDUÇÃO DE GALHA) em seu tabuleiro. Caso, nas 5 cartas iniciais o jogador não conseguir comprar a carta 'OVIPOSIÇÃO' ou 'INDUÇÃO DE GALHA', ele deve trocar de 1 a 5 cartas no baralho.

3. Depois de iniciar o jogo com a carta 'OVIPOSIÇÃO' ou a carta 'INDUÇÃO DE GALHA', o jogador escolhe entre as cinco cartas que tem em mãos se deseja trocar 1-2 no baralho ou se quer usar uma das cartas em mãos. A jogada é completada quando cada jogador coloca uma carta no tabuleiro e compra outra carta do baralho (ao final da jogada, o jogador deve estar sempre com 5 cartas em mãos).

4. O jogador, na hora de sua jogada e antes da troca de cartas no baralho, pode interferir no ciclo de vida dos jogadores adversários por meio das cartas que representam os fatores bióticos e abióticos. Ao ser atacado com um dos fatores bióticos, por exemplo: 'PREDADOR / PARASITÓIDE' ou abióticos 'SECA / FOGO', o jogador adversário tem seu ciclo de vida interrompido. Na sua vez de jogar, deve usar a carta de defesa correspondente: 'LIGNINAS ou CHUVA' e caso não tenha tais cartas, deve trocar de 1-5 cartas das que tem em mãos na tentativa de obter a carta de defesa correspondente. Caso não consiga efetuar a defesa, deve aguardar a próxima rodada para uma nova tentativa.

5. A jogada de ataque e de defesa se completa com a compra de 1 carta do tabuleiro, recompondo as 5 cartas em mãos.

6. O objetivo final é completar as etapas do ciclo de vida, ou seja, completar todas as casas do tabuleiro. O jogador / indutor que primeiro completar seu ciclo de vida é o vencedor.

7. Os cartões colocados no tabuleiro podem seguir a ordem do ciclo de vida ou não (caso o (a) professor (a) opte por tornar a atividade mais rápida).

Figura 1: Tabuleiro do jogo Por dentro das galhas.

Figura 2: Cartas utilizadas no jogo Por dentro das galhas.

OPORTUNIDADES DE APLICAÇÃO

O jogo foi aplicado em 2019 durante o 39^{0} Encontro Regional de Botânicos (ERBOT) organizado pela Regional Minas Gerais, Bahia e Espírito Santo da Sociedade Botânica do Brasil e no Espaço do Conhecimento da Universidade Federal de Minas Gerais (UFMG), durante a exposição “Docência Negra: Trajetórias e Vivências Plurais na UFMG” (Figura 3).

Nestas oportunidades, o público espontâneo foi formado por crianças, jovens e adultos, que independentemente da faixa etária, demonstrou entusiasmo e curiosidade sobre os temas galhas, insetos e suas plantas hospedeiras. Dessa forma, acreditamos que o jogo pode ser utilizado em espaços formais nos níveis de ensino fundamental, médio e ensino superior. O professor ou mediador pode variar os temas a serem abordados e a contextualização com o conteúdo didático que se pretende trabalhar, utilizando o jogo como elemento motivador.

Figura 3: Aplicação do jogo Por dentro das galhas na exposição Docência Negra: Trajetórias e Vivências Plurais na UFMG.

Para os espaços formais de aprendizagem, sugerimos que durante a aplicação do jogo, o (a) professor (a) circule entre os grupos e anote os elementos do jogo que mais chamaram a atenção dos alunos e os questionamentos referentes ao vocabulário. Após a aplicação, o (a) professor (a) utilizará estes elementos para relacioná-los aos conteúdos didáticos, usando o princípio de abordar a prática antes da teoria, de modo a aproximar os alunos de um conteúdo científico distante da sua rotina diária ou mesmo acadêmica. O estudo multidisciplinar de temas ambientais é crucial para a formação de cidadãos cuja percepção das condições ambientais permita o entendimento das relações de causa/efeito que condicionam a vida no espaço (geográfico) e no tempo (histórico), utilizando essa percepção para posicionar-se criticamente (MEC, 2021). As atividades que se seguem ao jogo têm por princípio levar à construção e consolidação do conhecimento a partir dos elementos levantados pelos alunos (Quadro 1).

Etapas	Atividades		Temas conceituais	Multidisciplinaridade	Objetivos dos PCN
1	Perguntas estimuladoras	(a). Quais estratégias de ataque e defesa você utilizou durante o jogo? Por que você as escolheu? (b). Qual estágio do ciclo de vida você considera mais interessante? Por que esta fase te chamou a atenção? Quais estágios do ciclo de vida você considera mais vulnerais aos fatores bióticos e abióticos?	- Ciclos de vida - Ciclos biogeoquímicos - Fatores abióticos - Fatores bióticos - Morfologia de insetos - Morfologia de plantas	Ciências Biologia Geografia Química	- Observar, registrar e comunicar algumas semelhanças e diferenças entre diversos ambientes, identificando a presença comum de água, seres vivos, ar, luz, calor, solo e características específicas dos ambientes diferentes; - Estabelecer relações entre características e comportamentos dos seres vivos e condições do ambiente em que vivem, valorizando a diversidade da vida.
2	Produção de textos	Relatar as etapas do ciclo de vida do galhador individualmente ou em grupos.	- Ciclos de vida - Interações ecológicas	Português Redação	- Comunicar de modo oral, escrito e por meio de desenhos, perguntas, suposições, dados e conclusões, respeitando as diferentes opiniões e utilizando as informações obtidas para justificar suas ideias.
3	Expedição de caça às galhas	Fotografar ou desenhar as plantas hospedeiras e suas galhas em passeios por áreas verdes	- Interações ecológicas - Morfologia de insetos - Morfologia de plantas	Artes Ciências e Biologia Geografia	- Organizar e registrar informações por meio de desenhos, quadros, esquemas, listas e pequenos textos, sob orientação do professor.
4	Mural fotográfico (físico ou virtual)	Montar de forma coletiva em sala de aula ou em plataforma digital (Facebook, Instagram, Canva® (https://www.canva.com) ou Padlet® (www.padlet.com)) um mural com as imagens obtidas na etapa de “Caça às Galhas”.	- Ciclos de vida - Interações ecológicas - Morfologia de insetos - Morfologia de plantas	Ciências e Biologia Artes Comunicação	- Comunicar de modo oral, escrito e por meio de desenhos, perguntas, suposições, dados e conclusões, respeitando as diferentes opiniões e utilizando as informações obtidas para justificar suas ideias.

Quadro 1 – Atividades didáticas propostas para a construção e consolidação do conhecimento após a aplicação do jogo.

CONSIDERAÇÕES FINAIS

A clareza dos objetivos e a organização da aplicação do jogo didático são essenciais para seu desenvolvimento e garantem o incentivo e a interatividade entre jogadores. Jogos de competição, como o "Por dentro das galhas" trazem a oportunidade de uso, por parte dos alunos de múltiplas habilidades como raciocínio, tomada de decisões, relacionamento interpessoal e respeito mútuo. Além disso, criam um ambiente informal que auxilia no processo de ensino e aprendizagem, enriquecendo as interações pedagógicas e estimulando o protagonismo do aluno e o prazer de aprender, uma vez que é ele quem decidirá qual estratégia utilizará para completar o ciclo de vida do galhador. A eficiência do jogo para fins didáticos pode ser avaliada pelo grau de envolvimento dos jogadores, e pela sua apropriação da terminologia técnico-científica ilustrada no tabuleiro e nas cartas. Além disso, o jogo proposto extrapola as paredes da sala de aula e traz para o alunado a oportunidade de conhecer um sistema planta-animal que, por vezes, não é descrito nos livros e materiais didáticos clássicos. Através do estudo das galhas, os alunos poderão conhecer e compreender interações ecológicas, morfologia da planta, morfologia de insetos, fatores bióticos e abióticos, ciclos de vida e ciclos biogeoquímicos.

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Capítulo 2

Galhas:
Planta-Inseto X Ciência-Educação

Publicado na Botânica Pública

Recursos didáticos

Galhas: Planta-Inseto x Ciência-Educação

Reisila Simone Migliorini Mendes (UEMG), Rosy Mary dos Santos Isaias (UFMG)

Galhas são pequenas verrugas, bolotas ou outras formas diferentes que crescem nas plantas. No interior das galhas estão os seres vivos, em geral insetos, responsáveis por sua formação e de onde conseguem obter alimento e proteção. Elas são formadas pela multiplicação de tecidos das folhas, caules ou outras partes das plantas em resposta ao estímulo do inseto galhador. Esta interação inseto-planta é um tipo de parasitismo.

No cenário educacional, a Base Nacional Comum Curricular (BNCC) prioriza cinco campos de atuação social para a área de Linguagens e suas Tecnologias. O campo de estudo e pesquisa visa desenvolver "habilidades relacionadas à análise, síntese, reflexão e problematização no contexto de estudo e da produção e divulgação científica". Esse campo contribui para a disseminação da divulgação científica (Brasil, 2018). Já os Parâmetros Curriculares Nacionais para o Ensino Médio (PCNEM) trazem orientações a respeito da utilização da divulgação científica em uma perspectiva do ensino contextualizado em que os estudantes possam reconhecer os aspectos da ciência em sua realidade (Brasil, 1999).

A pandemia da Covid-19 mudou a realidade educacional e impulsionou a produção de dispositivos de interpretação ambiental e ferramentas passíveis de serem utilizados à distância. Tais dispositivos atuam como facilitadores da conectividade entre o professor e os estudantes, servindo como mediadores do processo de ensino-aprendizagem.

Disseminação de conhecimento por meios digitais

As mídias sociais permitiram o aumento da conectividade das pessoas, e tornou-se essencial utilizar tais mídias como ferramentas para a comunicação e disseminação/difusão do conhecimento científico de forma acessível e ágil (Bueno, 2010). A divulgação científica tem deixado de ser restrita a revistas especializadas, tais como a Ciência Hoje e a Scientific American, e atualmente acontece por meio de diversas modalidades, como livros infantis, vídeos curtos no Instagram, aplicativos e outros. O uso das redes sociais como instrumento para disseminar o conhecimento gerado pela produção científica diminui a distância entre a pesquisa e o público geral (Costa, 2019). A potencial democratização do conhecimento científico proporcionada pelo universo digital representa um rompimento de barreiras (Navas et al., 2020) e traz agilidade para compartilhar conteúdos da ciência.

Ainda, no contexto de facilitadores da disseminação do conhecimento permeado pelo ensino remoto, o uso de vídeos de animação se mostra eficaz para explicar de forma simples as descobertas realizadas por meio de

pesquisas científicas sendo uma forma de divulgação que vem crescendo nos últimos anos (Silva et al., 2021) e pode também, ser uma excelente estratégia para despertar o interesse dos estudantes na sala de aula presencial.

Como proposto neste trabalho, produzimos um vídeo didático, um aplicativo e um livro paradidático visando explorar formas de compartilhar o conhecimento científico com o público em geral, criando pontes entre o fazer científico que ocorre na academia e os estudantes da educação básica. O objetivo foi criar ferramentas acessíveis, divertidas e educativas, capazes de disseminar informações sobre o universo das galhas de forma clara e objetiva para professores em formação, mas também podendo alcançar diferentes públicos e contribuir para a popularização da ciência.

Conversando com o Grupo Galhas

O vídeo intitulado "**Conversando com o Grupo Galhas**" (Figura 1) foi produzido utilizando o programa *PowToon – The visual communication* plataforma – de aces- so gratuito, tendo como mote um bate-papo entre pesqui- sadores. O vídeo foi utilizado em sala de aula, do ensino remoto, tendo como primeiro passo a contextualização, ex- plicando como ele se relaciona com o conteúdo que será estudado. Em seguida, foram propostas questões que incentivaram a reflexão e o debate sobre o tema apresentado. O público-alvo foi, em média composto, por 30 graduandos do 3º período do curso de licenciatura em Ciências Biológicas da UEMG/Ibirité por semestre (2019-2021).

O vídeo de divulgação foi produzido em parceria com a equipe do

Figura 1. Cenas do vídeo de divulgação científica disponibilizado no perfil @MadGal_UFMG do Instagram. A. Apresentação da pesquisadora entrevistada. B. Imagem do local de coleta na pesquisa. C. Observações em campo. D. Análises laboratoriais com o microscópio. Clique na imagem para assitir.

@MadGal_UFMG (Laboratório de Anatomia Vegetal da UFMG) e apresenta a rotina da pesquisadora e as galhas estudadas em *Byrsonima* spp. O vídeo desperta o interesse do público em geral pelas galhas e pela pesquisa científica, tendo alcançado 180 curtidas no Instagram. Entretanto, uso do vídeo em aulas remotas e presenciais como disparador de interesse nas aulas da educação superior atendeu o objetivo de chamar a atenção dos futuros professores para o mundo das plantas, como introdução para o conteúdo de Anatomia Vegetal e as interações das plantas com fatores bióticos (insetos galhadores) e abióticos, estimulando o olhar para a anatomia vegetal ecológica. Com uma linguagem acessível e visualmente atrativa, o vídeo foi capaz de compartilhar informações de forma clara e objetiva, tornando o aprendizado mais dinâmico e estimulante.

O aplicativo Gallocation no Ensino e para a Ciência Cidadã

O aplicativo ***Gallocation*** (Figura 2) foi produzido na plataforma Google Appsheet de acesso gratuito. Mas ainda está em fase de teste e em breve será disponibilizado na Play Store e na Apple Store. O público-alvo é formado por caminhantes e amantes da natureza e seu uso estimulará a produção de imagens e informações sobre a localização das galhas no ambiente natural. O registro das galhas no aplicativo permitirá contribuir para pesquisas científicas em andamento com da- dos preciosos difíceis e, por vezes, impossíveis ao pesquisador coletar sozi- nho. Sabe-se que a participação voluntária e consciente de diversos cidadãos reconfigura a ciência, de uma atividade fechada para uma ativida- de aberta democratizando a ciência. No *Gallocation* serão adotados os prin- cípios da Ciência Cidadã (Irwin, 1995) e será fomentada a participação

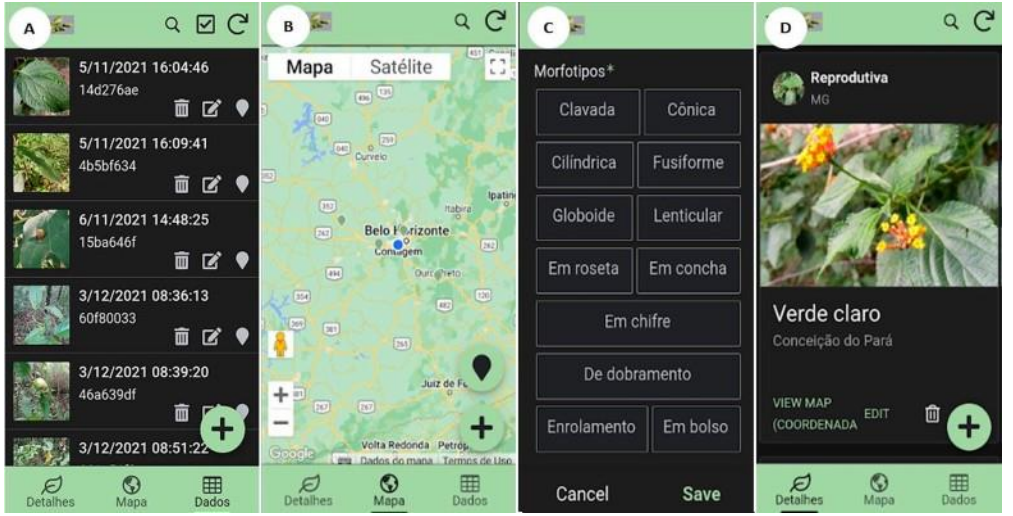

Figura 2. Telas do protótipo do aplicativo *Gallocation*. A. Dados coletados e armazenados na nuvem. B. Mapa com os pontos de localização das plantas hospedeiras e suas galhas. C. Tela para marcação do morfotipo (nome dado ao formato da galha). D. Detalhes da coleta de dados.

pública na produção do conhecimento e a transformação na maneira como as pessoas enxergam a ciência e o mundo. O app no formato atual foi usado simultaneamente por estudantes (n = 30) do 5º período do curso de licenciatura em Ciências Biológicas da UEMG/Ibirité, que cadastraram galhas encontradas nas trilhas do campus e em parques estaduais.

A meta com o app *Gallocation* será formar um banco de dados robusto que permita ao pesquisador avaliar a qualidade ambiental, bem como elencar novas áreas de estudos para a pesquisa Botânica visando desenvolver estratégias de conservação. Os resultados serão divulgados nas redes sociais, na perspectiva de valorizar a participação dos cidadãos sempre que tivermos descobertas relevantes no campo científico.

O aplicativo foi testado em aulas de campo, nas quais uma das atividades foi a Caça às Galhas (Figura 3 e 4).

Figura 3. Atividade de Caça às Galhas. A. Estudantes do curso de Ciências Biológicas da UEMG/Ibirité durante uma expedição. B. Galhas globoides caulinares (estrutura arredondada no caule). C. Galha lenticular intralaminar (estrutura abaulada dos dois lados da folha). D. Galha claviforme com projeções (estrutura em forma de bastão com protuberâncias no ápice). Fotografias: Reisila Simone Migliorini Mendes.

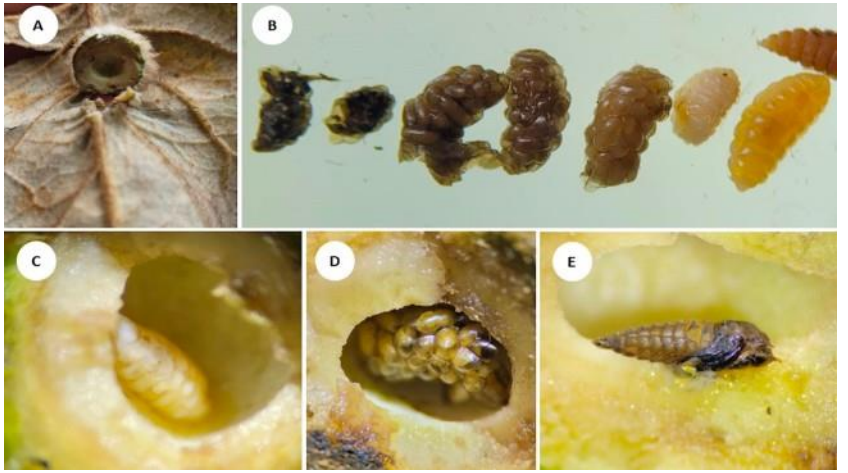

Figura 4. Galhas e insetos observados na Caça às Galhas. A. Galhas globoides foliares (estruturas arredondadas na folha) abertas manualmente para evidenciação da fauna associada. B-D. Larvas parasitadas (fases do desenvolvimento do inseto). E. Pupa (uma das fases do desenvolvimento do inseto antes da vida adulta). Fotografias: Reisila Simone Migliorini Mendes.

Até março de 2023, encontram-se cadastradas 47 galhas e suas hospedeiras em diferentes locais de Minas Gerais. Durante as análises em campo, os estudantes dissecaram as estruturas e demonstraram surpresa com a, até então, estrutura desconhecida das galhas. As perguntas permearam as interações ecológicas, ciclos de vida, alterações anatômicas e a capacidade do galhador em alterar a morfogênese (processo biológico de formação dos tecidos e dos órgãos) dos órgãos vegetais:

"Como ele entra na folha?"
"A planta hospedeira não se defende?"
"O parasitoide sabe onde encontrar o galhador?"
"As alterações nos tecidos vegetais ocorrem devido à capacidade de desdiferenciação dos parênquimas?"

Estas perguntas permitiram o ambiente educacional propício para apresentação e discussão dos temas afins – ecologia (estudo das relações entre seres vivos e o meio ambiente), morfologia (estudo da forma dos órgãos das plantas), anatomia (estudo dos tecidos que formam o corpo das plantas) e citologia (estudo das células) vegetal.

Os dispositivos educativos de interpretação ambiental desenvolvidos se configuraram como ferramentas eficazes para a popularização da ciência e para a aproximação entre a academia e a sociedade. O desenvolvimento e uso destes dispositivos em parceria com futuros educadores ampliou a perspectiva educacional da pesquisadora e a prática docente de todos os envolvidos. A disponibilização dos dispositivos nas mídias sociais e no site do *Neotropical Gall Group* (www.neotropicalgallgroup.com) permitirá ampliar a disseminação do conhecimento científico produzido pelo grupo de pesquisa.

O Livro infantil como ponte entre a ciência e a natureza

O livro infantil paradidático '**Arthur no oculto mundo das galhas**' (Figura 5) foi produzido no programa Canva de acesso gratuito, em parceria com uma discente do curso de licenciatura em Ciências Biológicas da UEMG/Ibirité está disponível no site

Figura 5. Capa do livro 'Arthur no oculto mundo das galhas'. O livro está disponível no site do *Neotropical Gall Group* (https://www.neotropicalgallgroup.com/didatic-gall-world). Clique na imagem para acessar.

www.neotropicalgallgroup.com. No livro foram apresentadas as aventuras de dois exploradores mirins que partem em uma caminhada exploratória e, descobrem as galhas foliares enquanto estão colhendo frutos de murici (*Bysronima coccolobifolia*).

O livro explora o universo infantil que é permeado por perguntas e curiosidades que buscam descobrir o mundo, como as crianças utilizam de sua imaginação para explorar o ambiente ao seu redor. Os personagens do livro, o murici e a larva do galhador associado conversam com as crianças sobre o que são galhas, herbívoros galhadores e planta hospedeira, despertando o interesse nas dinâmicas ecológicas e da importância da curiosidade no desenvolvimento cognitivo. Este livro permitirá aos estudantes do ensino fundamental atravessar a ponte formada entre o conhecimento acadêmico e a natureza.

Conectando a Ciência, o Ensino e o Ambiente

Avaliamos que os dispositivos de interpretação ambiental produzidos se constituem como facilitadores da divulgação científica e do ensino contextualizado para que os estudantes possam reconhecer a ciência em sua realidade, conforme proposto tanto pelas BNCC quanto pelos PCNEM. Ademais, cumpre, o papel de ponte entre o conhecimento acadêmico e a formação de professores, colocando a pós-graduação em um papel ativo na formação de cidadãos críticos e conscientes, capazes de compreender a importância da ciência e sua aplicação no cotidiano.

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CONSIDERAÇÕES FINAIS

O presente estudo sobre as galhas em *Byrsonima* spp. permitiu as seguintes conclusões:

1. Em *B. coccolobifolia*, o Cecidomyiidae galhador tem ciclo de vida univoltino e a indução da galha é sincronizada com o primeiro evento de brotamento foliar. A ocorrência de uma segunda fenofase de brotamento de forma assíncrona à oviposição pelo Cecidomyiidae galhador, configurando uma janela de oportunidade para a planta hospedeira escapar do parasitismo.
2. Não foi observado impacto negativo na floração e frutificação de *B. coccolobifolia* devido ao ciclo de desenvolvimento da galha, o que refuta a segunda hipótese.
3. No sistema *B.coccolobifolia*-Cecidomyiidae o investimento estrutural na formação de tecidos especializados para a nutrição do indutor é aparentemente maior do que para a formação de tecidos protetores, o que é aparentemente compensado pelo acúmulo de moléculas o metabolismo secundário no compartimento tecidual interno da galha, garantindo defesa química à galha.
4. No sistema *B.coccolobifolia*-Cecidomyiidae, as moléculas energéticas são acumuladas no protoplasma das células do tecido de armazenamento comum e do tecido nutritivo típico, indicando que o suprimento adequado de água durante a estação chuvosa tem um efeito positivo no desenvolvimento da galha.
5. A dinâmica dos arabinanos e galactanos nas paredes celulares dos tecidos tanto das galhas de inseto quanto de fungo em *Byrsonima* spp. facilita o transporte de metabólitos primários do tecido da hospedeira em direção à galha.
6. No sistema *B. variabilis*-Pucciniales, as proteínas dão suporte à instalação, crescimento e desenvolvimento dos fungos da ferrugem e os açúcares redutores são mobilizados sob a forma de glicogénio, que juntamente com os lipídios constituem as reservas energéticas dos aeciosporos.
7. No sistema *B. variabilis*- Pucciniales, a inesperada imunomarcação dos componentes das paredes celulares das células vegetais nas paredes celulares dos fungos da ferrugem fornecem provas inéditas do papel integrador de alguns componentes das paredes celulares vegetais no processo biológico de colonização patogénica dos tecidos foliares.

Nesse sentido, a hipótese central de que espécies congenéricas de *Byrsonima* podem apresentar alterações semelhantes nos padrões de desenvolvimento e metabolismo da planta hospedeira em resposta ao desenvolvimento das galhas associadas foi

parcialmente corroborada, tendo em vista que a especialização tecidual foi observada somente nas galhas induzidas pelo Cecidomyidae galhador. Entretanto, as alterações histoquímicas e imunocitoquímicas em função da instalação e desenvolvimento das galhas de inseto e de fungo da ferrugem são muito semelhantes no que diz respeito à distribuição dos metabólitos primários e à dinâmica das paredes celulares.

A conexão entre o conhecimento produzido na academia e a formação de professores realizada por meio da produção de dispositivos de interpretação ambiental nos permitiram caminhar rumo às diretrizes tanto das BNCC quanto dos PCNEM. Estes destacam a importância da disseminação da divulgação científica e do ensino contextualizado para que os estudantes possam reconhecer a ciência em sua realidade. A presente tese cumpre o papel de contribuir para que a pós-graduação tenha um papel ativo na formação de cidadãos críticos e conscientes, capazes de compreender a importância da ciência e sua aplicação no cotidiano.

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Anexo

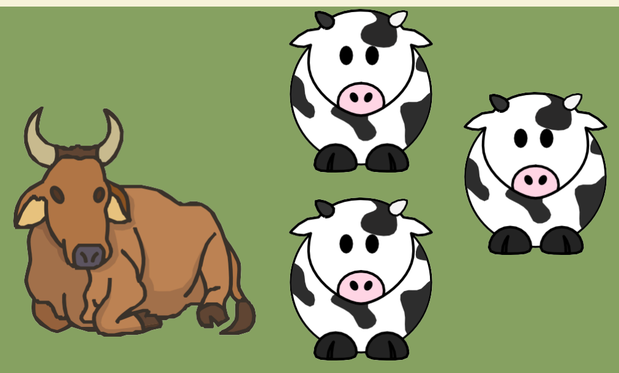
ARTHUR NO OCULTO MUNDO DAS GALHAS

Reisila Migliorini
Cristiane Petrini
Rosy Isaias

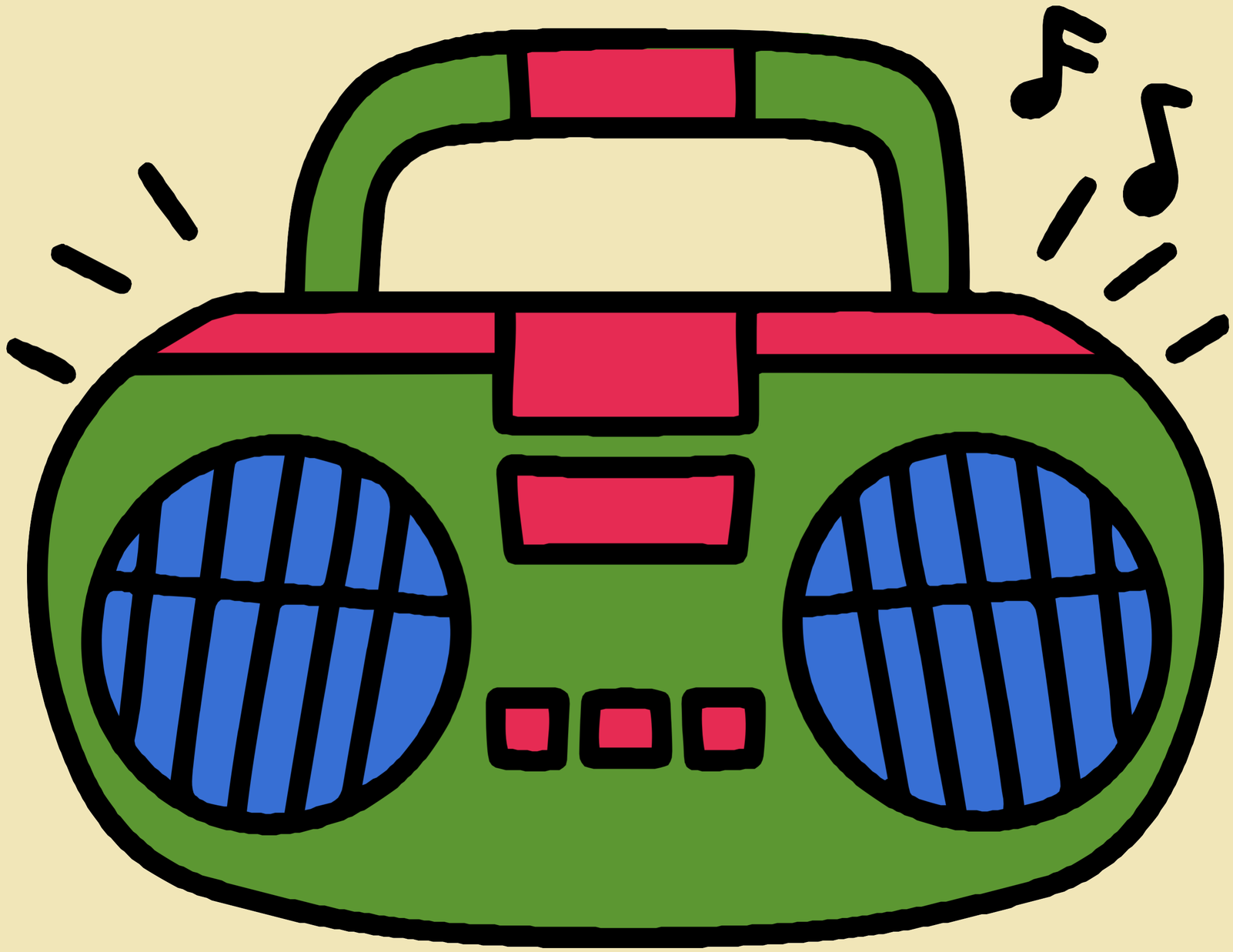
ARTHUR NO OCULTO MUNDO DAS GALHAS

O interior de Minas Gerais abriga uma cidadezinha batizada de Conceição do Pará. O povo conta que foi da família do capitão Jerônimo que o vilarejo nasceu. Foi na máquina Carda de tecer algodão que lá no início, o vilarejo foi batizado com o nome de Cardosos.

Arthur é morador de Conceição do Pará e adora pescar na beira do rio. Na fazenda do seu pai, ele acorda cedo e ajuda a cuidar do rebanho do gado, na alimentação do cavalo e na colheita do milho dentado.



Arthur é um garoto muito curioso e gosta de montar e desmontar eletrônicos. Todas as vezes que a sua mãe vai para a cidade grande, ele liga lembrando da pilha do controle ou da antena do rádio.



As tardes do mês de fevereiro são ensolaradas e floridas, Arthur e seu amigo Samuel estão empolgados, pois estão de férias da escola e agora está sobrando tempo para brincar.
Todas as tardes, depois do almoço, Arthur chama seu amigo Samuel para explorar a natureza.

Na sua pochete, Arthur carrega um kit que batizou com o nome de pintesoulupa, que é a junção de pinça, tesoura e lupa.

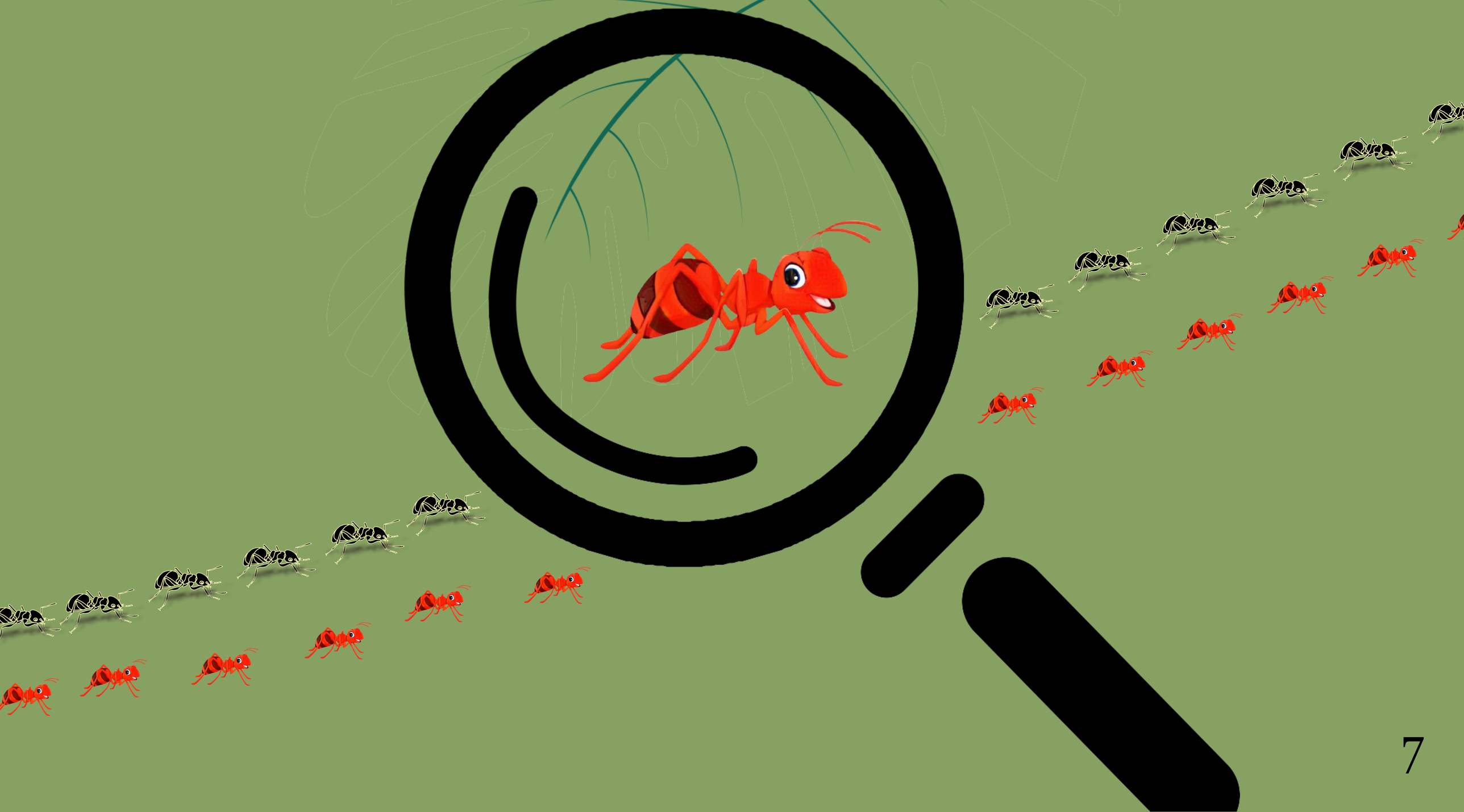
_Oi Arthur!
_ Oi Samuel!
Ontem fomos à beira do rio Pará, e hoje, onde vamos explorar?

Samuel com a sua lupa na mão responde empolgado:

_ Podemos ir para o campo aberto, também trouxe meu kit e lá poderemos observar os animais pequenos.

Saíram entusiasmados e, em todos os formigueiros paravam. Com a lupa observavam as formigas de pertinho.

Eram formigas pretas, vermelhas, grande e pequena. Com seus super-kits observavam e manuseavam os pequenos insetos com cuidado e curiosidade.



Dava para ver que no corpinho das formigas
existem pelinhos e também observar seus olhos e
suas anteninhas.
As joaninhas não escapavam da visão dos meninos,
pois o aumento das lupas exibiam suas bolinhas
pretas, que ficavam bem maiores. Eles também
podiam ver com detalhes, o rostinho das joaninhas.

As flores e as folhas pequeninas chamavam a atenção e eles observavam cada detalhe, por dentro e por fora, na parte inferior e superior.

_Olha Arthur o pé de Murici está com muitas flores e frutos.

_ É verdade Samuel! Achei que ele havia morrido, pois da última vez que o vimos estava sem nenhuma folha, apenas com os galhos.

_Vamos pegar algumas frutas para sua mãe fazer suco gelado, vai ajudar a refrescar.

Os meninos cataram todos os frutos que estavam no chão. Mas o Arthur, olhando para a árvore, viu que tinham muitos frutos amarelinhos e pomposos reluzindo de tão maduros.

_ Samuel, vou subir no pé para pegar os
frutos que estão nos galhos mais altos.
_ Isso! Você sobe e vai me jogando os frutos
que eu pego aqui embaixo.

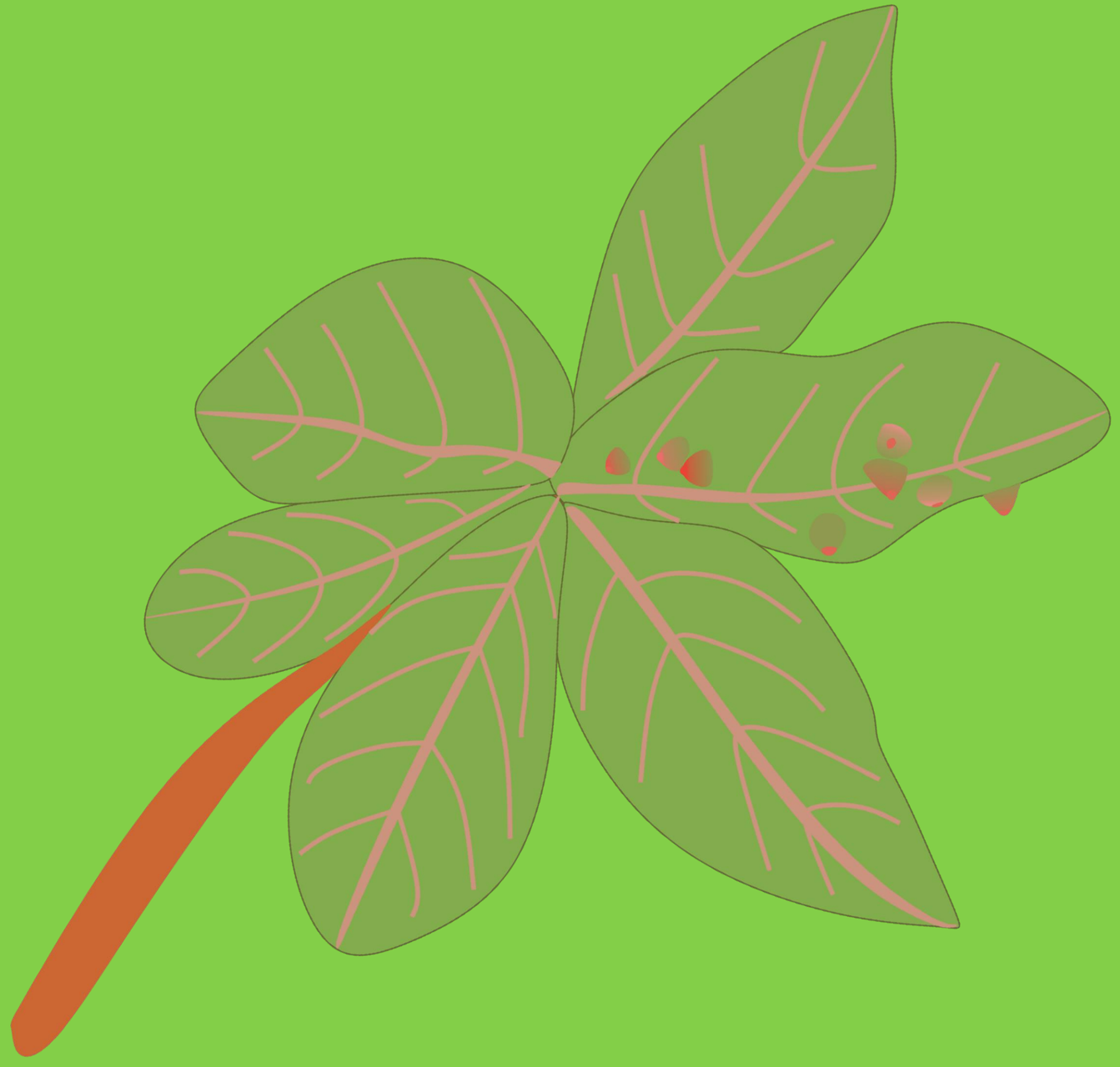
Arthur empolgado passava de um galho para o outro pegando o máximo de frutos possível.

_ Samuel, Samuel! Me passa a minha lupa por favor, encontrei algo diferente aqui nas folhas.

_ Calma! Vou subir aí.

Com as lupas em mãos, os garotos começaram a observar umas bolotas diferentes que estavam presentes nas folhas da árvore do murici.

Arthur tirou uma dessas bolotas da folha e observando mais de perto percebeu que ela atravessava a folha e tinha um furinho embaixo. Então pensou: é oca!



Usando a tesoura, Arthur abriu a bolota e os dois garotos se surpreenderam.

_ Samuel!!! Tem uma larvinha aqui dentro.

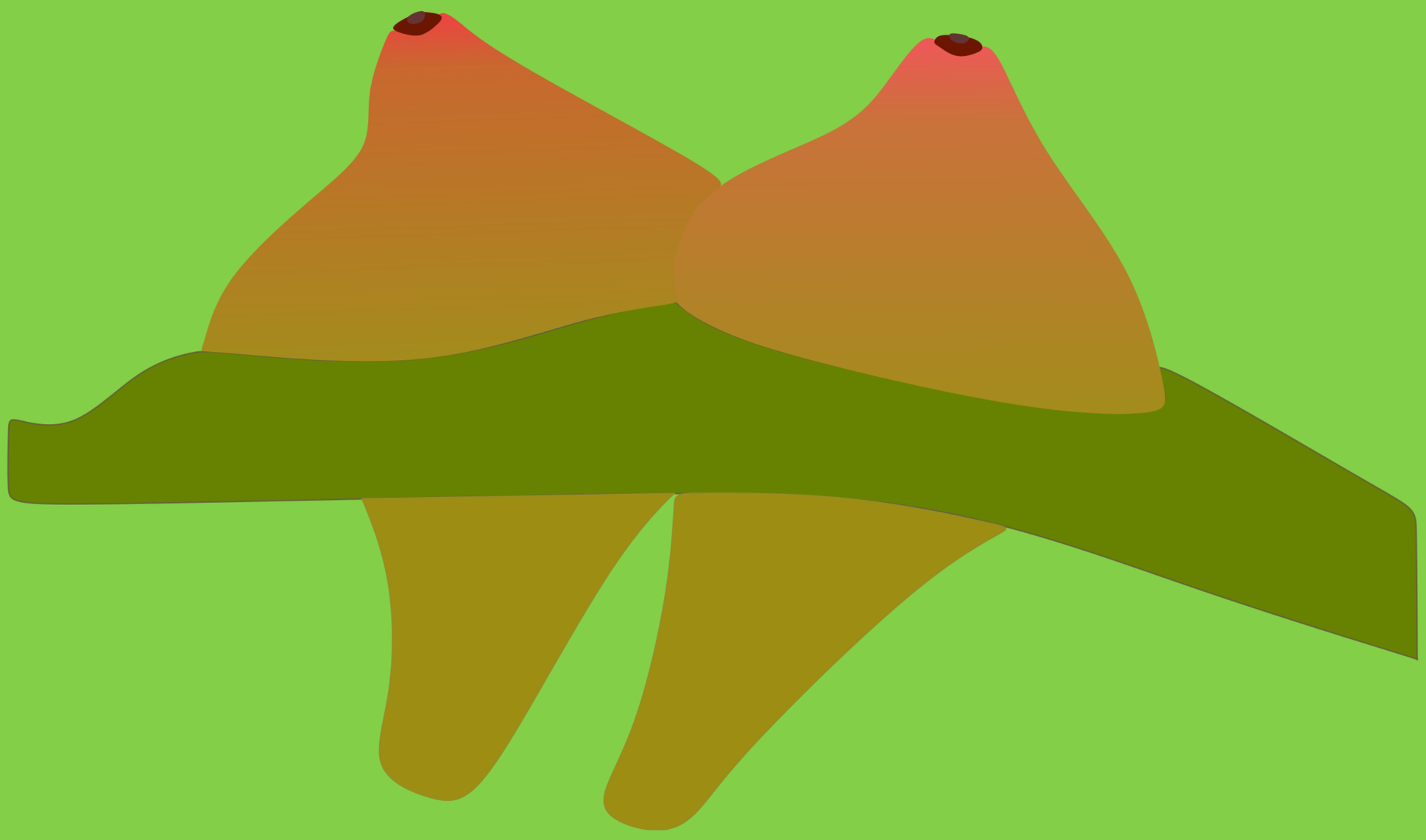
Samuel se apressou em olhar usando sua lupa

_ Uuauuu Arthur, como essa larva foi parar aí dentro? Será que nasceu da árvore?

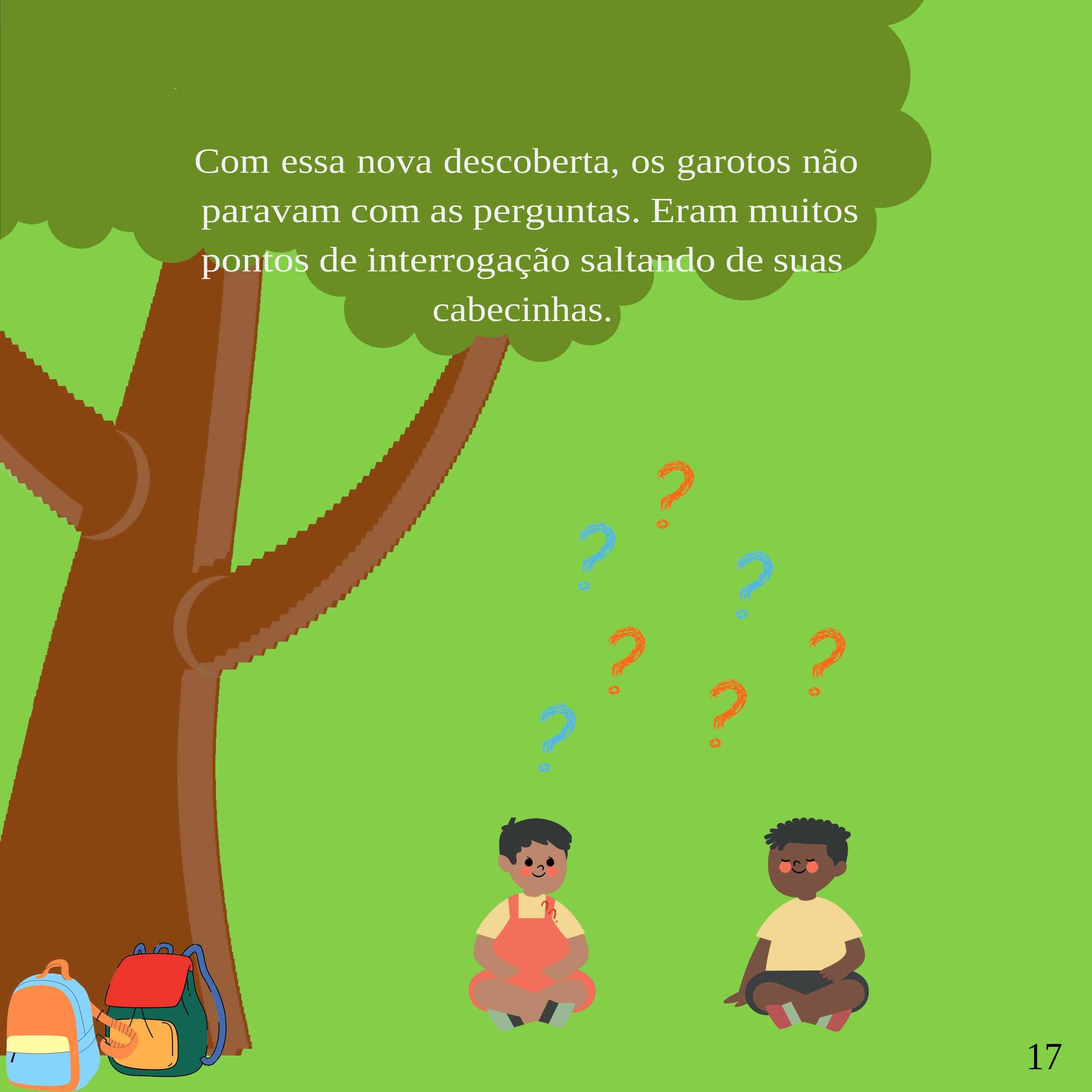
_Vamos abrir outras! Exclama Arthur.

_ Arthur, nessa aqui tem ovinhos por cima.

_ E nessa também!



Com essa nova descoberta, os garotos não paravam com as perguntas. Eram muitos pontos de interrogação saltando de suas cabecinhas.



Eis que de repente, viram uma luz brilhante e escutaram uma voz um pouco rouca. Olharam para todos os lados e não viram ninguém, mas novamente ouviram a voz.

_ Olá meus amiguinhos.
Os garotos novamente olharam para os lados
e não viram ninguém.
_ Oi meninos! Sou eu, o Murici.
_ Meu Deus! Você fala? Perguntou Arthur.
Samuel permaneceu em silêncio, com os
olhos arregalados, chocado.

_Apenas quando o portal dos porquês é aberto, continuou a árvore.
Nós plantas e criaturas de outros reinos conseguimos nos comunicar com os seres humanos. Assim conseguimos investigar e solucionar dúvidas no mundo das crianças.

_ Uaauuu! Exclama Samuel.
Então você poderá nos ajudar a solucionar este mistério?
_ Claro que sim, disse a árvore. Para nos ajudar, vou chamar o nosso amigo Larvito para explicar esse processo.

_Olá meninos, meu nome é Larvito e estou muito feliz que tenham aberto o portal dos porquês.

_ Obrigado Larvito! Eu e Samuel estamos muito empolgados, disse Arthur.

Primeiramente vou explicar o que são essas bolotas presentes nas folhas do nosso amigo Murici. Essas bolotas são chamadas de galhas e acontecem quando um outro ser, que pode ser um fungo, inseto ou até mesmo uma bactéria interage com as folhas ou outras partes da planta onde eles resolvem morar.

Neste caso aqui do Murici, as galhas são formadas porque um inseto resolveu morar aí nas folhas. Este inseto é um mosquitinho muito apressado e é responsável pela indução das galhas nas folhas do Murici. Como ele é pequenino e muito ágil, ainda não sabemos seu nome.

Esse mosquitinho misterioso coloca os ovos dele na folha, suas larvas eclodem, se alimentam, e mudam o tipo de crescimento das folhas, assim crescem o que chamamos de galhas.

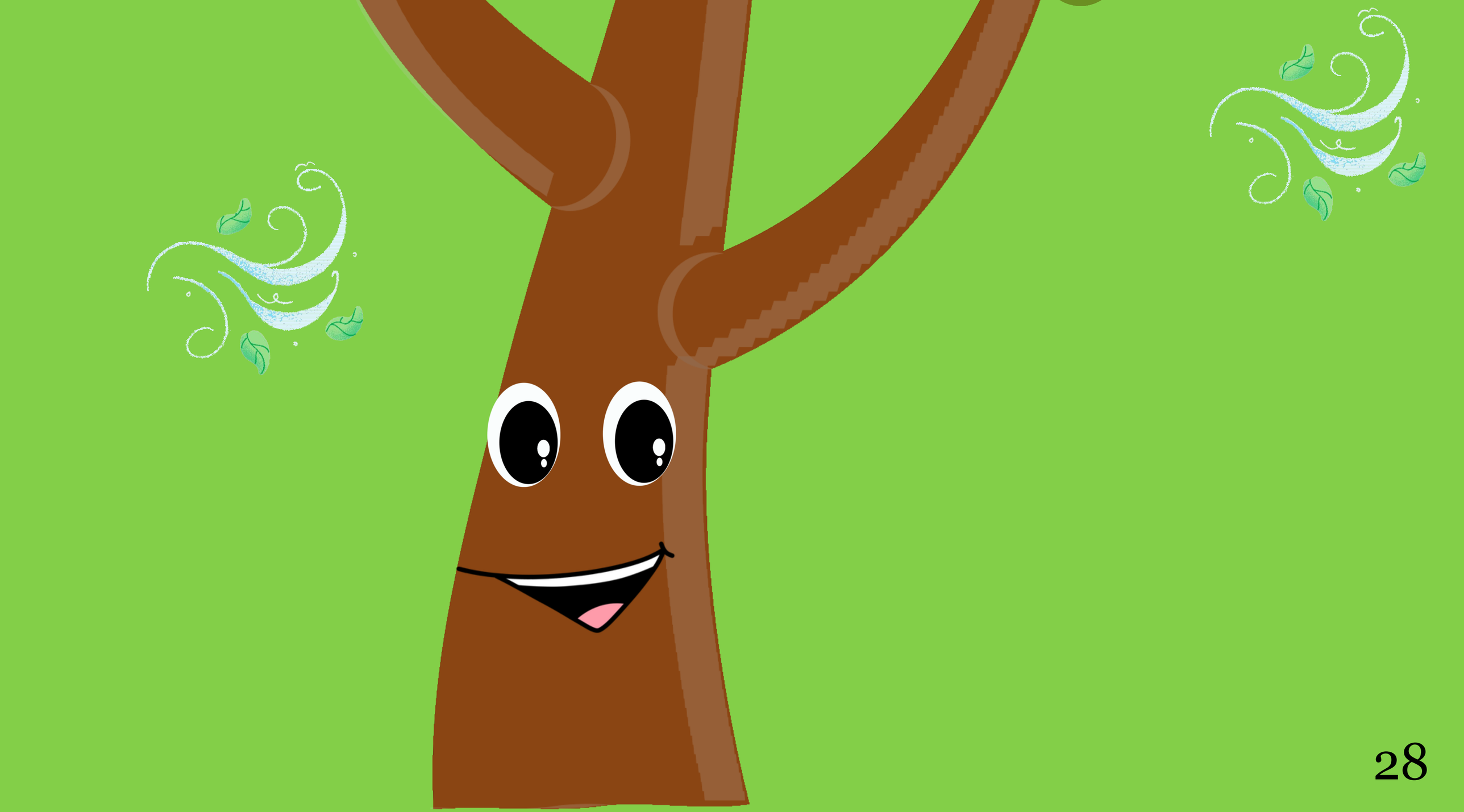
O mosquitinho usa a folha do Murici como fonte de alimento, mas também se abriga e se protege dentro dos tecidos da folha até concluir seu ciclo reprodutivo.

Que legal Larvito, muito obrigado pelos ensinamentos. Estamos muito felizes de ter desvendado esse mistério.

— Eu que agradeço. É muito bom encontrar meninos curiosos como vocês, responde o Larvito. Sempre que o portal dos porquês é aberto ficamos muito satisfeitos de poder ajudar.

Uuuuu, vuvuvuvuvu, uuuuuuu, vuvuvvuvuv
Ouve-se o barulho do balançar das árvores e o
Murici se pronuncia.

_ Venho como mensageiro do portal dos porquês para incumbir o Arthur e o Samuel de uma missão muito importante.
Vocês terão que me visitar mais vezes para encontrar o mosquito indutor dessa galha e ajudar os cientistas a darem um nome para a espécie.



Assim, vamos incluir o nome dele no nosso acervo de conhecimento. Outras crianças que, porventura, venham abrir o portal, ficarão mais sabidas.

Samuel e Arthur olharam um para o outro e se perguntaram: será que tudo isso foi um sonho? De qualquer forma, voltaram para casa muito satisfeitos e foram perguntar a mãe do Arthur sobre as galhas e como eles poderiam cumprir sua nova missão.

Arthur e Samuel são crianças curiosas e nesta história os dois amigos embarcaram em uma descoberta extraordinária para entender o mundo das galhas.

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