UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL

Perfil histoquímico e composição da parede celular durante o desenvolvimento de galhas induzidas por *Bystracoccus mataybae* (Eriococcidae) em *Matayba guianensis* (Sapindaceae)

Ana Flávia de Melo Silva

Orientador: Prof. Dr. Denis Coelho de Oliveira

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COMISSÃO EXAMINADORA

Presidente: _____
Prof. Dr. Denis Coelho de Oliveira – Orientador
Universidade Federal de Uberlândia – UFU

Examinadores: _____
Prof. Dr. Bruno Garcia Ferreira
Universidade Federal do Rio de Janeiro – UFRJ

_____
Profa. Dra. Ana Silvia Franco Pinheiro Moreira
Universidade Federal de Uberlândia - UFU

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"Se quer chegar rápido, vá sozinho. Se quer ir longe, longe vá em grupo."

(Provérbio Africano)

SUMÁRIO

RESUMO

SILVA, A.F.M. 2019. **Perfil histoquímico e composição da parede celular durante o desenvolvimento de galhas induzidas por *Bystracoccus mataybae* (Eriococcidae) em *Matayba guianensis* (Sapindaceae).** Dissertação de Mestrado. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Biologia Vegetal 98p.

Bystracoccus mataybae (Eriococcidae, Hemiptera) é um inseto galhador que induz galhas em *Matayba guianensis* Aubl. (Sapindaceae), espécie vegetal abundante no Cerrado. O galhador é partenogenético e possui três instares ao longo do seu desenvolvimento, com ocorrência no mesmo hospedeiro e em um ciclo sincronizado. Galhas em caules são induzidas por ninfas de primeiro ínstar (crawlers) na estação seca. No período de brotação das folhas, a ninfa do segundo instar se move para os folíolos para induzir um novo ciclo. Nos folíolos, a segunda ninfa se torna adulta, engravida e morre depois que os crawlers saem das galhas foliolares e se movem para o caule. Assim, o objetivo do estudo foi compreender a dinâmica dos componentes estruturais e químicos em galhas de *Matayba guianensis.* O estudo estrutural e químico das galhas com diferentes estágios do inseto galhador mostrou que galhas induzidas pela ninfa de primeiro instar de *B. Mataybae,* no caule, apresentaram baixa complexidade estrutural em comparação com as galhas de segundo instar induzidas nos folíolos. A compartimentação dos compostos primários e secundários foi determinada precocemente por ninfas de segundo instar em folíolos jovens e, poucas alterações ocorrem durante os demais estágios de vida do inseto. Em relação a composição péctica e protéica das paredes celulares, houve alterações dos folíolos não galhados para as galhas e durante o desenvolvimento da galha. Pectinas com diferentes níveis de metilesterificação foram mais presentes em galhas jovens. O processo de lignificação começou em galhas jovens e parece estar relacionado não apenas com o mecanismo de dissipação do estresse, mas também no crescimento e alongamento da parede celular. Dessa forma, alterações estruturais e químicas nas galhas de *M. guianensis* ocorrem dentro dos potenciais morfogenéticos da planta hospedeira, dependendo de cada sítio de indução do galhador.

Palavras-chave: Compostos Metabólicos; Eriococcidae; Morfogênese; Parede Celular; Perfil químico.

ABSTRACT

SILVA, A.F.M. 2019. **Histochemical profile and composition of the cell wall during the development of galls induced by *Bystracoccus mataybae* (Eriococcidae) in *Matayba guianensis* (Sapindaceae).** Dissertação de Mestrado. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Biologia Vegetal 98p.

Bystracoccus mataybae (Eriococcidae, Hemiptera) induce galls on *Matayba guianensis* Aubl. (Sapindaceae), an abundant plant species of the Cerrado. The galling insect is parthenogenetic and has three instars throughout its development, all of them occurring on the same host in a synchronized cycle. Stem galls are induced by first instar nymphs (crawlers) at the dry season. When the leaf sprouting begins, the second instar nymph moves to the leaflets to induce a new gall cycle. At the leaflets, the second nymph becomes adult, gets pregnancy and die after the crawlers get out of the leaflet galls and move to stem. Thus, the objective of the study was to understand the dynamics of the structural and chemical components in *Matayba guianensis* galls. The structural and chemical study in different insect stages revealed that galls induced by the first instar nymph of *B. mataybae* in stem presented low complexity compared to leaflet galls induced by the second instar nymphs. The compartmentalization of the primary and secondary compounds was determined early by the second instar nymph at the beginning of leaflet gall development, and they change little during the other stages of the galling insect. Changes were observed in the pectic and protein cell wall composition from non-galled leaflet to gall and during gall development. Pectins with different methyl esterification degrees were more present in young galls. The lignification process started in young galls and seems to be related not only as mechanism of stress dissipation but also assist in the growth and stretching of the cell wall. Thus, all the structural and chemical changes in *M. guianensis* galls occur within the morphogenetic potentials of host plant dependent of the induction sites.

Keywords: Metabolic compounds; Eriococcidae; Morphogenesis; Cell wall; Chemical profile.

INTRODUÇÃO GERAL

Galhas são estruturas neoformadas a partir da hipertrofia e hiperplasia de células, tecidos e órgãos vegetais, e podem ser induzidas por fungos, bactérias, vírus, ácaros, nematódeos e insetos (Mani 1964). A galha é considerada uma associação parasítica que desencadeia a formação de um novo órgão vegetal (Shorthouse et al. 2005), com características estruturais e fisiológicas distintas às do órgão hospedeiro (Oliveira et al. 2016). Essa associação proporciona ao galhador abrigo, alimentação e proteção contra inimigos naturais e fatores abióticos (Price et al. 1986; Rohfritsch e Anthony 1992). Embora vários organismos sejam capazes de induzir galhas, aquelas induzidas por insetos são consideradas as mais complexas em termos morfológicos (Raman 2007, Isaias et al. 2014).

A morfogênese das galhas é dependente do estímulo químico e alimentar contínuo do galhador (Mani 1964; Meyer e Maresquelle 1983; Bronner 1992), sendo a relação entre insetos galhadores e plantas hospedeiras espécie-específica (Shorthouse et al. 2005). Essa especificidade está relacionada não somente ao seu hospedeiro, mas também aos limites impostos pelo órgão onde a galha será induzida (Dreger-Jauffret e Shorthouse 1992; Floate et al. 1996; Oliveira et al. 2016). Essas especificidades se refletem na conformação estrutural de cada galha, com a sua ocorrência condicionada ao reconhecimento pelos galhadores de características fenológicas e fisiológicas da planta, manipulando-a de modo a suprir suas necessidades nutricionais e a manter o funcionamento e desenvolvimento de sua estrutura (Bronner 1992; Oliveira et al. 2006; Moura et al. 2008; Oliveira et al. 2010; Oliveira e Isaias 2010; Isaias et al. 2014; Oliveira et al. 2016). Desta forma, o inseto galhador manipula a planta hospedeira para o direcionamento de metabólitos primários para o seu suprimento metabólico (Hartley 1998), variando o tipo de substância e o local de armazenamento de acordo com os taxa e a forma de alimentação do inseto.

Proteínas, lipídios e carboidratos são as substâncias primárias comumente armazenadas em galhas (Ferreira et al. 2017). Tais substâncias podem estar distribuídas funcionalmente nos tecidos da galha formando gradientes nutricionais responsáveis pela manutenção da maquinaria celular da galha e alimentação do inseto galhador (Bronner 1992; Oliveira e Isaias 2010, Oliveira et al. 2010). A presença de tecido nutritivo típico ao redor da câmara larval é comum em galhas induzidas por Cecidomyiidae (Diptera), Hymenoptera e Lepidoptera, com maior acúmulo de carboidratos nas galhas induzidas por Cecidomyiidade e de lipídios naquelas induzidas por Hymenoptera e Lepidoptera (Raman e Dhileepan 1999; Raman 2007; Vieira e Kraus 2007; Bedetti et al. 2013; Ferreira e Isaias 2013; Vecchi et al. 2013; Ferreira et al. 2017). Em galhas induzidas por sugadores, como no sistema *Aspidosperma australe* - *Pseudophacopteron* sp. (Apocynaceae-Hemiptera), o inseto galhador se alimenta diretamente no floema (Malenovsky et al. 2015) e, consequentemente, não há formação de um tecido nutritivo típico. O mesmo pode ser observado em galhas induzidas pelo inseto sugador *Euphalerus ostreoides* (Hemiptera) em folíolos de *Lonchocarpus muelhbergianus* (Fabaceae) (Oliveira et al. 2006).

Além dos metabólitos primários, nitidamente relacionados à nutrição do galhador, as galhas apresentam variações nos compostos do metabolismo secundário, como compostos fenólicos, alcalóides e terpenoides (Hartley 1998; Bragança et al. 2017). Essas substâncias podem atuar na proteção do inseto contra o ataque de parasitoides e de predadores, além de reduzir a competição por alimento ao inibir o ataque de outros insetos fitófagos (Janzen 1977; Cornell 1983). Além disso, podem atuar na sinalização e estímulo ao crescimento da galha, como é o caso de alguns derivados fenólicos que agem sinergicamente com as auxinas (Bedetii et al. 2014). Esse processo atua na inibição da AIA-oxidases, aumentando assim a ação das auxinas envolvidas no processo de hipertrofia celular que ocorre durante a formação da galha (Fosket 1994). Em galhas induzidas por Cecidomyiidade em *Piper arboreum* Aubl.

(Piperaceae) os metabólitos secundários se acumulam apenas no parênquima cortical externo. As células externas formam compartimentos fotossintéticos e respiratórios defensivos, enquanto as células internas assumem papel nutricional, demostrando a divisão funcional dos tecidos de galhas (Bragança et al. 2017).

Os mecanismos de diferenciação celular em galhas ocorrem por meio de um balanço sinérgico e complexo entre as células que estão em divisão e expansão ao longo do seu desenvolvimento (Oliveira et al. 2010). Assim, a dinâmica e composição da parede celular tem papel fundamental no desenvolvimento da estrutura da galha (Formiga et al. 2013; Oliveira et al. 2014; Carneiro et al. 2014). A composição da parede está relacionada com o controle do reforço, flexibilidade, mobilidade e porosidade da parede, adesão entre as células e a capacidade de manter a hidratação em momentos de estresse para órgãos vegetais em geral, inclusive em galhas (Formiga et al. 2013; Oliveira et al. 2014; Magalhães et al. 2014). A obtenção de novas formas e crescimento nas galhas do sistema *Baccharis dracunculifolia* - Psyllidae (Asteraceae) foi demonstrada, por exemplo, pelo arranjo das microfibrilas de celulose ao longo do seu desenvolvimento (Magalhães et al. 2014). Nesse mesmo sistema a presença das cadeias laterais de "β-D-galactanos" e "α-L-arabinanos" ligados a pectinas do tipo Ramnogalacturonanos do tipo I na parede celular possibilitaram a flexibilidade necessária para rediferenciação de células da lâmina foliar e formação da galha de dobramento marginal (Oliveira et al. 2014).

Mudanças na forma dos órgãos e tecidos vegetais ocorrem durante o crescimento e desenvolvimento, sendo resultado de divisões celulares, modificações estruturais e reorganização de componentes da parede celular (Meijer e Murray 2001; Rose 2003; Albersheim et al. 2010). Destes componentes, as pectinas são os polissacarídeos mais abundantes da classe de macromoléculas presentes na matriz da parede celular vegetal e na lamela média, onde têm a função de regular a adesão intercelular (Willats et al. 2001). Além desta função, múltiplas evidências indicam o papel importante de pectinas no crescimento

vegetal, desenvolvimento, morfogênese, defesa, estrutura da parede, sinalização, expansão celular e porosidade da parede, entre outros (Ridley et al. 2001; Willats et al. 2001; Rose 2003; Albersheim et al. 2010). Os polímeros pécticos são classificados dentro de três principais domínios: homogalacturonanos (HGs), o mais abundante grupo de polissacarídeos pécticos (~65% da pectina), ramnogalacturonanos II (RG-II), pectinas estruturalmente mais complexas e que compreende aproximadamente 10% das pectinas existentes na matriz da parede, e ramnogalacturonanos I (RG-I), que representam de 20-35% da matriz péctica (Ridley et al. 2001). É geralmente aceito que HGs são sintetizadas em uma forma altamente metil-esterificada (Albersheim et al. 2010), cujo grau resulta a variedade de estados deste composto. Em algumas paredes celulares, há muitos domínios onde o grau de esterificação é modificado, sendo o grau de esterificação e as mudanças na molécula péctica importantes nas propriedades funcionais da parede celular vegetal, principalmente durante o crescimento e desenvolvimento dos tecidos (Knox 1997). Junto aos HGs e RGs, glicoproteínas (Showalter 1993), arabinogalactanos proteínas (AGPs), são encontrados em múltiplas formas associadas com a parede celular ou a membrana plasmática (Pennell et al. 1989). Pela sua localização no tecido, elas podem desempenhar papel importante no crescimento e diferenciação celular. Os AGPs têm aspecto mucilaginoso e estão relacionadas com a adesão celular (Cosgrove 1997), crescimento, nutrição, proliferação celular (Majewska-Sawka e Nothnagel 2000), e prevenção da morte celular programada (Mastroberti e Mariath 2008). Os AGPs formam um gel nos sítios de injúria nas células constituindo uma barreira física contra a invasão celular (Cassab 1998).

Baseado no exposto, percebe-se que alterações estruturais e químicas ocorrem em galhas de diferentes *taxa* de indutores. Assim, neste trabalho, estudos histoquímicos, imunocitoquímicos, e de espectroscopia Raman foram utilizados para compreender a dinâmica dos componentes estruturais e químicos em galhas de *Matayba guianensis*. Para tal, a

dissertação foi subdividida em dois capítulos, ambos em formato de artigo científico, assim distribuídos:

1. Early gall inducer developmental stages define final gall structural and histochemical profiles - The case of *Matayba guianensis* galls induced by *Bystracoccus mataybae*.
2. Chemical composition of cell walls in galls induced by *Bystracocus mataybae* (Ericoccidae) on *Matayba guianensis* (Sapindaceae) assessed by immunocytochemistry and Raman spectrometer analysis.

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CAPÍTULO I

Early gall inducer developmental stages define final gall structural and histochemical profiles - The case of *Matayba guianensis* galls induced by *Bystracoccus mataybae*

Manuscrito submetido (Botany)

Early gall inducer developmental stages define final gall structural and histochemical profiles - The case of *Matayba guianensis* galls induced by *Bystracoccus mataybae*

Ana Flávia de Melo Silva[1], Vinícius Coelho Kuster[2], Uiara Costa Rezende[1] and Denis Coelho de Oliveira[1*]

[1] Universidade Federal de Uberlândia, Instituto de Biologia, Campus Umuarama, CEP 38402-020, Uberlândia, Minas Gerais, Brazil. ana.f.melo2015@gmail.com; uiara.ucr@gmail.com

[2] Universidade Federal de Goiás, Regional Jataí, Instituto de Biociências, Campus Cidade Universitária, CEP 75801-615, Jataí, Goiás, Brazil. viniciuskuster@ufg.br

* Corresponding author: denisoliveira@ufu.br. Telephone +55 34 32258557

Abstract

Gall morphotypes depend on continuous chemical and feeding stimuli of the gall inducer, which is the promoter of specialized systems. The galling insects manipulates host plant tissues in order to target primary and secondary metabolites, with the chemical arsenal and the storage place varying according to the insect's feeding habit. We selected the *Matayba guianensis* (Sapindaceae)-*Bystracoccus mataybae* (Eriococcidae) system for the current study based on the occurrence of different instars that can induce different morphological patterns. Here, we investigate if the structural and histochemical profiles of gall tissues are dependent on the instars of the galling insect. Standard anatomical analyses were carried out, as well as histochemical evaluation of reactive oxygen species and primary and secondary metabolites. Some structural changes were induced on the stem by the first instar nymph, while deeper changes induced by the second instar nymph on the leaflet, indicating that the host plant tissues impose more limits on gall development and complexity than the distinct stages of insect development. Compartmentalization of the primary and secondary compounds is established from the beginning of insect development by second instar nymphs on leaflets, and they change little during the next stages.

Key words: Gall Morphotype, Metabolic compounds, Insect instars, anatomical anylses.

Introduction

Insect gall morphogenesis depends on the continuous chemical and feeding stimuli of the gall inducing organism (Mani 1964; Meyer and Maresquelle 1983; Bronner 1992), highlighting the plant-insect interactions as a very specialized systems in nature (Shorthouse et al. 2005). The gall structure develops by host cell hypertrophy, tissue hyperplasia, and cell re-differentiation, which create a new plant organ with peculiar shape and functionality (Shorthouse et al. 2005; Raman 2007; Isaias et al. 2014; Oliveira et al. 2016). Galls provide a favorable microenvironment for the galling insects, protecting against desiccation and attack of natural enemies, and providing more adequate nutrition (Stone and Schönrogge 2003). In this respect, the distribution of primary and secondary metabolites in galls occurs in response to galling insect feeding activity. Consequently, the histolocalization of these substances can be an excellent tool for understanding the mechanism of inducer's nutrition and defense against natural enemies (Bragança et al. 2017; Isaias et al. 2018; Kuster et al. 2019 *in press*).

Some taxa of galling insects can develop a typical nutritive tissue around the larval chamber, with primary metabolite accumulation used in the galling diet (Bronner 1992; Ferreira et al. 2017). The cecidomyiid galls usually accumulate carbohydrates, while galls induced by Hymenoptera and Lepidoptera usually accumulate lipids (Raman and Dhileepan 1999; Raman et al. 2007; Vieira and Kraus 2007; Bedetti et al. 2013; Ferreira and Isaias 2013; Vecchi et al. 2013; Ferreira et al. 2017; Rezende et al. 2018). In contrast, galls induced by phloem-sucking insects do not develop a true nutritive tissue since feeding activity may be restricted to the phloem cells (Bronner 1992; Ferreira et al. 2017). Nonetheless, some galls of phloem-sucking insects can accumulate primary metabolites in their tissues, as is the case for galls of *Pseudophacopteron aspidormermi* (Hemiptera) on *Aspidosperma australe* and induced by *Euphalerus ostreoids* (Hemiptera) on *Lonchocarpus muelhbergianus* (Fabaceae) (Oliveira et al. 2006; Oliveira and Isaias 2010). The galls can also accumulate secondary metabolites such

as phenolic compounds, alkaloids, and terpenes (Hartley 1998; Bragança et al. 2017). These metabolites supposedly protect the galls against natural enemies (Bennett and Wallsgrove 1994; Formiga et al. 2009) and promote the scavenging of oxidative stress molecules (Isaias et al. 2015; Isaias et al. 2018).

The histochemical approach has been used to clarify tissue organization on galls through the localization of primary and secondary metabolites, revealing differences and patterns of metabolite compartmentalization and functions (Bragança et al. 2017; Kuster et al. 2019 *in press*). Therefore, changes in the metabolic profile have been tested during gall development in different systems (e.g. Oliveira et al. 2006; Carneiro and Isaias 2015). However, the developmental stages of gall inducing organisms were based mostly on gall size (i.e. young, mature stages). Recent studies have raised the idea that gall phases can be marked by changes in the inducer instars (see Pfeffer et al. 2018), which leads to a new perspective and question in gall studies: Are structural and histochemical changes in the gall triggered by the development of galling insect instars? For this, galls induced by *Bystracoccus mataybae* (Hemiptera) on leaflets and stems of *Matayba guianensis* (Sapindaceae) were used as a model to answer this question for the first time, helping to elucidate whether the age of galling insects is determinant in manipulate plant tissue for gall developmental patterns.

Material and methods

Host plant-galling insect system

The galling insect *Bystracoccus mataybae* (Eriococcidae, Hemiptera) (Hodgson et al. 2013) that induces galls on leaflets of *Matayba guianensis* Aubl. (Sapindaceae) is parthenogenetic and has three instars throughout its development, all of them occurring on the same host in a synchronized cycle. When the leaf falls (dry season), the first instar nymph

moves from the leaflets to the branches, while the second nymph instar induces galls in new sprouting leaflets, turning into adults and becoming pregnant (Pfeffer et al. 2018).

Study area and sampling

Collections were made at Estação Ecológica do Panga (19° 10'S, 48°24'W) and Fazenda Experimental do Glória (18º57'S, 48º12'W), Uberlândia, Minas Gerais State, Brazil. Ten individuals of *Matayba guinanesis* with galls induced by *B. mataybae* were selected in each population. Non-galled stem, non-galled leaflets and galls were collected from July 2017 to March 2018 for structural and histochemical analyses. Samples of (i) young and mature non-galled leaflets (n = 10) and galls induced by *B. mataybae* (n = 10), considering the different developmental stages of gall inducer: (ii) stem galls –induced by the first nymph (named as crawler) (Figure 1A), (iii) young leaflet galls - second instar nymph (Figure 1B) and, (iv) mature galls - adult stages or adult with eggs and/or crawlers (Figure 1C), were collected as described by Pfeffer et al. (2018) and Hodgson et al. (2013).

First-instar nymphs were collected on the stem galls from July to August, young leaflets and galls induced by the second instar nymph were collected from August to September, while adult insects were collected in September and October. From November to February were collected leaflet galls containing adult female with eggs and/or crawlers. Histochemical tests were performed at different times in galls with adult insects, when the adult was not pregnant and when it presented eggs and/or crawlers. Analyses were also carried out on senescent galls (Figure 1D) in order to observe possible changes in the metabolic accumulation sites and, the presence or absence of primary and secondary substances.

Histological analysis

Structural features were examined in samples fixed in 0.1 mol. L^{-1} Karnovsky's solution in phosphate buffer, pH 7.2 (2.5% glutaraldehyde and 4.5% formaldehyde in 0.1 M phosphate buffer) (Karnovsky 1965), dehydrated in an ethanol series and embedded in 2-hydroxyethylmethacrylate (Historesin®, Leica Instruments, Germany). Transverse 5 μm-thick sections were obtained with a rotary microtome (YD-315 model, China), stained with 0.05% toluidine blue, pH 4.6 (O'Brien et al. 1965) and mounted with Entellan®.

Histochemical analysis

For detection of primary and secondary metabolites, only fresh samples were used, and handmade sections were done using razor blades, followed by immersion in specific reagents. For protein detection, the samples were immersed in 0.1% bromophenol blue in a saturated solution of magnesium chloride in 70% alcohol (Mazia et al. 1953). For lipids and starch, samples were submitted to a saturated solution of Sudan III in 70% alcohol (Sass 1951) and Lugol's solution (1% iodine-potassium iodide solution) (Johasen 1940), respectively. Reducing sugars were detected by Fehling's reagent, which consists of the combination of solution "A" (II copper sulfate 6.93% w:v) and "B" (sodium potassium tartrate 34.6% and 12% sodium hydroxide m:m:v), with subsequent heating of the slide containing the samples to pre-boiling (Sass 1951). The sections were treated with Ehrlich's solution to determine the sites of auxins accumulation (Leopold and Plummer 1961). Ruthenium red solution was used to determine the presence of pectins (Jensen 1962). Control tests were performed in all cases, comparing the material with blank sections.

Secondary metabolites such as phenolics, alkaloids, and terpenoids were tested. Total phenolics were detected with 2% ferrous sulfate in 10% formalin (v/v 1:1) (Gahan 1984) and Dragendorff's reagent was used for alkaloid detection, followed by washing in 5% sodium

nitrite (Svendsen and Verpoorte 1983). For the detection of flavonoids, the samples were pre-fixed in 0.5% caffeine and sodium benzoate in 90% butanol and then incubated in 1% *p*-dimethylaminocinnamaldehyde (DMACA) (Feucht et al. 1986). Terpenoids were detected with 1% α-naphthol and 1% dimethyl-*p*-phenylenediamine in phosphate buffer solution, pH 7.2 (NADI test) (David and Carde 1964). Structural lignin was detected using 2% phloroglucinol in acidified solution (Johansen 1940). Control tests were performed in all cases by comparing the material to blank sections.

For evaluation of the sites reactive oxygen species (ROS) accumulation, the samples were immersed in DAB reagent (3,3 'diaminobenzidine) in the dark (Rossetti and Bonatti 2001). All the structural and histochemical sections were photographed with Leica® ICC50HD camera coupled to DM500 photomicroscope.

Results

Structural traits of non-galled leaflets and galls

The non-galled stem showed an uniseriate epidermis covered with a thick cuticle (Figure 2A) or already with periderms. The cortical tissues are predominantly parenchymatic with fibers in small groups (Figure 2A). The vascular system has a secondary growth (Figure 2A). Fibers surround the secondary phloem (Figure 2A) and form a complete circle. The first instar nymphs (crawlers) induce galls on the stem surface (Figures 1A and 2B) by the increase of phellogen activity and consequently cell hypertrophy and periderm hyperplasia, especially the phelloderm (Figure 2B).

The young leaflets exhibit a dorsiventral chlorophyllous parenchyma with secretory cavities immersed in the parenchyma and vascular bundles undergoing differentiation (Figure 2C). The mature leaflets are hypostomatic, with uniseriate epidermis, covered with thick cuticle, and a dorsiventral mesophyll (Figure 2D). A uniseriate layer of hypodermis occurs under the

adaxial epidermal surface (Figure 2D). The palisade parenchyma is compact and has 3-4 cell layers (Figure 2D), while the spongy parenchyma contains large intercellular spaces and about 6-7 cell layers (Figure 2D). In the middle of the chlorophyll parenchyma there are collateral vascular bundles (Figure 2D).

Galls induced by the second instar of the galling insect on young leaflets are globoid, intralaminar, green, with an adaxial surface opening (Figure 1B). The young galls have a uniseriate epidermis with ordinary cells of isodiametric shape, a thin cuticle and non-glandular trichomes. The gall cortex is homogeneous and consists of parenchyma cells of various sizes (Figure 2E), with the occurrence of tissue hyperplasia and cell hypertrophy. New vascular bundles differentiate and are then displaced by the hyperplasia of the parenchyma tissue, taking position near the nymphal chamber (Figure 2E).

The adults of *B. mataybae* (third instar) are reddish and build a second nymphal chamber (Figure 2F) below the adaxial surface where they start to place the eggs. The galls (Figure 1C), kept covered by the uniseriate epidermis and the cortex, can be divided into outer and inner compact cortex types. The outer cortex is composed of homogenous parenchyma (Figure 2E), while the inner one occurs around the nymphal chamber and is mostly formed by sclerenchyma cells (Figure 2F). Collateral vascular bundles are placed near the nymphal chamber (Figure 2E). The senescence phase (Figure 1D) occurs when the offspring leave the progenitor's body and the adult female dies and becomes black.

Histochemical characterization of non-galled organs and galls

The tests for primary, secondary and reactive oxygen species (ROS) are listed in Table 1, as well as the results for all non-galled tissues and gall instar phases.

Non-galled stem and stem galls induced by the first instar nymph

In the non-galled stem, auxins, proteins, total phenolics, and flavonoids were detected in the parenchyma cells. Terpenoids and lignin were detected only in cortical parenchyma cells, while the alkaloids are detected also in the phloem, and central parenchyma cells. Reactive oxygen species (ROS) prevail in cortical and phloem cells.

In stem galls induced by the first instar nymph (Figure 3, Table 1), reducing sugars (Figure 3A) and proteins (Figure 3B) occur in cells of the cortical parenchyma, while auxin (Figure 3C) is concentrated in the periderm and cortical cells, where total phenolics (Figure 3D), alkaloids (Figure 3E) and terpenoids (Figure 3F-G) are also found. The ROS showed a positive reaction on the periderm, cortical parenchyma cells, phloem, and primary xylem (Figure 3H-J). Structural polymers such as pectins (Figure 3K) and lignins (Figure 3L) were found in the cortical parenchyma.

Non-galled leaflet and galls with gall inducer in the second instar nymph

Non-galled leaflets showed a positive reaction for reducing sugars and auxins in mesophyll cells, and proteins only in cells of epidermis and vascular bundles. Alkaloids and flavonoids were detected in the palisade parenchyma, but terpenoids occurred in the adaxial epidermis only. The epidermis and parenchyma cells of vascular bundles showed a positive reaction for pectins. Lignin and phenolic compounds were detected only in mature leaflets in the cell walls of the pericyclic fibers and mesophyll cells, respectively. ROS were detected in the chlorophyllous parenchyma, as well as in the vascular bundles of non-galled leaflets.

The leaflet galls induced by the second instar (Figure 4, Table 1) showed a positive reaction for reducing sugars (Figure 4A) and auxins (Figure 4B) in the outermost cell layers towards the inner cortex, with a negative gradient toward the nymphal chamber. Proteins (Figure 4C) occurred in the epidermis, especially in the cortex near the nymphal chamber and

vascular bundles. Lipids (Figure 4D) were detected in the epidermis and few lipid droplets were detected in cortical cells (Figure 4E). Terpenoids (Figure 4F), alkaloids (Figure 4G) and flavonoids (Figure 4H) occurred more intensely in the outer cortical parenchyma and weakly in the cells near the nymphal chamber. Lignin (Figure 4I) was part of the sclerenchyma cell walls that surround the nymphal chamber and vascular bundles. Pectins (Figure 4J) were detected in the cell walls of all gall tissues, more intensely in the cell walls that surround the nymphal chamber and in the outer layers of the cortex. ROS (Figure 4K) were more evident in the outer cortex cells but occurred in all cells of the gall, with more labeling in the vascular bundles, especially in the xylem cells, and in the cells that surround the second nymphal chamber.

Leaflet galls with galling in the third instar (adults)

The chemical compounds detected histochemically in the galls induced by the first and second instar nymphs were also detected in leaf galls with the adult insect (Figure 5A-L). Starch (Figure 5A) was an exception, as a few grains occurring in the cortex cells near the nymphal chamber, only in the adult gall. Phenolics did not occur in galls induced by the second instar nymph but were detected in adult galls (Figure 5G). There were no differences in the location of the metabolites between the pregnant and non-pregnant females.

Senescent galls – when the crawlers come out

The first instar nymph comes out of the adult female that died leading to the gall senescence. The same metabolites detected histochemically in the previous stages of gall development were found in senescent galls, except for terpenoids, which are absent in senescent galls (Figure 6, Table 1). It was possible to observe greater lignification near the nymphal chamber and in some cortical areas throughout the gall (Figure 6I). All cortical cells of the gall

were labeled with ROS, except areas of sclerenchyma that surround the nymphal camera (Figure 6J).

Discussion

Despite changes in the host stem and leaflets morphology induced by the phenotype manipulator, both galls on *M. guianensis* showed similar histochemical profiles according to the developmental stage of the galling insect. This histochemical profile is determined early by the second instar nymph at the beginning of leaflet gall development. An exception is noted for starch, which appears only in the mature galls (i.e. third instar), when the galling insects are adults. Although the gall inducer mouth apparatus seems to be determinant for gall shape and tissue specialization (Bronner 1992; Ferreira et al. 2017), the host morphogenetics and the developmental phase of the insect seem to be relevant. Nevertheless, the early histochemical pattern definition supports the idea that the gall configurations can be determined since the beginning of galling insect development.

The stem galls induced by the first instar nymph of *B. mataybae* showed low complexity compared to leaflet galls induced by the second instar nymphs. These stem galls exhibit a single nymphal chamber, which is open throughout the occupation period. Although gall is open, gall tissues around the first instar nymph seems to be an adaptive feature to keep the galling in better conditions of moisture and temperature than those provided by the environment, acting as a micro-habitat (Lill and Marquis 2003). The differences between stem and leaflet galls indicate the greater importance of host morphogenetic constraints in the determination of patterns than galling insect developmental stages. The host organ potentialities probably restrict gall primary and secondary traits. In this context, the extended phenotype concept (Dawkins 1982; Stone and Cook 1998) can be revised here, considering that the same

insect species, a parthenogenetic one, induces different morphotypes in the same host plant species.

Regarding the histochemical differences between stem and leaflet galls, it is important to emphasize that stem galls do not contain starch. The absence of these metabolites may be related to the behavioral habits of the insect, which does not feed during this developmental phase (Gulan 2005). However, galls induced by other phloem-sucking (Hemiptera) and scraping-sucking (Cecidomyiidae) insects can store starch when the gall stops feeding and consequently reduces gall tissue metabolism (Bronner 1992). The configuration of substances can also be limited by the potential of stem tissue to produce, allocate and accumulate them. The primary metabolite compartmentalization in leaflet galls is indistinct among the developmental stages of *B. mataybae*. One of these histochemical traits is the centripetal gradient of reducing sugars detected in the outer cortex among all the instar changes of the insect. This production and accumulation of carbohydrate occur in *Calliandra brevipes* galls (Fabaceae), indicating the relevant role of carbohydrates as metabolic resources in some galls (Detoni et al. 2010). Starch is the second carbohydrate resource for galls, observed as few grains near the nymphal chamber in the adult female phase, indicating low cell metabolism (e.g. Oliveira et al. 2017). Starch can be enzymatically cleaved into sucrose, fructose and glucose during gall development and used as a metabolic cell resource (Oliveira and Isaias 2010a).

The few lipid droplets found in the leaflet gall cortex, induced by different insect instars of the galling insect *B. mataybae*, probably is not related to insect feeding needs, but rather to be related to the maintenance of the cellular machinery. Lipids represent a high energy reserve related to the maintenance of gall structure, as shown for the *Lonchocarpus mulherbergianus – Euphalerus ostreoides* (Oliveira et al. 2006) and *Lantana camara – Aceria lantanae* (Moura et al. 2008) systems. Protein synthesis and storage is common in nutritive tissues of other galling-inducing groups as Cecidomyiids (Oliveira et al. 2010, 2011a; Ferreira

and Isaias, 2014) and Cynipids (Bronner 1992). The proteins accumulated in vascular bundles and perivascular parenchyma cells, as found in the *B. mataybae* leaflet galls, was also reported for *Nothotrioza cattleiani* (Triozidae) - *Psidium cattleianum* (Myrtaceae) galls (Carneiro and Isaias 2015). Carneiro and Isaias (2015) suggested that these phloem-sucking insects can induce a redifferentiation of nutritive tissue around the vascular system, and this may be related to the well-developed ROS scavenging apparatus (Carneiro and Isaias 2015). In addition, the increase of proteins in gall tissues seems to be a good indicator of high oxidative stress (Schonrogge et al. 2000).

The outermost layers of the gall cortex also has ROS, which can be related to high metabolite activity by the photosynthesis process, as shown for the Cecidomyiidae - *Piper arboreum* system (Bragança et al. 2017). ROS, especially hydrogen peroxide, are the main stressing factors described for gall tissues. They can also act on molecular signaling and contribute to gall tissue morphogenesis (Oliveira et al. 2011; Isaias et al. 2015). ROS detection on the chlorophyllous parenchyma of non-galled leaflets and during all instar changes of *B. mataybae* galls indicates that ROS are intrinsic substances for cell metabolism, but the high ROS labeling on the gall cells reveals that the biological activity of the galling insect is also a stressing agent for the plant tissue. Adult galls show ROS on vascular bundles and facing their upper and lower tissue layers, which may be associated with an increase of the insect's sucking activity.

In view of the oxidative stress generated in the gall, some mechanisms of stress dissipation are necessary for tissue homeostasis (Isaias et al. 2015), such as phenolic compound production (Bedetti et al. 2014; Suzuki et al. 2015; Oliveira et al. 2017). The phenolic accumulation into cells from the non-galled leaflets in all instars of *B. mataybae* galls (stem and leaflet phases) may indicate that phenolics are the main ROS dissipation for the plant and gall tissues. The occurrence of total phenolics in the chlorophyllous parenchyma and around the

nymphal chamber can be a response to ROS production by photosynthesis and to damage from insect feeding, respectively, as already reported in a recent review published by Kuster et al. (2019 *in press*). Phenolic derivatives such as lignin can be an additional mechanism of stress dissipation in the cell walls of gall tissues (Akhtar et al. 2010; Isaias et al. 2015). Lignin synthesis depends on ROS generation, especially the hydroxyl radicals, which are consumed and dissipated while lignin is deposited on the cell walls (Grace and Logan 2000; Blokhina et al. 2003). Thus, the lignin on the perivascular and sclerenchyma cell walls from the first to the last instar of the insect could be the secondary path for ROS dissipation in *B. mataybae* galls.

Recent studies have shown that some phenolic derivatives can act on the signaling and stimuli for gall growth, when synergistically associated with the auxins (Bedetti et al. 2014). This process acts on the inhibition of AIA-oxidases, thus increasing the action of auxins and leading to cell hypertrophy during gall formation (Fosket 1994), as demonstrated in *Piptadenia gonocantha* galls (Bedetti et al. 2014). For the *B. mataybae* leaflet galls, the histochemistry results support this function, since the auxin and total phenolics observed showed an ample synchronic presence inside the cells, mainly in the cortical parenchyma and near the nymphal chamber. The presence of alkaloids and terpenoids during leaflet gall instars may have some defensive function against the attack of parasitoids and predators, reducing competition for food by inhibiting the attack of other phytophagous insects (Janzen 1977; Cornell 1983).

Main considerations

The phenotype manipulator *B. mataybae* changes differently the morphogenetic patterns of stem and leaves of *M. guianensis*. Despite deep structural differences between stem and leaflet galls, there were no significant changes in the establishment of the histochemical profile. In addition, the instar changes in the leaflet gall were not the triggers of structural or

histochemical changes, indicating that the host organ potentialities probably restrict the primary and secondary traits of the galls. Thus, the studies on *Bystracoccus mataybae-Matayba guianensis* system indicate that the host potentialities can be as important as galling stimuli in gall structural and chemical definition.

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Table 1. Histochemical results of non-galled and galled tissues of stem and leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*.

Metabolite groups	**Non-galled organs**			**Galls**			
	Stem	Leaflets		Stem	Leaflets		
		Young	Mature	1° instar	2° instar	Adult	Senescent
				Primary metabolites			
Starch	-	-	-	-	-	+	+
Proteins	+	+	+	+	+	+	+
Lipids	+	-	-	+	+	+	+
Reducing sugars	+	+	+	+	+	+	+
Pectins	-	+	+	+	+	+	+
Auxin	+	+	+	+	+	+	+
				Secondary metabolites			
Alkaloids	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	-
Phenolics	+	-	+	+	-	+	+
Lignins	+	-	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
				Others			
ROS	+	+	+	+	+	+	+

+ = positive; - = negative

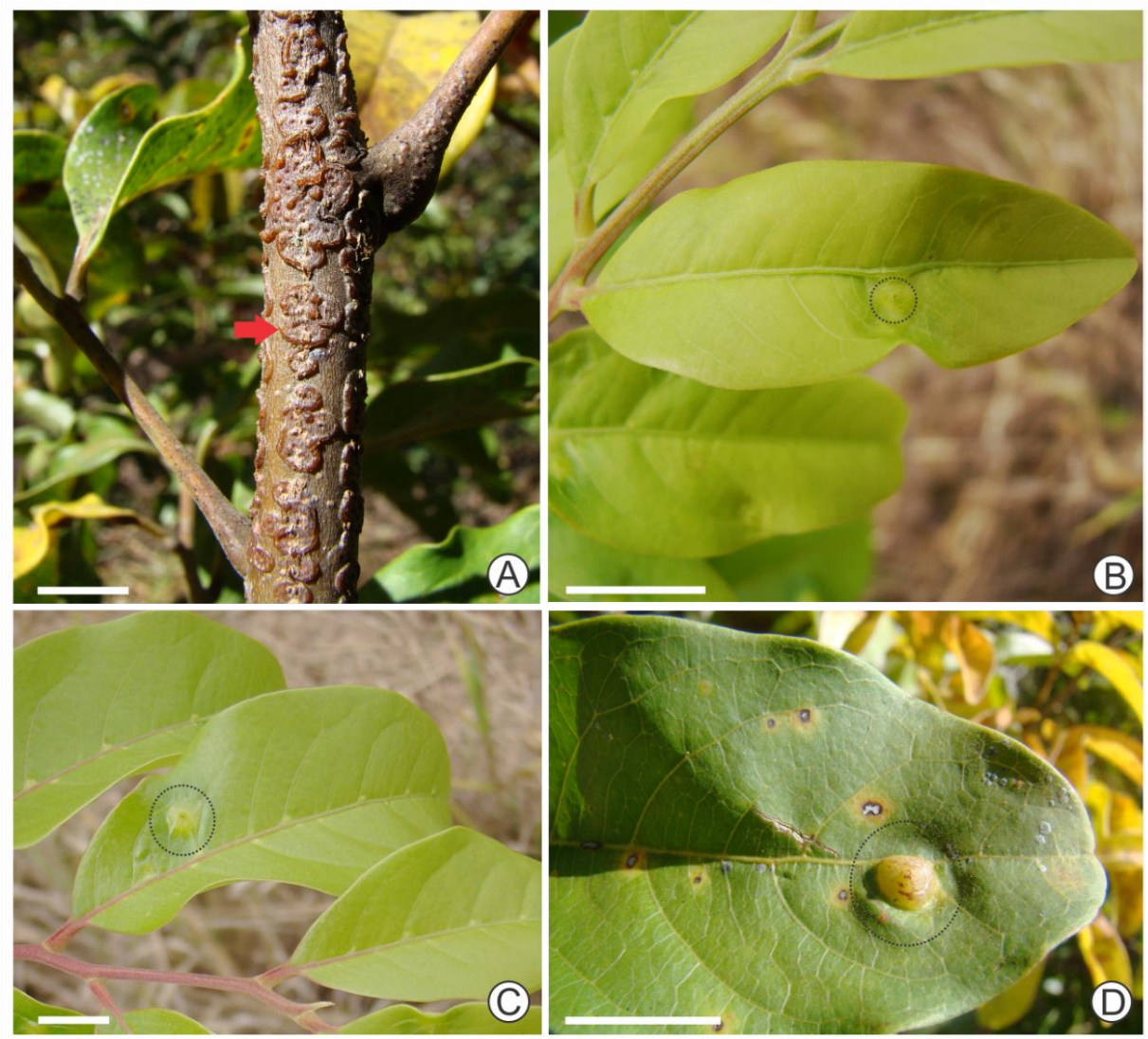

Figure 1. Galls induced by *Bystracoccus mataybae* on *Matayba guianensis*. A- Stem galls induced by galling insect at first nymph (crawler); B- Young leaflet galls - second instar nymph; C- Mature galls - adult stages; D- Senescent galls. *Scale bar:* 1 cm

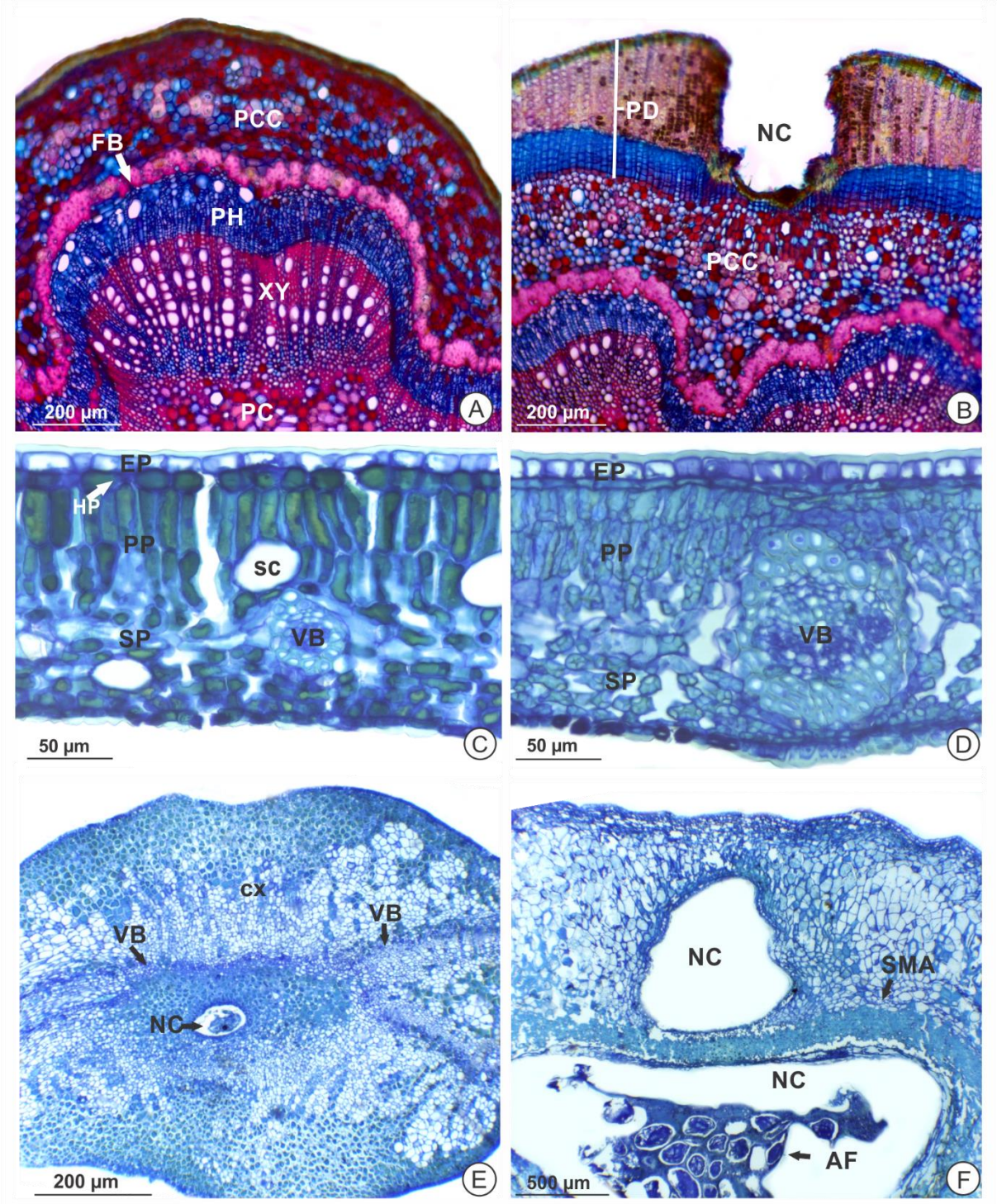

Figure 2. Anatomical features of non-galled and galled tissues of stem and leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A- Non-galled stem. Stem in secondary growth. Epidermis (arrow); B- Gall with first instar in the stem; C- Young non-galled leaflets with many secretory cavities; D- Mature non-galled leaflets; E- Gall with second instar in the leaflets. Vascular bundles in differentiation (arrows); F- Gall with third instar in the leaflets; the second

(cont.)

nymphal chamber is highlighted. *Abbreviations:* AF- Adult female with eggs; CX- cortex; Ep- Epidermis; FB- Fibers; HP- Hypodermis; NC- Nymphal chamber; PC- Parenchymatous cell; PCC- Parenchymatous cortical cells; Periderm; PP- Palisade parenchyma; SC- Secretory cavities; SMA- Sclerenchyma; SP- Spongy parenchyma; VB- Vascular bundle; XY- Xylem.

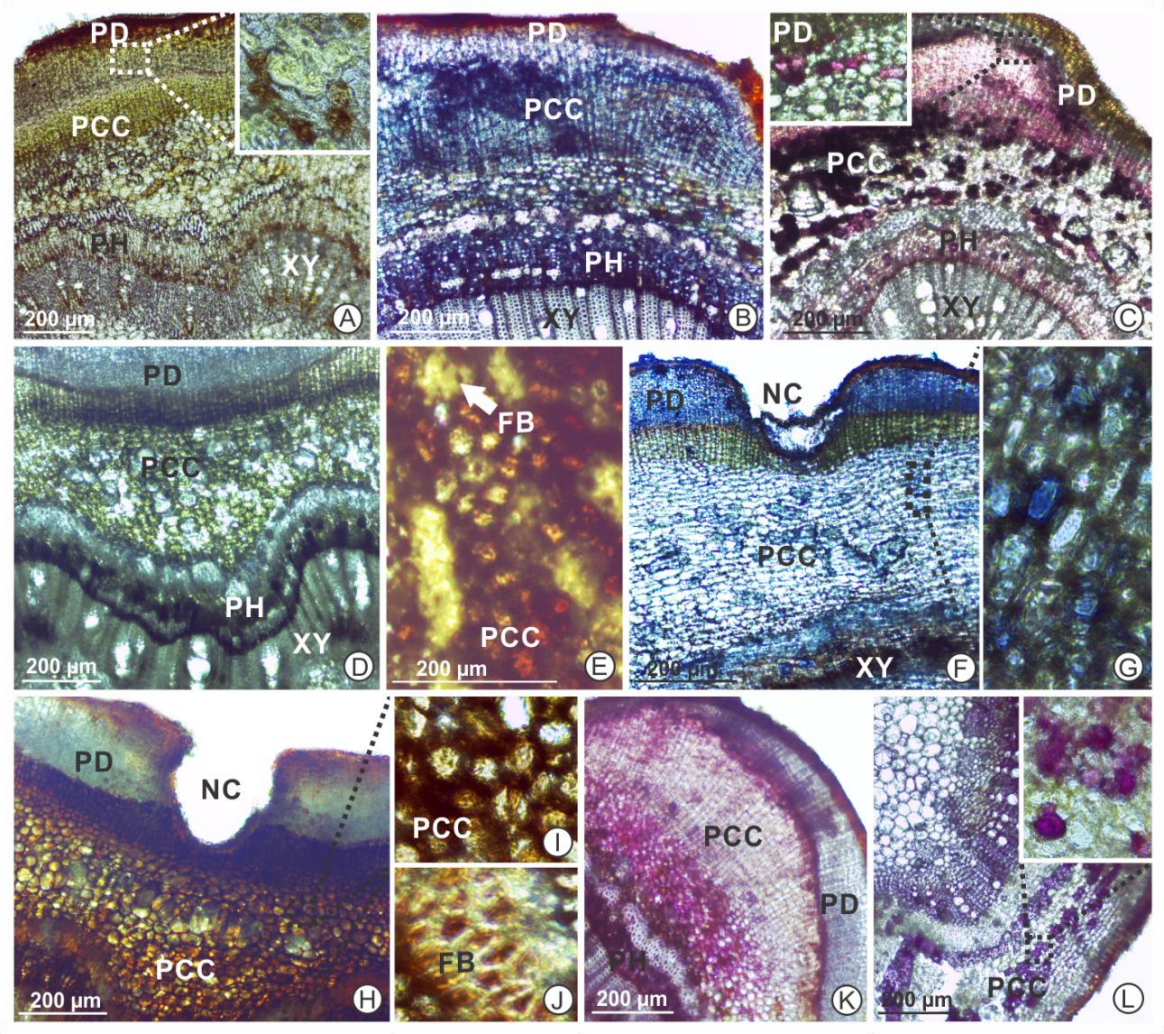

Figure 3. Positive histochemical results for galls with first nymph on the stem of *Matayba guianensis* induced by *Bystracoccus mataybae*. A-C- Primary metabolites; A- Reducing sugars in the cortical parenchyma cell; B- Proteins through the cortical parenchyma and central cells, phloem and xylem; C- Auxins in cortical parenchyma cells near the periderms; D-F- Secondary metabolites; D- Total phenolics in the cells of the cortical parenchyma; E- Alkaloids in the cortex with fibers (arrow); F- Terpenoids; G- Details of terpenoids in the cortex; H- ROS; I- Details of ROS in the cortical parenchyma cell; J- Details of ROS in the fibers; K-L- Structural polymers; K- Pectins in the cortical parenchyma cell walls; L- Lignins in the cortical parenchyma cortical cells. *Abbreviations:* FB- Fibers; NC- Nymphal chamber; PCC- Parenchymatous cortical cells; PD- Periderm; PH- Phloem; XY- Xylem.

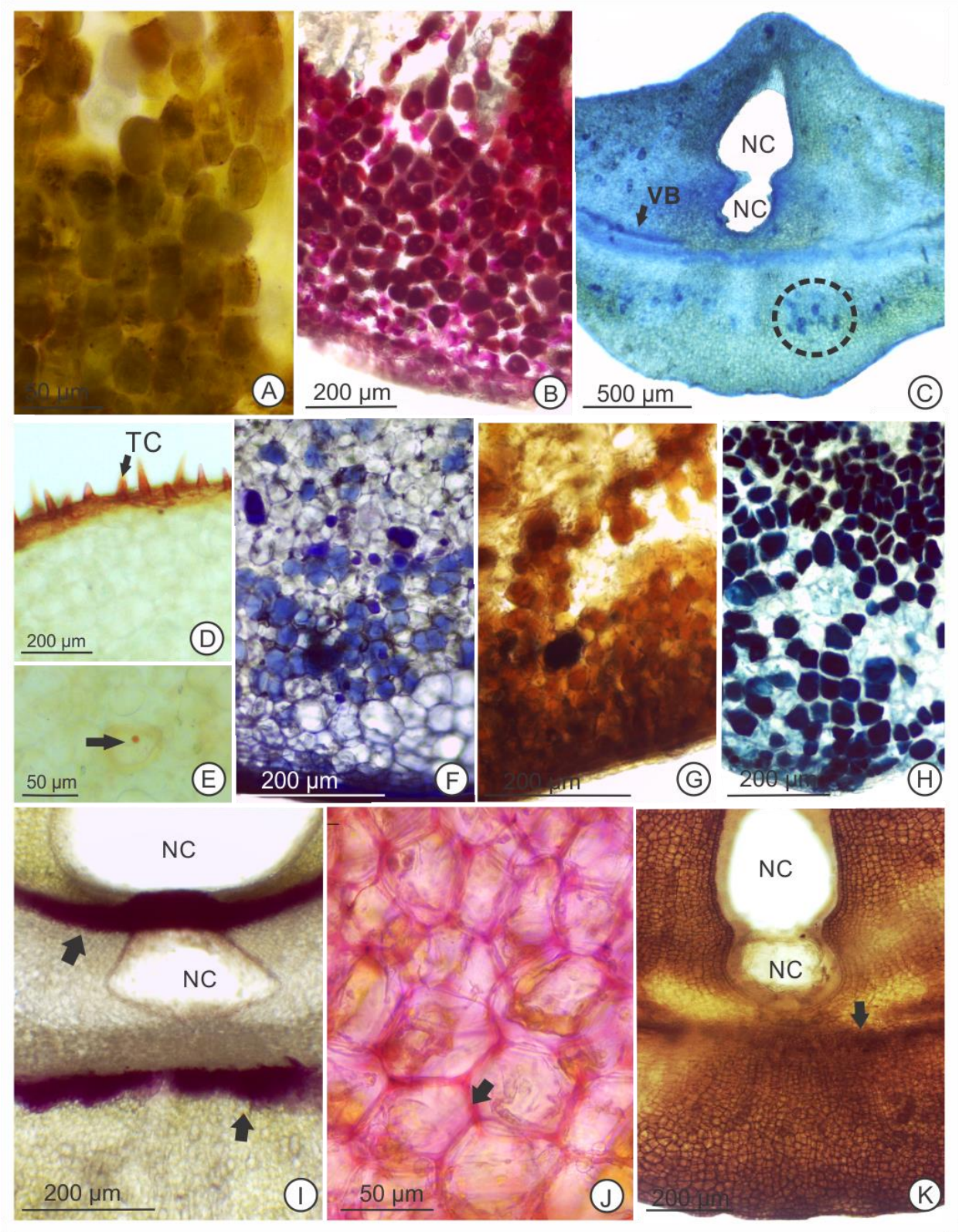

Figure 4. Histochemical positive results for a gall with a second instar nymph in the leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A-F- Primary metabolites; A- Reducing sugars in the outer cortex; B. Auxins on outermost cortex; C- Proteins in through the

(cont.)

gall tissues, especially in the cortex (circle); D- Lipids on the epidermis; E- A lipid droplet in the cortex (arrow); F-H- Secondary metabolites in the outer cortical parenchyma; F- Terpenoids; G- Alkaloids; H- Flavonoids; I-J- Structural polymers (arrow); I- Lignin in cells of the sclerenchyma (arrows); J- Pectin in cells walls of the cortex (arrow); K- ROS in all cells of the cortex, darker markings in the middle of the gall, region of the vascular bundles (arrow). *Abbreviations:* NC- Nymphal chamber; VB- Vascular bundle; TC- Trichomes.

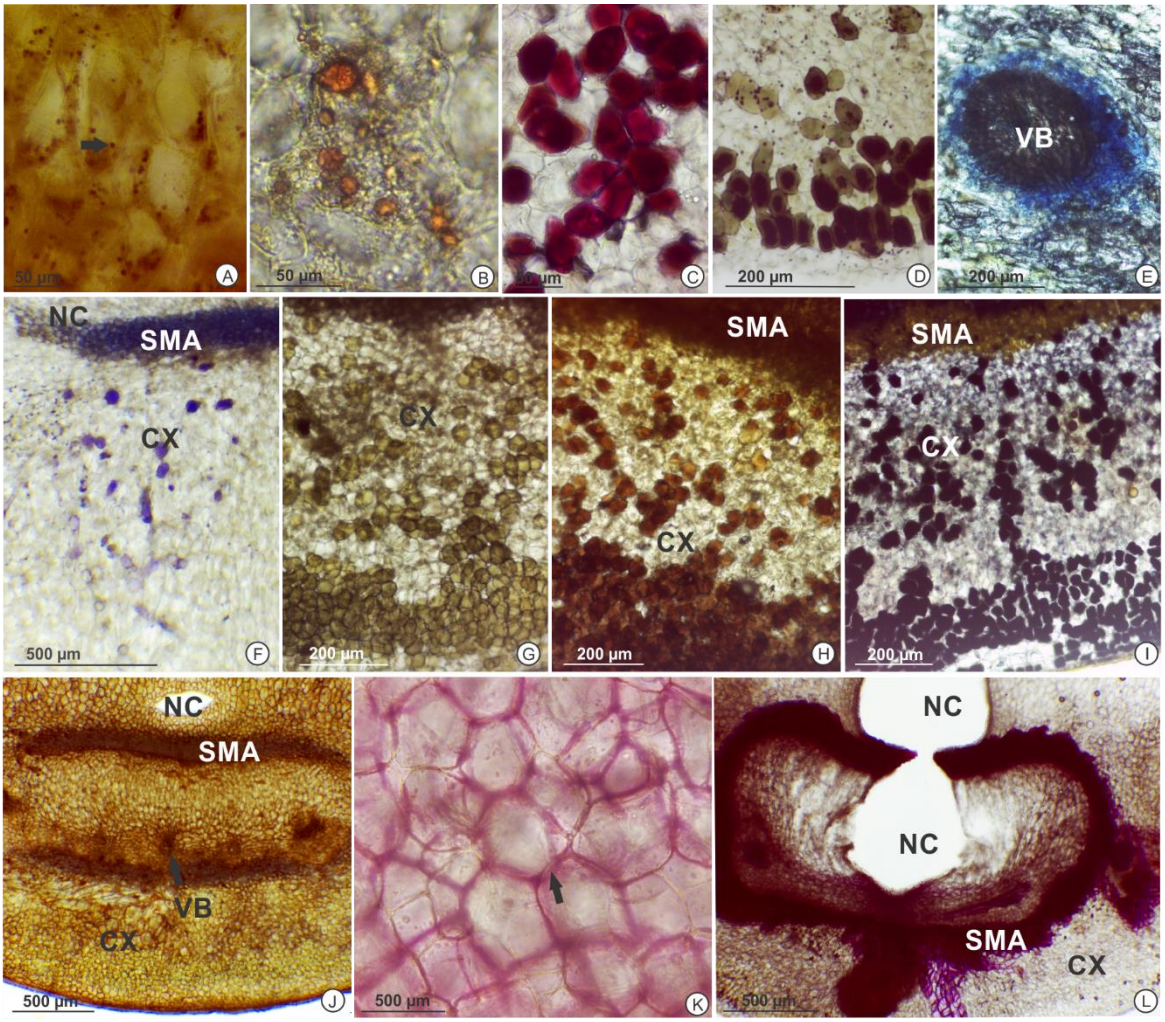

Figure 5. Positive histochemical results for a gall with a third instar nymph on the leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A-E- Primary metabolites; A- Starch in the cortex near the nymph chamber; B- Lipids in cortical cells; C- Auxins in the outermost cortex; D- Reducing sugars in the cortical cells; E- Proteins in the vascular bundles; F-I- Secondary metabolites in the cortical cells; F- Terpenoids in the cortical cells near the nymph chamber and in the sclerenchyma; G- Total phenolics; H- Alkaloids; I- Flavonoids; J- ROS with dark labeling in the vascular bundles (arrows) and sclerenchyma that surround the chamber of the insect; K-L- Structural polymers; K- Pectin in the cortical cell walls (arrow); L- Lignins in the sclerenchyma and vascular bundles. *Abbreviations:* CX- Cortex; NC- Nymphal chamber; VB- Vascular bundle; SMA- Sclerenchyma.

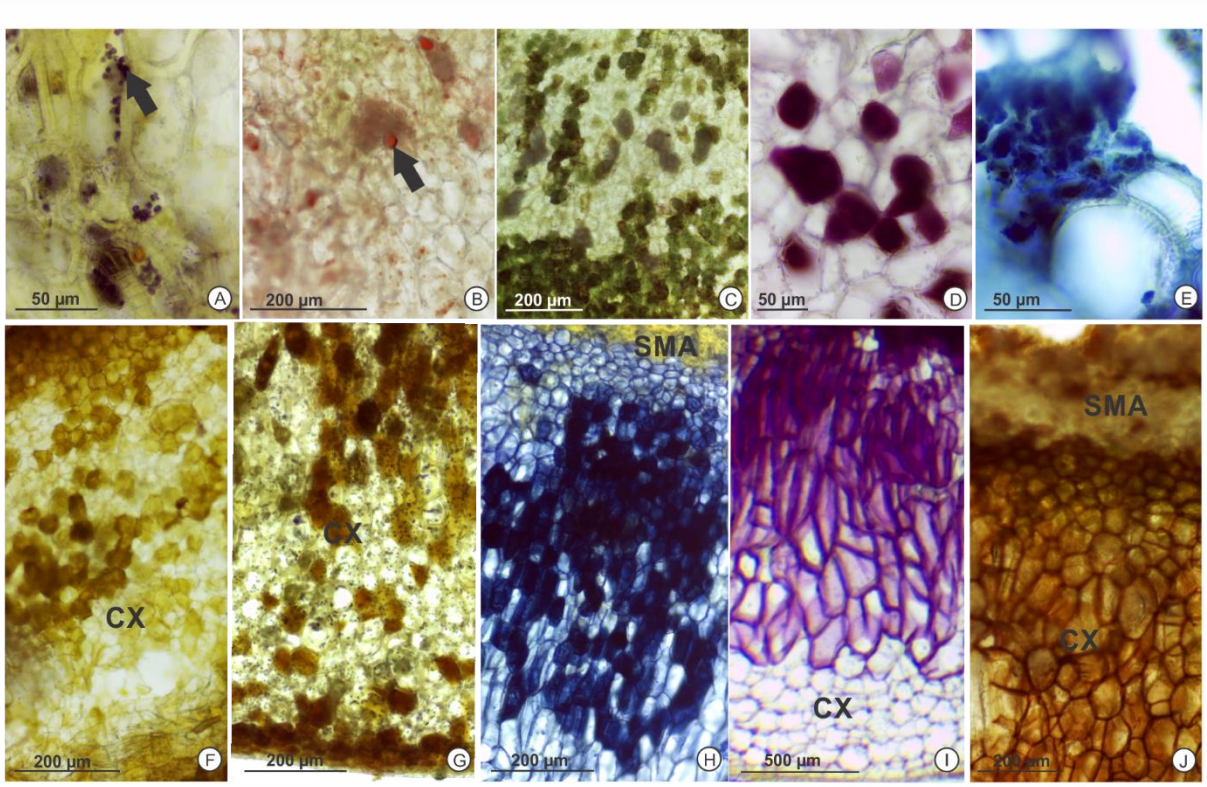

Figure 6. Positive histochemical results for the senescent gall phase on the leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A-E- Primary metabolites; A- Starch in the cortical cells (arrow); B- Lipids in the cortical cells (arrow); C- Reducing sugars in the outermost cortex; D- Auxins; E- Proteins in the cells of the vascular bundles; F-G- Secondary metabolites in the cortical cells; F- Total phenolics; G- Alkaloids; H- Flavonoids; I- Lignin in the median cortex; J- ROS – cortical cells labeled by ROS and cells of the sclerenchyma without labeling. *Abbreviations:* CX- Cortex; SMA- Sclerenchyma.

CAPÍTULO II

Chemical composition of cell walls in galls induced by *Bystracoccus mataybae* (Ericoccidae) on *Matayba guianensis* (Sapindaceae) assessed by immunocytochemistry and Raman spectrometer analysis

Abstract

Changes in the shape of the organs and tissues occurs during growth and development of plants as a result of cell division, elongation, structural modification, and reorganization of cell wall components. As a phenotype manipulator, the galling insects induce a new plant organ in the host plants, manipulating the structure and metabolism of host tissues to build the gall. The structure of galls depends on the new arrangement of cellulose microfibrils, changes in the degree of pectin methyl-esterification, and the distribution of compounds in the cell wall to allow growth and elongation of the cells. Herein, in addition to immunocytochemical approach, Raman spectroscopy was used as a new tool for investigation of the chemical composition of cell wall components during the development of galls induced by *Bystracoccus mataybae* (Eriococcidae) on leaflets of *Matayba guianensis* (Sapindaceae). Non-galled leaflets and galls showed variation in pectin and protein distribution in tissues by immunocytochemistry analyses. Epitopes of (1→4) β-D- galactans, and (1→5) α-L-arabinans were evident in the tissues of the young and senescent galls. The degree of methyl-esterification of homogalacturanans increases from young to the senescent galls. Variations in the distribution of cellulose, pectins and lignins were recorded by the Raman spectrometer, where bands of pectin and lignin were more evident in mature and senescent galls. Spectra from different regions of the cell walls showed that chemical composition changes during the development of the gall, letting to specific cell wall shape and functionalities.

Keywords: Gall development, Morphotype, Cell wall components, Pectins, Lignins.

Introduction

The development of plant organs depends on the patterns of cell divisions, growth and elongation. Thus, understanding the dynamics of cell wall organization can give us new insights and perspectives about the acquisition of new cell wall properties, tissue functions (Meijer and Murray 2001; Rose 2003; Albersheim et al. 2010) and interactions with biotic agents (Sarkar et al. 2009). In the primary cell walls (e.g. cellulose, hemicelluloses and pectins), the cellulose microfibrils are immersed in a matrix of hemicellulose, pectins, and proteins (Cosgrove 2005; Amar et al. 2010; Albersheim et al. 2010), which form a three-dimensional network responsible for its permeability, as for exchange of nutrients, catabolites and chemical signals between the cells (Albersheim et al. 2010). During the primary cell wall formation, cellulose and non-cellulosic elements are deposited on the surface of older microfibrils, which are more arranged more longitudinally and parallel in response to cell wall stretching (Albersheim et al. 2010).

Pectins are non-cellulosic structural elements, divided in many different groups, as homogalacturonans (HGs), rhamnogalacturonans I (RGI), rhamnogalacturonans II (RGII), xylogalacturonans (XGA) and apiogalacturonans (AP) (Ridley et al. 2001; Willats et al. 2001; Mohnen 2008; Caffall and Mohnen 2009; Wolf et al. 2009; Albersheim et al. 2010). HGs are linear polymers of (1→4)-α-galacturonic acid esterified in different degrees with methyl and acetyl groups. The RGI is composed by repeating subunits of (1→2)-α-L rhamnosyl- (1→2)-α-D-galacturonyl disaccharides with variable side chains of arabinans and galactans (Wydra and Beri 2006). The RGII consists of many unusual polysaccharides and are not present in large amounts in cell wall (O'Neill et al. 2004). The arabinogalactans proteins (AGPs) are another important component of the cell wall, a class of the hydroxyl-proline-rich glycoprotein family, which play a role in plant growth and development (Gao and Showalter 2000).

During formation of the secondary cell walls, lignins are deposited. Lignins are a complex polymers formed by a variety of different types of covalent bonds derived from oxidative coupling. This oxidative coupling comes from three different types of phenolic precursor units, p-coumaryl, coniferyl, and sinapyl alcohols (Arjyal et al. 2000; Lewis and Yamamoto 1990), that binds and anchors cellulose microfibrils and may prevent the cellular damage (Ralph et al. 2004). In the secondary cell walls, there are sublayers named S1, S2 and S3, which may be differed among in the chemical composition and the alignment of cellulose microfibrils (Agarwal 2006). In fact, components of cell wall are expected to be realigned, over or dawn expressed during the interactions with biotic stressors, as the galling insects for example.

As a phenotype manipulator, the galling insects induce a new plant organ in the host plants (Shorthouse et al. 2005), manipulating the tissue composition and metabolism to build the gall (Mani 1964; Oliveira et al. 2014; Oliveira et al. 2016). The cell hypertrophy and tissue hyperplasia are the main convergent processes in gall formation and, sometimes, cell redifferentiation (Oliveira et al. 2010; Isaias et al. 2014). In galls, the changes in cell growth and elongation depend on the new arrangement of cellulose microfibrils (Magalhães et al. 2014), as well as changes in the level of pectin methyl-esterification and distribution in cell walls (Formiga et al. 2013; Oliveira et al. 2014; Carneiro et al. 2015). The HG methyl-esterification act in many physical cell wall properties, such as deposition of highly methyl-esterified HGs in young galls (Carneiro et al. 2015; Teixeira et al. 2018). The processes of de-methyl-esterification of the HGs can occur during gall development (Carneiro et al. 2015) or be impaired (Teixeira et al. 2018). In addition, analyses in some arabinogalactan proteins (AGPs) in cell walls have been revealed contradictory results during gall development, since they are being detected

in young (Oliveira et al. 2014), mature (Oliveira et al. 2014) or senescent (Formiga et al. 2013; Carneiro et al. 2014; Teixeira et al. 2018) gall phases.

Until now, the dynamics and composition of cell walls during gall development have been accessed using immunocytochemical analyses. These analyses have proven to be an excellent tool to understand the impact caused by the galling insect in the host tissue and the new functionalities established in the gall tissues. Herein, in addition to immunocytochemistry approach, the Raman spectroscopy has been used as a new tool in the research of plant cell walls, basing on detect on molecular vibrations (Atalla and Agarwal 1985; Gierlinger 2017). The Raman technique provides a very efficient means for the investigation of the chemical composition of each cell wall components, as well as the distribution of the different polymers (Agarwal 2006), microstructure and compounds interaction (Gierlinger 2017). To the best of our knowledge, Raman spectroscopy has not yet been applied in gall development studies and it could provide a novel type of understanding about chemical changing in cell wall to build the gall. In this context, we aimed to investigate the composition and dynamics of cell walls during the development of galls induced by *Bystracoccus mataybae* (Eriococcidae) on leaflets of *Matayba guianensis* (Sapindaceae) using both immunocytochemistry and Raman spectroscopy approach.

Material and methods

Sampling area and study model

The samples were collected at the Estação Ecológica do Panga (19° 10'S, 48°24'W) and at the Fazenda Experimental do Glória (18º57'S, 48º12'W) at Uberlândia city, Minas Gerais State, Brazil. Thus, 10 individuals of *Matayba guinanesis* with galls induced by *Bystracoccus mataybae* were selected for the current study. *Bystracoccus*

mataybae (Eriococcidae, Hemiptera) induce galls on leaflets of *Matayba guianensis* Aubl. (Sapindaceae), an abundant shrub occurring in the Cerrado (Sano et al. 2008). *B. mataybae* has an univoltine life cycle, synchronized with vegetative plant phenology. This galling insect induce two different gall morphotypes on the same host plant, on the leaflets and on the stem. Stem galls are induced by first instar nymphs (crawlers) at the dry season. When the leaf sprouting begins, the second instar nymph moves to the leaflets to induce a new gall cycle. At the leaflets, the second nymph becomes adult, gets pregnancy and dies after the crawlers get out of the leaflets galls and move to stem (Pfeffer et al. 2018).

Herein, we just used non-galled leaflets and leaflet galls collected from August 2017 to March 2018 for immunocytochemistry and Raman spectroscopy analyses. The samples (n = 10 per developmental stage) were selected based on different stages of development: (i) Young and mature non-galled leaflets; (ii) young leaflet galls - with second instar nymph; (iii) mature galls - with adult female stages or/and adult females with eggs and/or crawlers; and (iv) senescent galls – when the adult female is not alive.

Immunocytochemical analysis

Fresh samples were washed in 50% methanol and shaken on an orbital shaker (3 times of 30 min) to remove phenolic compounds (Singleton et al. 1999). After that, all samples were fixed in 70% alcohol, followed by freehand-cut transverse sections. To prevent crosslinking, block solution (3% powdered milk in phosphate buffered saline (PBS) - pH 7.1) was used as first step of incubation, during 30 minutes, followed by incubation with primary monoclonal antibodies LM1, LM2, LM5, LM6, LM19 and LM20 (Centre for Plant Sciences, University of Leeds, UK) (1:10 dilution in milk/PBS for 1-2 h), for detection of pectins and proteins of cell wall, summarized in Table 1. In

sequence, the samples were washed in PBS, and then incubated in a secondary FITC antibody (Sigma®, USA) (1: 100 in 3% milk / PBS) for 2 hours in the dark. Control was conducted by suppressing incubation with the primary antibody. All samples were mounted in 50% glycerin. A fluorescence microscope (Leica® DM4000B) and a monochromatic camera (DFC3000 G, Leica®) with a CCD sensor were employed to obtain the images.

Raman Spectroscopy

Sample preparation

Samples of fresh leaflet galls were embedded in Polyethylenglycol (PEG) (50% melted at 60 ºC - 50 % deionized water, v: v), followed by fully water evaporation in heating plate at 55-60º (Gierlinger et al. 2012). After from 24 to 48 hours, the fragments were embedded in pure PEG (Kraus and Arduin 1997) and sectioned in a rotary microtome (YD-315 model, China) at thickness of 18 µm in transverse section. The PEG was removed of the samples after washing with water to avoid misinterpretation and false results.

Raman Measurements

Raman spectra were recorded using a LabRAM HR Evolution Raman spectrometer (Horiba scientific – New Jersey) equipped with a confocal microscope (Olympus BX41). Parenchyma cell walls of galls in different stages of development were obtained by using the serial mapping, polygon type. Based on 686 spectrums per area/stage, Raman images of a transverse section of galls were calculated by integrating different bands attributed to the functional groups of plant cell walls of *Matayba guianensis* galls. They were calculated by integration over the wavenumber range 1562-1692 cm^{-1} (C, aromatic ring and C=O stretch of lignin) (Agarwal and Ralph 1997), 840-

880 cm^{-1} (D, α-1-4 glycosidic bond in pectin) (Synytsya et al. 2003) and 978-1778 cm^{-1} (cellulose) (Agarwal and Ralph 1997). For the measurement, a 40x magnification objective was used. A 532 nm laser with 150 mW power and 40 seconds exposure time was accumulated in 3 times to remove the signal-to-noise ratio (Agarwal 2006). The operating parameters were kept constant for all measurements. The spectra were processed using the LabSpace 6 from Horiba Scientic/map software package and background corrected prior to analyses.

Results

Immunochemistry

The epitopes of pectins and AGPs were labeled differently in the distinct developmental stages of galls induced by *B. mataybae* on leaflets of *M. guianensis* and in the distinct gall tissue compartments, as well as from the non-galled leaflet tissues (Table 2).

Non-galled leaflets- Epitopes of high methyl-esterified HGs, recognized by LM20, were moderately labeled in the mesophyll of the young leaflet (Figure 1A), while the epitopes of (1→5) α-L-arabinans, recognized by LM6, were moderately labeled in cell walls of adaxial epidermis, in the chlorophyllous parenchyma and xylem (Figure 1B). Epitopes of low methylesterified HGs, recognized by LM19, showed moderate to intense labeling in few cell walls of the parenchyma, xylem and abaxial epidermis (Figure 1C). In the vascular bundles of young non-galled leaflets, the epitopes of β-D- galactans, recognized by LM5, were intensely labeled on the cell walls of xylem (Figure 1D). In the mature leaflets, some cell walls of xylem were labeled for (1→4) β-D- galactans (Figure 1E), (1→5) α-L-arabinans (Figure 1F), and low and high methyl-esterified HGs (Table 2).

Young galls- Epitopes of (1→4) β-D-galactans, recognized by LM5, were moderately labeled on the cell walls of parenchyma (Figure 2A) and epidermis (Figure 2A). Epitopes of (1→5) α-L-arabinan, recognized by LM6, showed weak labeling in the cell walls of parenchyma (Figure 2B). Epitopes of low methyl-esterified HGs, recognized by LM19, were moderately detected in the cell walls of epidermis, parenchyma and vascular bundles (Figure 2C). Epitopes of high methyl-esterified HGs, recognized by LM20, were moderate detected in cell junctions of the parenchyma (Figure 2D), while intense labeling was detected by (1→4) β-D- galactans in cell walls in the vascular bundles (Figure 2E). The epitopes of AGPs, recognized by LM2, were moderately labeled in cell walls of parenchyma (Figure 2F).

Mature galls- Epitopes of high methyl-esterified HGs were weakly labeled in parenchyma cell walls (Figure 2G), while epitopes of low methyl-esterified HGs were absent in this stage of development. The epitopes of AGPs were weakly detected in parenchyma and sclerenchyma cell walls (Figure 2H). Epitopes of (1→5) α-L-arabinan were moderately detected in the cell walls of parenchyma and xylem (Figure 2I).

Senescent galls- The epitopes of (1→4) β-D- galactans, (1→5) α-L-arabinans and high methyl-esterified HGs were labeled in parenchyma cell walls of senescent galls. The (1→4) β-D- galactans were labeled intensely in sclerenchyma cell walls, delimiting the nymphal chamber (Figure 3A). Moderate labeling of (1→5) α-L-arabinans was found in parenchyma cell walls (Figure 3B), while weak detection of high methylesterified HGs occurred in cell walls of the parenchyma and sclerenchyma (Figure 3C). The epitopes of extensins, recognized by LM1, were moderately detected in inner part of parenchyma and the sclerenchyma cell walls (Figure 3D). In the vascular bundles, AGPs (Figure 3E) and (1→5) α-L-arabinans (Figure 3F) were labeled in xylem cell walls.

Raman Spectroscopy

A simple image and plot calculations based on the integrals of the marker bands of plant cell wall polymers gave us a structural overview of the cell walls on *Matayba guianensis* galls. There were changes in the chemical composition and distribution of cell wall components in the parenchyma cells of galls during their development, differing in relation to intensity of occurrence (Figure 4). Young galls showed almost for carbohydrates bands at 978 -1778 cm^{-1} (Figure 4B-C). The band at 840-880 cm^{-1} recognized pectins in highest intensity in mature gall phase (Figure 4B-D). The strong lignin band at 1562-1692 cm^{-1} is assigned to aryl stretching vibrations of lignin and revealed high intensity in senescent galls (Figure 4B-E). Associated with each pixel in Raman, there is a spectrum representing a molecular fingerprint at every mapped position. Details about the molecular composition in different regions of the cell (e.g. cell corner, middle lamella and inner part of the cell) in parenchyma cell walls of gall at different stages were obtained by the extraction of a single spectrum. The spectra obtained showed several signals of pectins, cellulose and lignins. The assignments of the most prominent bands are given in Table 3.

The young gall spectrum (Figure 5A) showed peaks of 866 cm^{-1} and 998-1076 cm^{-1}, which indicate a high content of pectins and cellulose, especially in the middle lamella, respectively. The lignin was recognized by the band 1214 cm^{-1} and 1327 cm^{-1} in the inner cell wall of young galls. The spectrum of mature gall (Figure 5B) has pectins at bands of 861 cm^{-1}, higher intensity of cellulose at 1451cm^{-1}, and lignin band at 1273 cm^{-1} in the region of middle lamella and at band 1608 cm^{-1} in the inner cell wall. Spectrums of senescent galls (Figure 5C) showed contributions of pectins at 850 cm^{-1} and cellulose at bands at 977-1336 cm^{-1}, as well as higher intensity of lignin at 1557 cm^{-1} and 1657 cm^{-1}.

Discussion

The biotic stress induced by the *B. mataybae* galling insect on leaflets of *M. guianensis* changes the chemical composition and distribution of the structural polymers on cell walls during the development of galls. The higher deposition of (1→5) α-L-arabinans and (1→4) β-D- galactans on non-galled leaflets and galls was evident, especially in parenchyma and xylem cell walls, indicating their high functionality to cell development in *Matayba guianensis* non-galled and gall tissues. The almost absence of HG epitopes with low and highly methyl-esterified groups on sclerenchyma cell walls indicates that they are not associated with its gain of shape and functionalities on this gall system. Despite this, these epitopes appear to be related to structural changes of the parenchyma cells, as reported for other galls (Carneiro et al. 2015; Oliveira et al. 2014; Oliveira et al. 2016). Moreover, Raman spectrometer showed that chemical profile is different among parenchyma cell wall sites and between the development of gall.

Immunocytochemical profile

The deposition of pectins highly methyl-esterified, recognized by LM20, mainly in the junctions of parenchyma cell walls of the young gall cortex can act on adhesion, cell elasticity and porosity of the cell wall (Bouton et al. 2002; Willats et al. 2001). Moreover, their labeling in the median lamella of mesophyll cells is related to well-transport of photosynthetic products (Giannoutsou et al. 2013). The moderate intensity of reaction of LM20 during the mature and senescence gall phases also contributes to cell wall loosening and expansion, as reported in the mesophyll of *Vigna sinensis* (Sotiriou et al. 2016).

Epitopes of low methyl-esterified HGs, recognized by LM19, are linked by calcium bridges that form cell wall matrix gels with high viscosity (Willats et al. 2001;

Jarvis et al. 2003; Wolf et al. 2009). The deposition of these epitopes seems to develop a key role as a barrier to cell wall detachment during intercellular space formation (Giannoutsou et al. 2013). Therefore, the occurrence of these epitopes in epidermis, parenchyma and vascular bundles in the young non-galled and gall tissues fit with the previously role reported. The degree of methyl-esterification of HGs changed from young to senescent gall phases and occurs by the action of the pectin methylesterases (PMEs). The PMEs are responsible to de-methyl-esterification of the HGs by removing the methyl groups, making the pectin polymers firmer and, strengthening cell wall structure (Knox et al. 1990; Sabba and Lulai 2005). The activity of PMEs in galls can be impaired, as observed in the *Baccharis dracunculifolia* - *Baccharopelma dracunculifoliae* system (Oliveira et al. 2014) and *Clinodiplosis* - *Croton floribundus* system (Teixeira et al. 2018), as well as for *M. guianenis* leaflet galls. Then, in *M. guianensis* galls, the nymph also may block the PME activity, resulting in the total absence of epitopes of low methyl-esterified HGs in the mature and senescent gall phases. The occurrence of highly methyl-esterified HGs, recognized by LM20, in cell wall contacts may aid the detaching cell wall areas (Orfila et al. 2001; Willats et al. 2001, 2004; Ordaz-Ordiz et al. 2009), as reported in some senescence organs (Albersheim et al. 2010). Because of that, in *M. guianensis* leaflet galls, some cell walls lose some adhesion in mature and senescent phases.

The presence of (1→4)-β-D-galactans, (1→5) α-L-arabinans and HGs in the senescent *M. guianensis* galls should maintain the mechanical stability of the cell walls (Gao and Showalter 1999; Schindler et al. 1995; Sabba and Lulai 2005), as well as it contributes to the increase of the porosity necessary for the final signaling of the cell cycle (Oliveira et al. 2014). The epitopes of (1→4)-β-D-galactans, recognized by LM5, have been reported in mature cells (e.g. pericarp of *Lycopersicon esculentum*) (Orfila and Knox 2000), keeping some cell wall extensibility. The labeling of these epitopes in vascular

bundles of *M. guianensis* non-galled and young gall tissues allow more cell wall flexibility, which permits vascular system growth (Oliveira et al. 2014). The (1→5) α-L-arabinans, recognized by LM6, allow maintenance of cell wall hydration and, then, are responsible for cellular turgor control (Jones et al. 2003). The increase of LM6 labeling in senescent gall phase, associated to maintenance of the methyl-esterified status of HGs, may indicate the conservation of juvenile features in some tissue compartments of *M. guianensis* gall in the final phase of development.

In relation to the glycoprotein content of cell walls, the AGPs, recognized by LM2, are involved in cell wall formation (Mastroberti and Mariath, 2008) and can act as a protective zone that preventing cell disruption during changes in cell walls (Lamport et al. 2006), ensuring flexibility. As previously reported, the AGPs have contradictory results for galls, since they may occur in all gall stages (Formiga et al. 2013; Carneiro et al. 2014; Oliveira et al. 2014; Teixeira et al. 2018). However, here they were more intensely labeled in young *M. guianensis* gall and fit with the cell adhesion, elongation, and cellular proliferation roles (Majewska-Sawka and Nothnagel, 2000; Seifert and Roberts, 2007). The extesins, recognized by LM1, reinforce the plant cell wall (Sabba and Lulai 2005) and usually occur in mature tissues, after cessation of growth (Cassab 1998). The cell wall reinforcement was reported for *Clinodiplosis* - *Croton floribundus* system (Teixeira et al. 2018) in senescent gall and in mature pocked galls of *Baccharis reticularia* (Formiga et al. 2013). The *M. guianensis* leaflet galls showed similar results of *Clinodiplosis* - *Croton floribundus* system (Teixeira et al. 2018), allowing both structural reinforcement and growth control.

Chemical composition by Raman analysis

The cellulose is a crystalline polymer and its orientation varies in the different layers of the cell wall, changing the intensity in different cell wall layer sites (Agarwal 2006). The cellulose wave number positions by Raman spectroscopic are well known (Wiley and Atalla 1987), have been detected for cellulose bands in *M. guianensis* galls at 989 cm^{-1} and 1097 cm^{-1}, assigned to C-H and C-H_2 asymmetric stretching vibration of the C-O-H glycosidic linkage (Wiley and Atalla 1987; Ji et al. 2013; Gierlinger et al. 2013), and at 1336 cm^{-1} due to deformation vibration (δ(HCC) and δ(HCO)) of the macromolecule (Agarwal 1999; Zeise et al. 2018). The presence of this polymer was found in the middle lamella of *M. guianensis* young galls and can ensure the adhesion of a cell to its neighbors (Mateu et al. 2014), while low peaks were found in senescent galls, because regions of highest lignin concentrations are known to be locations of low cellulose concentrations (Meier 1985).

Pectin bands are sensitive to the process of acetylation (CH_3-C(=O)) and methylation (CH_3) that occurs within its structure (Synytsya et al. 2003). Together, both reactions can enhance the effect of each other and leads to decrease in free hydroxyl (OH) content (Fillippov 1980) and the weakening of hydrogen bonds, which are responsible to stabilizes the pectin conformation (Synytsya et al. 2003). Moreover, the peaks at bands around 862 cm^{-1} (maximum) may be associated with increases of acetylation, while decrease of the wave number 850 cm^{-1} (minimum) may be related with the methylation (Synytsya et al. 2003). In *M. guianensis* leaflet galls, bands at 866 - 861 cm^{-1} were detected in young and mature phases. At 866 cm^{-1}, the acetylation could have destabilized the structure of the pectin and made it more less rigid, allowing the expansion of the cell walls at these stages of development. In the senescent phase, in band at 850 cm^{-1}, the process of methylation may allow the loosening of the cell walls of the gall and can be a

metabolic pathway to be explored. The middle lamella and cell wall corners are rich in calcium (Ca^{+2}) and pectates (Carpita and Gibeaut 1993), and these are the first sites to be lignified (Boerjan et al. 2003), which can explain the high distribution of pectin in mature phase (Figure 4) and its substitution for lignin in the senescent phase (Figure 4B). Besides that, pectins might be involved in the process mechanical/physical cellulose microfibrils aggregation in middle lamella (Thimm et al. 2009). Studies in celery collenchyma revealed that cellulose microfibrils are not in lateral contacts, among cells by hydrated matrix of polysaccharides (Kennedy et al. 2007).

The lignin structure varies in composition in the different taxa of plants, cell types, tissue layers and are influenced by developmental and environmental cues (Campbell and Sederoff 1996). Lignin deposition proceeds in different phases during gall development, but interesting bands at 1214 cm^{-1} and 1327cm^{-1} were found in young galls, in the inner cell wall. Primary cell walls are defined as growing unlignified tissues, however the increasing lignin content in the primary cell walls bring more mechanical stability to the cell wall (Richter et al. 2011). In maize coleoptile, cell wall stiffness and the growth structure can be controlled by lignification of the primary cell wall (Musel et al. 1997). Besides that, lignin was also reported in primary cell walls after wounding an attack of parasitic fungi (Lewis and Yamamoto 1990; Boudet, 2000). In *M. guinanensis* galls, cell wall lignification is an additional mechanism of stress dissipation (Oliveira et al. 2017). Reactive oxygen species, such as hydrogen peroxide, can dissemble the calcium channels with the microtubules (Mazars et al. 1997), resulting in calcium influx and consequently the destabilization of microtubules (Livianos et al. 2012). Biotic attack activates the calcium influx faster (Jeworutzki et al. 2010), changes the orientation of both microtubules and the microfibrils of cellulose, allowing the adjustment of the morphogenesis of plants (Nick 2014). Thus, the lignification process in *M. guianenis* galls

can occur both for the reduction of oxidative stress (as a mechanism of stress dissipation) and for morphogenesis of the gall. In the mature galls, bands at 1273 cm^{-1} and 1608 cm^{-1} were found by the stretching of the aryl ring. Bands of lignin were higher in the senescent galls, dominated by the strong band around 1595 cm^{-1} and at 1657 cm^{-1}, typical signals of lignin appear, caused by different stretching vibrations of the aryl ring of coniferyl aldehyde and coniferyl alcohol (Gierlinger, 2013; Zeise et al., 2018) in the region of the inner cell wall, place of deposition of secondary cell wall.

Main considerations

The chemical composition of the cell walls changes during the development of *M. guianensis* gall, letting to specific cell wall shape and functionalities. The investigation of the cell walls chemical composition and sites was assessed by two techniques and revealed the conservation of juvenile features even in the senescent galls. These properties may be related to the water binding capacity of the galacturonic acid residues and indicate that polymers containing such groups can hydrate and keep the cells turgid. The Raman also proved to be a good tool for the investigation of the gall lignification process, suggesting that the presence of lignin contributes to the growth and shape of *M. guianensis* gall since the young phase, even in primary cell walls where the lignification processes begins at cell junctions and median lamella.

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Table 1. Recognition of monoclonal antibodies and their epitopes utilized for labeling protein and pectin in cell walls of *Bystracoccus mataybae* - *Matayba guianensis* galls.

Monoclonal antibodies	**Epitopes**	**References**
LM1	Extensins	Smallwood et al. 1996
LM2	Arabinogalactan glycan (AGP)	Smallwood et al. 1996; Yates et al. 1996
LM5	(1→4) β-D- galactans	Jones et al.1997; Williats et al. 1999; Orfila and Knox 2000 and Andersen et al. 2016
LM6	(1→5) -α-L-arabinans	Williats et al. 1998; Williats et al. 1999; Orfila and Knox 2000; Orfila et al. 2001; Lee et al. 2005 and Verhertbruggen et al. 2009
LM19	Partially methyl-esterified homogalacturanan (HG)/ no ester	Verhertbruggen et al. 2009
LM20	Partially methyl-esterified HG	Verhertbruggen et al. 2009

Table 2. Intensity of reaction of monoclonal antibodies in non-galled leaflets and in different developmental stages of *Bystaracoccus mataybae* - *Matayba guianensis* galls.

Monoclonal antibodies	Stages	Epidermis	Parenchyma	Sclerenchyma	Vascular system
LM1	Young non-galled leaflets	-	-	-	-
	Mature non-galled leaflets	-	-	-	-
	Young gall	-	-	-	-
	Mature gall	-	-	-	-
	Senescent gall	+	+	+	-
LM2	Young non-galled leaflets	-	-	-	+
	Mature non-galled leaflets	+	-	-	-
	Young gall	-	++	-	-
	Mature gall	-	+	-	-
	Senescent gall	+	-	-	+
LM5	Young non-galled leaflets	-	-	-	+++
	Mature non-galled leaflets	-	-	-	++
	Young gall	+	++	-	+++
	Mature gall	-	-	-	-
	Senescent gall	-	++	+++	-
LM6	Young non-galled leaflets	+	+	-	++
	Mature non-galled leaflets	-	-	-	+
	Young gall	-	+	-	-
	Mature gall	-	+	-	+
	Senescent gall	-	++	-	++
LM19	Young non-galled leaflets	++	++	-	++
	Mature non-galled leaflets	-	-	-	++
	Young gall	-	+++	-	-
	Mature gall	-	-	-	-
	Senescent gall	-	-	-	-
LM20	Young non-galled leaflets	-	++	-	-
	Mature non-galled leaflets	-	-	-	+
	Young gall	-	++	-	-
	Mature gall	-	+	-	-
	Senescent gall	-	+	+	-

Intensity of the reaction: (-) negative, (+) weak, (++) moderate, and intense (+++).

Table 3. Band assignments in the Raman spectra of the data sets from parenchyma sections of galls of *Matayba guianensis* in different stages of development.

Stages of development	Band position (cm^{-1})	Tentative assignments to molecule	References
Young gall	866	Pectin	Synytsya et al. 2003
	989	δ (CH_2), Cellulose	Wiley and Atalla, 1987; Ji et al. 2013; Gierlinger et al. 2013
	1076	δ (CC) and δ (CO), Cellulose	Agarwal 1999
	1214	C- Aryl- O, Lignin	Agarwal 1999
	1327	Aliphatic O-H bend, and lignin. Possibly contribution from carbohydrate	Agarwal and Ralph 1997; Agarwal 1999
	1428	δ (O-CH_3), δ (CH_2), guaiacyl ring, lignin	Agarwal 1999
Mature gall	861	Pectin	Synytsya et al. 2003
	967	δ(CH_2), Cellulose	Agarwal 1999
	1086	δ (CC) and δ (CO), Cellulose	Agarwal 1999
	1273	Aryl- O or Aryl OH and Aryl O-CH_3; guaicyl ring mode with CO -group, lignin	Agarwal 1999; Agarwal et al. 2011
	1451	δ (O-CH_3), δ (CH_2), guaiacyl ring, lignin. Possibly contribution from carbohydrate	Agarwal 1999
	1608	Lignin	Agarwal and Ralph 1997; Gierlinger and Schwanniger 2007
Senescent gall	850	Pectin	Synytsya et al. 2003
	977	δ(CH_2), Cellulose	Agarwal 1999; Gierlinger et al. 2013
	1097	ν(CC) and ν (CO), Cellulose	Gierlinger et al. 2013
	1336	δ (HCC) and δ (HCO), Cellulose	Agarwal 1999; Zeise et al. 2018
	1457	δ (HCC) and δ (HCO), Cellulose	Agarwal 1999; Agarwal and Ralph 1997
	1595	ν (Aryl-ring); Lignin	Agarwal and Ralph 1997; Gierlinger and Schwanniger, 2007
	1657	ν. conj (Ring C=C) Coniferyalcohol; V(C=O) of conifeyaldehyde, Lignin	Agarwal 1999; Agarwal et al. 2011

Abbreviations: ν, stretching vibration; δ, deformation vibration; conj, conjugated

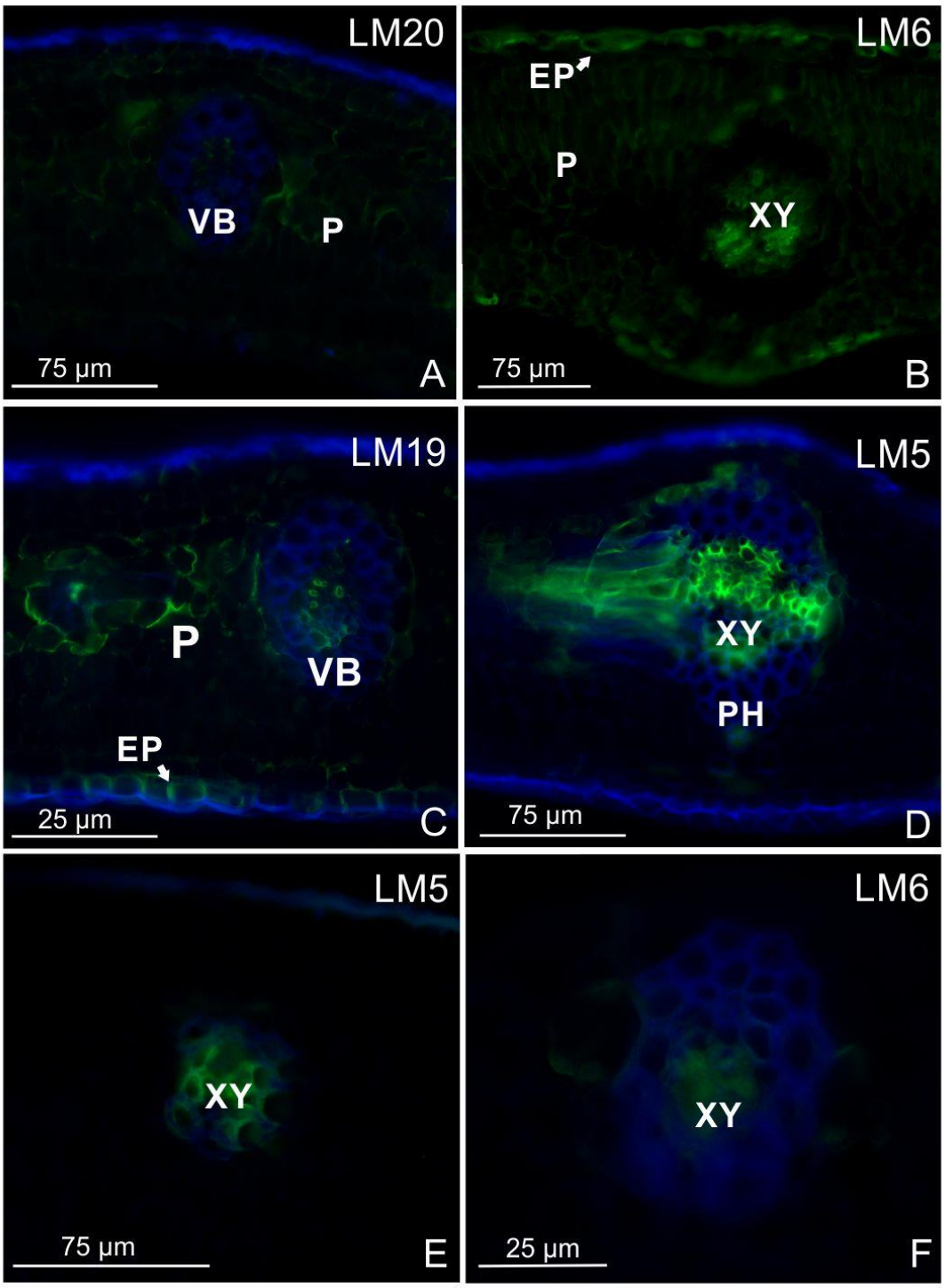

Figure 1. Immunocytochemistry results for pectic and protein epitopes of young and mature non-galled leaflets of *Matayba guianensis*. A-D- Young non-galled leaflets; E-F- Mature non-galled leaflets; A- Highly methyl-esterified HG recognized in parenchyma and in xylem cell walls; B- (1→5) α-L-arabinans labeled detected in adaxial epidermis,

(cont.)

parenchyma and xylem cell walls; C- Low methyl-esterified HG labeled in cell walls of parenchyma, xylem and abaxial epidermis; D- (1→4) β-D- galactans detected in cell walls of xylem. E- (1→4) β-D- galactans labeled in xylem cell walls. F- (1→5) α-L-arabinans labelled in xylem cell walls. *Abbreviations*: EP- Epidermis; P- Parenchyma; VB- Vascular bundles; PH- Phloem; XY- Xylem.

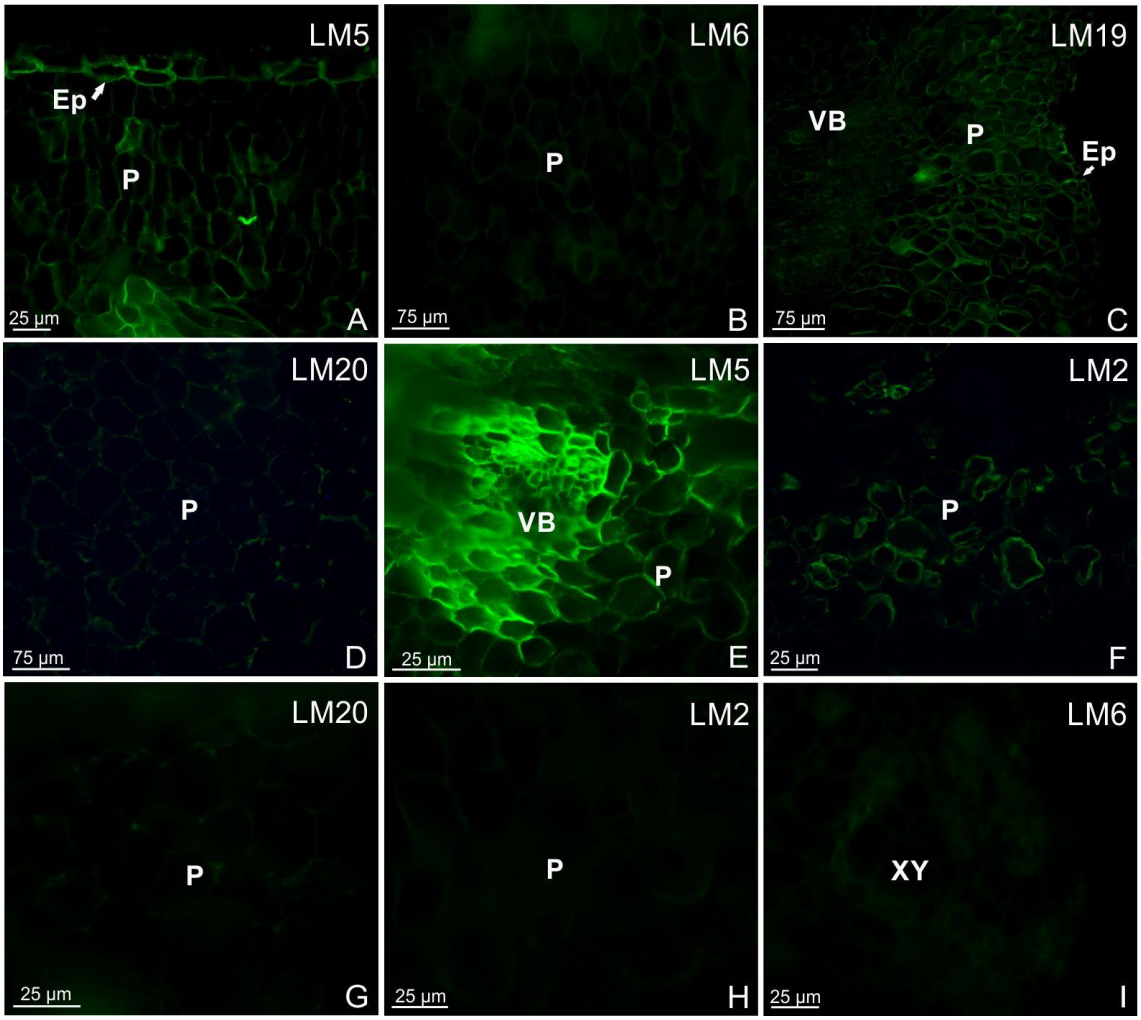

Figure 2. Immunocytochemistry results for pectic and protein epitopes of young and mature *Bystracoccus mataybae* - *Matayba guianensis* galls**.** A-F- Young galls; G-I- Mature galls. A- (1→4) β-D- galactans labeled in epidermis and in parenchyma cell walls. B- (1→5) α-L-arabinans detected in cells walls of the parenchyma. C- Low methyl-esterified HG in recognized in cell walls of the epidermis, parenchyma and vascular bundles. D- Highly methyl-esterified HG labeled in parenchyma cell walls. E- (1→4) β-D- galactans labeled in vascular bundles and in parenchyma cell walls. F- AGP detected in parenchyma cell walls. G- Highly methyl-esterified HG labeled in parenchyma cell walls. H- AGP detected in parenchyma cell walls. I- (1→5) α-L-arabinans labeled in cell walls of xylem. *Abbreviations*: EP- Epidermis; P- Parenchyma; VB- Vascular bundles; XY- Xylem.

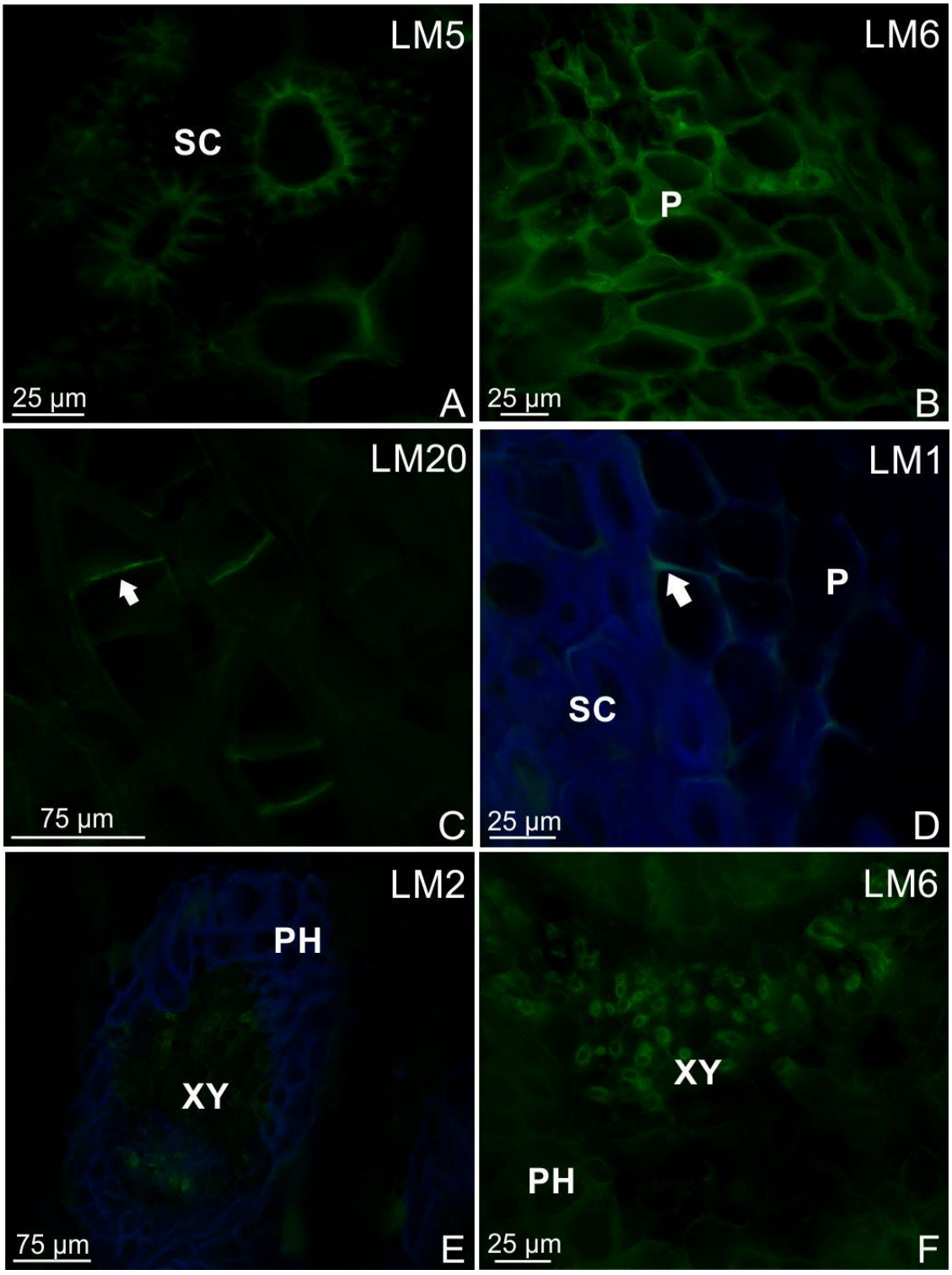

Figure 3. Immunocytochemistry results for pectic and protein epitopes of senescent *Bystaracoccus mataybae* - *Matayba guianensis* galls. A- (1→4) β-D- galactans labeled in sclerenchyma cell walls; B- (1→5) α-L-arabinans detected in parenchyma cell walls; C- Highly methyl-esterified HG recognized in parenchyma cell walls; D- Extensin labeled

(cont.)

in the junction of the cell walls of the sclerenchyma. E- AGP labeled in xylem cell walls; F- (1→5) α-L-arabinans labeled in xylem and phloem cell walls. *Abbreviations*: P- Parenchyma; PH- Phloem; SC- Sclerenchyma; XY- Xylem.

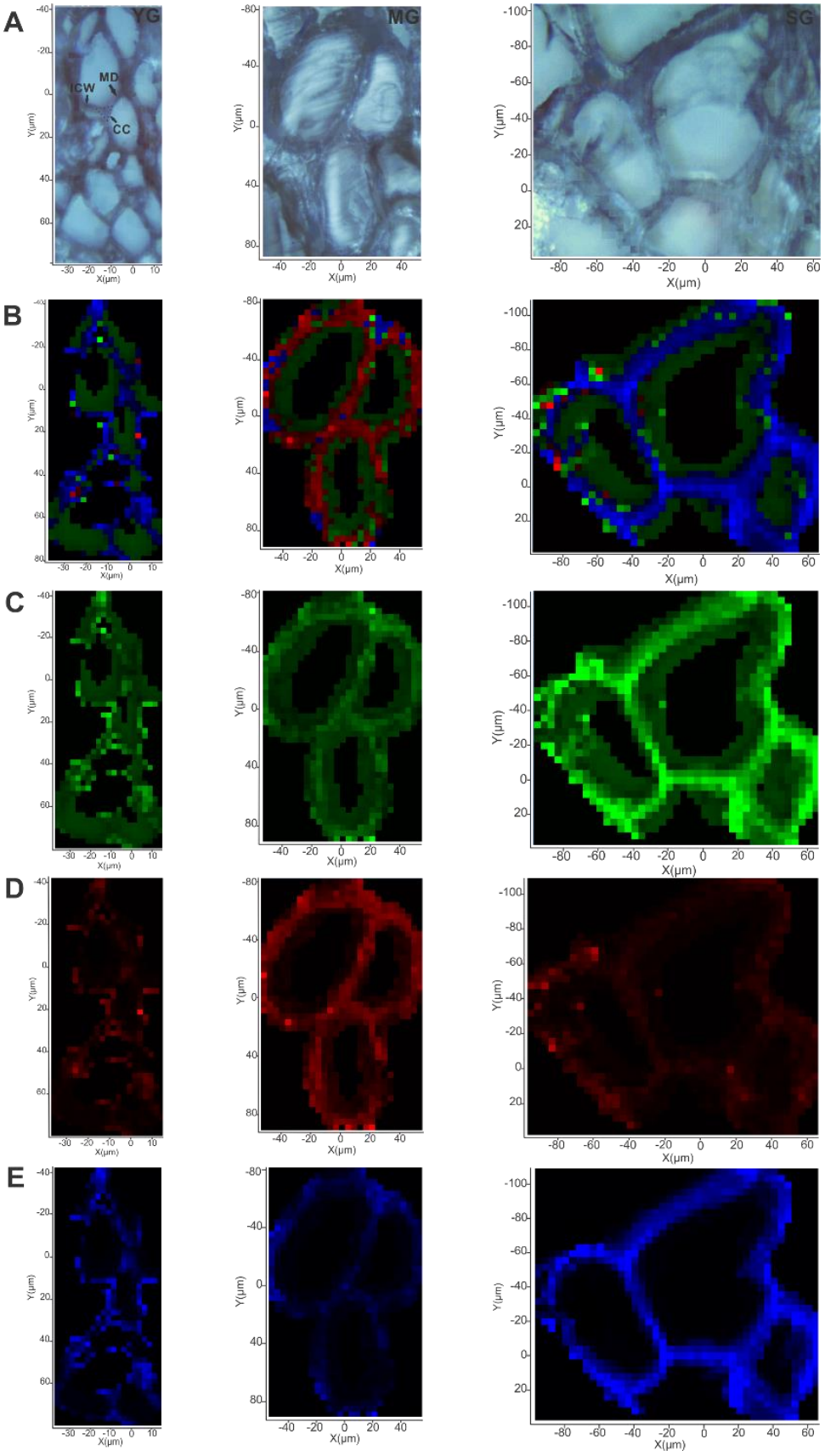

Figure 4- Raman images of a transverse section of the parenchyma cell wall of *Bystaracoccus mataybae* - *Matayba guianensis* galls. A- Bright fiels images of young,

(cont.)

mature and senescent gall; B- Chemical profile of the distribution cell wall components - cellulose (green), pectins (Red) and lignin (Blue); C- Distribuition of cellulose at bands of 978-1778 cm^{-1}; D- Pectins at bands of 840-880 cm^{-1}; E- Lignins at bands of 1536-1692. *Abbreviations*: CC- Cell corner, MD- Middle lamella; ICW- Inner cell wall; YG- young gall; MG- mature gall and SG- senescent gall.

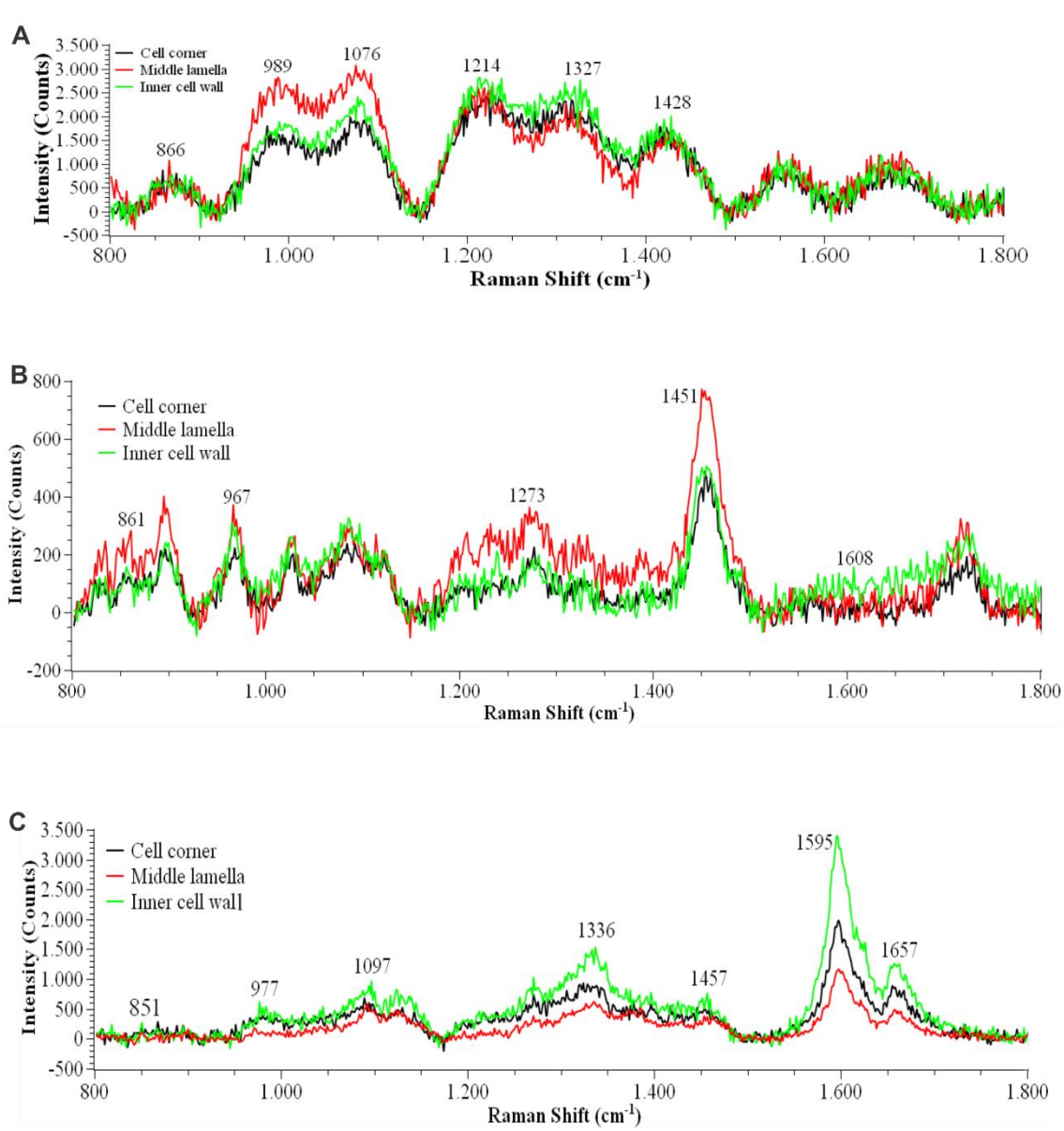

Figure 5. Spectral bands extracted from the parenchyma of mature *Bystaracoccus mataybae* - *Matayba guianensis* galls. Each spectrum represents individual locations of a cell wall region within a region band of a cell wall component. A-Young galls- Higher cellulose concentration is responsible for higher peaks at 989 cm^{-1} and 1076 cm^{-1}, especially in the middle lamella of the cell (red). B- Mature galls- Higher cellulose concetration is responsible for higher peaks at 1451 cm^{-1}, especially in the middle lamella of the cell (red). C- Senescent galls- Higher lignin concentration is responsible for higher peaks at 1595 in the region of deposition of secondary cell (green).

CONSIDERAÇÕES FINAIS

Nas galhas de *Matayba guinensis*, as alterações estruturais e químicas foram averiguadas por meio de estudos anatômicos, histoquímicos, imunocitoquímicos e de espectroscopia Raman que permitiram investigar como a compartimentalização dos compostos metabólicos e a dinâmica dos polímeros da parede interferem no crescimento e na estrutura final da galha. O perfil histoquímico foi similiar durante os instares de *B. mataybe* e definido ainda em galhas jovens, induzidas pela ninfa de segundo instar. A distribuição de componentes da parede celular variou dentro de regiões celulares e em diferentes tecidos de galhas em desenvolvimento, mostrando que as respostas geradas ocorrem dentro do potencial morfogenético do hospedeiro. Novos ensaios realizados com microespectroscopia Raman ainda são necessários para melhor compreender a intensidade dos diferentes tipos de pectinas e ligininas que podem ser detectados e quantificados por meio da técnica. E ainda buscar assinaturas espectroscopicas relacionadas a interação entre insetos galhadores e plantas hospedeiras.