INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA GOIANO – CAMPUS RIO VERDE
DIRETORIA DE PÓS-GRADUAÇÃO, PESQUISA E INOVAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE E CONSERVAÇÃO

GALLS ON *Sapium glandulosum* (Euphorbiaceae) INDUCED BY *Neolithus faciastus* (Hemiptera: Triozidae) IN A MULTITROPHIC CONTEXT: STRUCTURAL, HISTOCHEMICAL AND IMMUNOCYTOCHEMICAL CHANGES INDUCED BY THE INQUILINE

Autor: Maísa Barbosa Santos
Orientador: Dr. Vinícius Coelho Kuster
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RIO VERDE – GO
MARÇO – 2024

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Dissertação apresentada como parte das exigências para obtenção do título de MESTRE EM BIODIVERSIDADE E CONSERVAÇÃO no Programa de Pós-Graduação em Biodiversidade e Conservação do Instituto Federal de Educação, Ciência e Tecnologia Goiano – Campus Rio Verde - Área de Concentração: Conservação dos Recursos Naturais.

Manuscript formatted according to the submission guidelines of the Plant Biology Journal

RIO VERDE – GO
Março – 2024

Sistema desenvolvido pelo ICMC/USP
Dados Internacionais de Catalogação na Publicação (CIP)
Sistema Integrado de Bibliotecas - Instituto Federal Goiano

SM231g

SANTOS, MAISA
GALLS ON Sapium glandulosum (Euphorbiaceae) INDUCED BY Neolithus faciastus (Hemiptera: Triozidae) IN A MULTITROPHIC CONTEXT: STRUCTURAL, HISTOCHEMICAL AND IMMUNOCYTOCHEMICAL CHANGES INDUCED BY THE INQUILINE / MAISA SANTOS; orientador Vinícius Coelho Kuster; co-orientador Fernando Henrique Antoniolli Farache. -- Rio Verde, 2024.
44 p.

Dissertação (Mestrado em Biodiversidade e Conservação) -- Instituto Federal Goiano, Campus Rio Verde, 2024.

1. Natural enemies. 2. Guilds. 3. Immunocytochemistry. 4. Histochemistry. I. Kuster, Vinícius Coelho, orient. II. Farache, Fernando Henrique Antoniolli, co-orient. III. Título.

Responsável: Johnathan Pereira Alves Diniz - Bibliotecário-Documentalista CRB-1 n°2376

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- ☐ Artigo científico
- ☐ Capítulo de livro
- ☐ Livro
- ☐ Trabalho apresentado em evento
- ☐ Produto técnico e educacional - Tipo:

Nome completo do autor: Maísa Barbosa Santos

Matrícula: 2022102310840005

Título do trabalho:

Galls on Sapium glandulosum (Euphorbiaceae) induced by Neolithus faciastus (Hemiptera: Triozidae) in a multitrophic context: structural, histochemical and immunocytochemical changes induced by the inquiline

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Documentos 16/2024 - SREPG/CMPR/CPG-RV/DPGPI-RV/CMPRV/IFGOIANO

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TITULAÇÃO: Mestre em Biodiversidade e Conservação - Área de Concentração Conservação dos Recursos Naturais

APROVADA em 22 de março de 2024.

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- Vinícius Coelho Kuster, Vinícius Coelho Kuster - Professor Avaliador de Banca - Universidade Federal de Jatai (35840659000130), em 25/03/2024 18:11:29.
- Rosy Mary dos Santos Isaias, Rosy Mary dos Santos Isaias - Professor Avaliador de Banca - Universidade Estadual de Minas Gerais (65172579000115), em 25/03/2024 17:21:32.
- Denis Coelho de Oliveira, Denis Coelho de Oliveira - Professor Avaliador de Banca - Instituto Federal Minas Gerais (1), em 25/03/2024 11:59:47.

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AGRADECIMENTOS

Ao Instituto Federal Goiano – Campus Rio Verde e ao Programa de Pós-Graduação em Biodiversidade e Conservação pela minha formação e à CAPES pela concessão da bolsa.

Ao meu orientador, Prof. Dr. Vinícius Coelho Kuster, por estar sempre presente, pela paciência, por todo o auxílio e incentivo. Agradeço especialmente por ele ter contribuído com a minha formação acadêmica desde a graduação.

Ao meu coorientador, Prof. Dr. Fernando Henrique Antoniolli Farache, por toda ajuda, principalmente nas análises estatísticas.

Ao Dr. Alejandro Zaldívar-Riverón, da "Universidad Nacional Autónoma de México" por me ajudar na identificação do inquilino. Ao Prof. Dr. Denis Coelho de Oliveira por ajudar na imunocitoquímica e na discussão dos dados.

Um agradecimento especial para Elaine Cristina Alves Pereira (*in memoriam*) que insistiu muito para que eu fizesse este mestrado, por acreditar que eu conseguiria e ser a pessoa que me motivava.

Aos meus amigos e colegas do laboratório de anatomia vegetal pela ajuda com a realização deste trabalho e pela companhia. Um agradecimento especial para Ana Paula de Souza, Mateus Gomes Thomé, Luana Silva Flores, Rafaela Ferreira da Silva, Gabriel Rodrigues Gonçalves e Cleiberson dos Santos Paulino.

A Patrícia Dias, que me hospedou em sua casa durante o tempo que eu fiquei na UFU para a realização das análises de imunocitoquímica, bem como a Lucivânia que me hospedou em sua casa nas semanas que fiquei em Rio Verde.

À minha família, especialmente Lourdes Souza Gomes e Antônio Carlos Souza Santos, por me incentivarem a continuar estudando e me aperfeiçoando.

Ao Ghost e Tobias Forge que indiretamente tornaram minha jornada acadêmica mais leve por meio da música, sendo minha companhia nos momentos difíceis.

BIOGRAFIA

MAÍSA BARBOSA SANTOS, filha de Antônio Carlos Souza Santos e neta de Lourdes Souza Gomes, nascida em 26 de março de 1999, na cidade de Jataí, no estado de Goiás. O interesse pela biologia iniciou-se no ensino médio nas aulas de biologia, com o Prof. Pablo, a Prof. Lilian e a Prof. Adriana, e se concretizou quando visitei a UFJ (juntamente com a escola) para conhecer o curso de Ciências Biológicas. Então, em maio de 2017, eu ingressei no curso de bacharelado em Ciências Biológicas na Universidade Federal de Jataí. Me encantei pela botânica nas disciplinas de anatomia vegetal, fisiologia vegetal, morfologia vegetal e ecologia vegetal. Os excelentes professores que ministraram essas disciplinas foram extremamente importantes para que criasse gosto por essa área. Em 2018 eu fui monitora da disciplina de anatomia vegetal e em 2019 fiz iniciação científica no laboratório de anatomia vegetal. No ano de 2020, em meio a pandemia, eu defendi meu trabalho de conclusão de curso intitulado "Cell stretching patterns in young galls of *Dipteryx alata* (Fabaceae) define mature cell facts and need a large amount of primary metabolites", sob a orientação do Prof. Dr. Vinícius Coelho Kuster. Concluí o curso no ano de 2021. Em abril de 2022, iniciei o Mestrado no Programa de Pós-Graduação Biodiversidade e Conservação no Instituto Federal Goiano-Campus Rio Verde, sob a orientação do Prof. Dr. Vinícius Coelho Kuster, no qual desenvolvi a dissertação intitulada "Galls on *Sapium glandulosum* (Euphorbiaceae) induced by *Neolithus faciastus* (Hemiptera: Triozidae) in a multitrophic context: structural, histochemical and immunocytochemical changes induced by the inquiline".

SUMMARY

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ABSTRACT

SANTOS, MAISA BARBOSA. Instituto Federal Goiano – Campus Rio Verde-GO, março de 2024. **Galls on *Sapium glandulosum* (Euphorbiaceae) induced by *Neolithus faciastus* (Hemiptera: Triozidae) in a multitrophic context: structural, histochemical and immunocytochemical changes induced by the inquiline.** Orientador: Vinícius Coelho Kuster. Coorientador: Fernando Henrique Antoniolli Farache

Inquilines interact with the gall, feeding on its tissues and modifying it structurally and chemically. We selected globoid leaf galls induced by *Neolithus faciastus* on *Sapium glandulosum*, with the inquiline *Eurytoma* sp. In this work, we evaluated the anatomical, micromorphometric, histochemical and immunocytochemical modifications of the cell wall promoted in the galls after the entry of the inquiline, in the larval, pupa and adult stages. The presence of the inquiline leads to subtle histochemical changes in the galls, such as the apparent remobilization of some primary metabolites, such as starch, and the loss of secondary metabolites, such as alkaloids. The deposition of compounds on the cell wall changed especially in galls with inquiline larvae, forming a centrifugal gradient of compound labeling on the cell wall. Non-methylesterified HGs and extensins in the cortex of gall larvae and pupae increased the structural reinforcement of cell walls, which may be related to the physical pressure caused by the number of inquilines in the gall. (1 → 4)-β-D-galactans, (1 → 5) α-L-arabinans, extensins and RG-1 in the vascular bundles of inquiline galls may have ensured the flexibility and adhesion of their cell walls, necessary to support the high flow of metabolites provided by the remobilization of reserves. Herein, we demonstrate that *Eurytoma* sp. stimulated the tissues of the leaf galls of *S. glandulosum*, changing histological patterns to the composition of non-cellulosic compounds in the cell wall.

Keywords: Natural enemies, Guilds, Immunocytochemistry, Histochemistry.

RESUMO

SANTOS, MAISA BARBOSA. Instituto Federal Goiano – Campus Rio Verde-GO, março de 2024. **Galhas em *Sapium glandulosum* (Euphorbiaceae) induzidas por *Neolithus faciastus* (Hemiptera: Triozidae) em um contexto multitrófico: alterações estruturais, histoquímicas e imunocitoquímicas induzida pelo inquilino.** Orientador: Vinícius Coelho Kuster. Coorientador: Fernando Henrique Antoniolli Farache

Inquilinos interagem com a galha, alimentando de seus tecidos e modificando-a estrutural e quimicamente. Selecionamos galhas foliares globoides induzidas por *Neolithus faciastus* em *Sapium glandulosum*, e invadidas por *Eurytoma* sp. Neste trabalho avaliamos as modificações anatômicas, micromorfométricas, histoquímicas e imunocitoquímicas da parede celular promovidas nas galhas após a entrada do inquilino, nas fases de larva, pupa e adulto. A presença do inquilino leva a alterações histoquímicas sutis nas galhas como a aparente remobilização de alguns metabólitos primários, como o amido, e a perda de metabólitos secundários, como os alcalóides. A deposição de compostos na parede celular mudou especialmente em galhas com larvas inquilinas, formando um gradiente centrífugo de marcação de compostos na parede celular. HGs não metilesterificados e extensinas no córtex de larvas e pupas de galhas aumentou o reforço estrutural das paredes celulares, o que pode estar relacionado à pressão física causada pelo número de inquilinos na galha. (1 → 4)-β-D-galactanos, (1 → 5) α-L-arabinanos, extensinas e RG-1 nos feixes vasculares de galhas com inquilino pode ter garantido a flexibilidade e adesão de suas paredes celulares, necessárias para suportar o alto fluxo de metabólitos proporcionado pela remobilização de reservas. Aqui, demonstramos que *Eurytoma* sp. estimulou os tecidos das galhas foliares de *S. glandulosum*, alterando desde padrões histológicos até a composição de compostos não celulósicos na parede celular.

Palavras-chave: Inimigos naturais, Guildas, Imunocitoquímica, Histoquímica.

INTRODUCTION

Galls provide feed and adequate environmental conditions for their inducers, but may attract different guilds of natural enemies (Mani 1964; Sanver & Hawkins 2000), such as cecidophages, successors, parasitoids, symbionts, kleptoparasites, and inquilines (Luz & Mendonça-Júnior 2020). Inquilines are exclusively phytophagous, sedentary, and can coexist or kill the gall inducer (Brooks & Shorthouse 1997; Luz & Mendonça-Júnior 2020). Although inquilines cannot induce true galls, they can modify gall tissues or stimulate the production of new ones to improve their own diet (Brooks & Shorthouse 1997; Van Noort *et al.* 2007). In a general way, the natural enemies of gall inducers may impose selective pressures triggering new histological, cytological, and histochemical profiles in galls (Rezende *et al.* 2019), influencing the diversity of morphotypes (Stone & Schönrogge 2003; Bailey *et al.* 2009; Rezende *et al.* 2021) and consequently the protective arsenal (Bailey *et al.* 2009). As an example, increases in gall diameter, cortex thickness, and stiffness are associated with decreases in gall mortality rates from natural enemies' attacks (Fernandes *et al.* 1999; Luz *et al.* 2021). In addition to structural defense, secondary metabolites are generally histolocalized in the outer tissues of galls (Kuster *et al.* 2019), with a greater presence of phenolic compounds (Hartley 1992; Ikai & Hijii 2007; Rehill & Schultz 2012; Carneiro *et al.* 2014). Both chemical and structural defenses may increase with the presence of a natural enemy, such as inquilines, who can modify gall tissues.

Some examples of structural changes promoted by the presence of inquilines in galls have been reported in the literature, but studies with histological and histochemical analyses are rare (Rezende *et al.* 2021). Galls induced by *Diplolepis nodulosa* Beutenmüller (Cynipidae) on *Rosa blanda* Ait (Rosaceae) became enlarged and multi-chambered, with little resemblance to galls inhabited only by the inducer after the entry of the inquiline *Periclistus pirata* Osten Sacken, 1863 (Cynipidae) (Brooks & Shorthouse 1997). *Periclistus* sp. (Cynipidae) inquiline also caused a series of morphological changes in the *Diplolepis rosaefolii* Cockerell (Hymenoptera: Cynipidae) - *Rosa virginiana* Mill (Rosaceae) system, including an increase in the number of larval chambers, as well as changes in the type and proportion of tissues in the galls (Leblanc & Lacroix 2001). In the case of the *Schinus polygamus* (Cav.) Cabrera (Anacardiaceae) - *Calophya* aff. *duvauae* Scott (Hemiptera: Calophyidae) system, the Hymenoptera inquiline managed to produce new nutritious tissue for its benefit (Dias 2010). Another aspect that has been used in gall studies is the changes in the composition of the cell walls, through the use of

immunocytochemical analyses; however, this analysis has never been used to understand the changes in the composition of the cell wall promoted by the inquilines.

The cell wall is a complex and dynamic structure, made up of cellulose and hemicellulose, which can be associated with pectins, lignins, proteins, and lipid impregnations, among other components (Zhang *et al*. 2021). Pectin, in general, is the most abundant component of primary cell walls (Goldberg *et al.* 1989). They act in cell adhesion, especially in the middle lamella, cell porosity, expansion, flexibility, signaling, and defense (Jarvis 1984; Knox 1992; Albersheim *et al.* 1996; Willats *et al.* 2001). The three main pectic polysaccharides are (i) homogalacturonans (HGs), which are linear homopolymers abundant in the cell wall and composed of about 100 molecules of (1→4)-α-D-glucuronic acid (Willats *et al.* 2001). The degree of methylesterification of HGs determines the physical properties of the cell wall, interfering in the regulation of cell development (Knox 1997; Wolf & Greiner 2012; Voiniciuc *et al.* 2018); (ii) rhamnogalacturonans I (RG-I), which have a main chain composed of alternating residues of rhamnoses and galacturonic acids and long side chains composed of arabinans, galactans and arabinogalactan I; and (iii) rhamnogalacturonans II (RG-II), which are the least abundant and have a more complex structure than RG-I (Willats *et al.* 2001). We expected that the presence of inquilines would re-stimulate pectin biosynthesis and dynamics in the cell wall as they can induce new tissues.

Proteins are frequent in plant cell walls and can perform different functions (Showalter 1993; Cassab 1998; Jamet & Dunand 2020). Arabinogalactan glycoproteins (AGPs) occur in the cell wall and have a mucilaginous consistency and function in cell proliferation, adhesion, growth, elongation, and nutrition (Majewska-Sawka & Nothnagel 2000; Seifert & Roberts 2007). Extensins are also glycoproteins associated with the cell wall, with the role of reinforcing cell walls in mature tissues (Castilleux *et al.* 2018), as well as providing mechanical protection against pathogens (Chen *et al.* 2015). Hemicelluloses are non-cellulosic polysaccharides synthesized in the Golgi apparatus and transported to the cell wall by vesicles, being firmly attached to cellulose microfibrils when they reach the cell wall (Albersheim *et al.* 1996), with emphasis on xyloglucans, heteromannans and heteroxylans since they are frequent in plant cell walls. The dominant hemicellulose in the primary cell walls of most plants is xyloglucan, which appears to prevent microfibril sliding and thus limit cell expansion (Park & Cosgrove 2015). Heteromannans act as carbohydrate reserves in seeds, vegetative tissues, and endosperm cell walls (Buckeridge *et al*. 2000). Heteroxylans were detected in greater quantities in

secondary cell walls of xylem or fiber cells (Louback *et al.* 2021). Therefore, understanding changes in the composition of the cell wall can help us understand the impact that inquilines have on the galls they invade.

The globoid leaf galls of *Sapium glandulosum* (L.) Morong. (Euphorbiaceae) induced by *Neolithus faciastus* Scott, 1882 (Hemiptera: Triozidae) (Cardoso 2016; Rosa *et al.* 2024) are invaded by different stages (larva, pupa and adult) of *Eurytoma* sp. (Hymenoptera: Eurytomidae). We believe that the inquiline larval stage will promote changes in the structural and chemical profile of the globoid gall induced by *N. faciastus*, with gradual loss of gall layers in the following stages of development of *Eurytoma* sp., through feeding and gall necrosis (Rezende *et al.* 2019) in the innermost layers. Furthermore, it is expected that there will be stronger labeling of cell wall epitopes in the inner region of the cortex, as this is the inquiline feeding region, and also labeling of non-methylesterified HGs, indicating gall stiffening. To test this, we evaluated the micromorphometric, histochemical and immunocytochemical anatomical modifications of the cell wall promoted in the galls of *S. glandulosum* after the hatching of the inquiline larvae in the galls, as well as the changes caused throughout the other stages of its development.

MATERIAL AND METHODS

Multitrophic system and collection area

Sapium glandulosum (L.) Morong (Euphorbiaceae) (Figure 1A) can reach up to 18 m in height and 40 cm in diameter. Its leaves are simple, alternate, glabrous, stipulate, elliptical and produce white latex (Kruijt 1996). The galls induced by *Neolithus fasciatus* Scott, 1882, are globoid, intralaminar and occur on both sides of the host plant leaves (Fig. 1B, C). Females of *Eurytoma* sp. oviposit in the galls of *S. glandulosum*, inside the gall these eggs hatch and occupy the nymphal chamber of the gall inducer throughout their development, i.e., larval (Fig. 1D), pupal (Fig. 1E) and adult (Fig.1F). The definition of *Eurytoma* sp. as an inquiline was based on three main shreds of evidence as proposed by Luz & Mendonça-Júnior (2020): being phytophagous, colonizing the gall with a high number of individuals (≥10), and modifying existing tissues. The identification of the inquiline was carried out by Dr. Alejandro Zaldívar-Riverón, from the “Universidad Nacional Autónoma de México”.

S. glandulosum galls were collected between January and April of 2022 and 2023 from individuals (n=5) at the Campus Jatobá of the Universidade Federal de Jataí, and urban areas in the Jataí municipality, Goiás state (18°27'43" S/ 51°37'10" W), Brazil. The median portion of mature galls was collected under the following conditions (n= 5 per condition): (i) with the induction of *N. fasciatus* (in the 2nd or 3rd instars) and without the presence of *Eurytoma* sp. (Fig. 1C); (ii) galls without *N. fasciatus* and with *Eurytoma* sp. in the larval stage (Fig. 1D); (iii) galls without *N. fasciatus* and with *Eurytoma* sp. in the pupal stage (Fig. 1E); and (iv) galls without *N. fasciatus* and with *Eurytoma* sp. in the adult stage (Fig. 1F). The occurrence of *Eurytoma* sp. in *S. glandulosum* galls always led to the death of *N. fasciatus*, so the galls with the inquiline, in their different stages of development, no longer had the inducer.

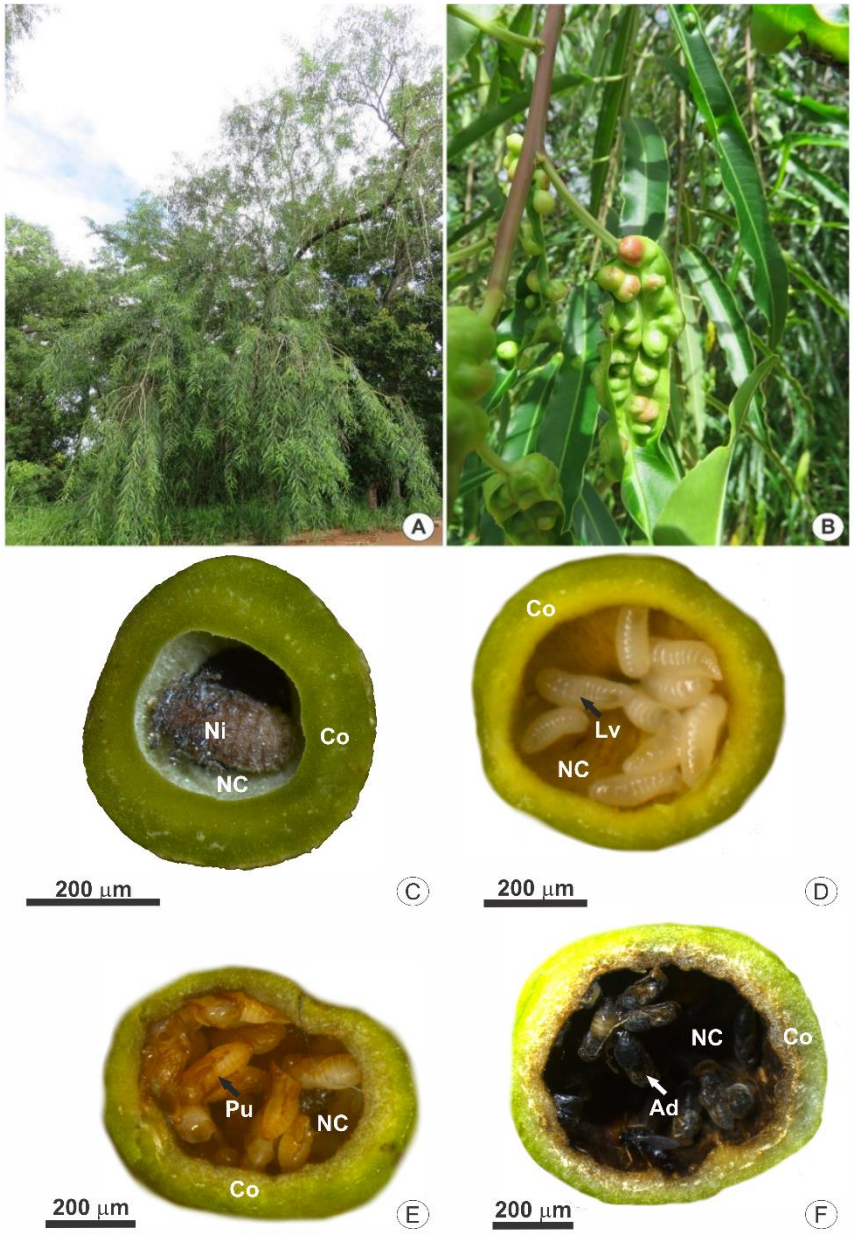

Fig. 1. General aspects of the host plant-galler-inquiline system. A- Adult individual of *Sapium glandulosum*; B- Globoid leaf galls; C- Mature gall with *Neolithus fasciatus* in the nymphal stage and without inquilines; D- Larval stage of the inquilines (*Eurytoma* sp.) in the nymphal chamber; E- Pupal stage of the inquilines in the nymphal chamber; F- Adult stage of the inquilines in the nymphal chamber. Co= Cortex; NC= Nymphal chamber; Lv= Larva, Pu= Pupae; Ny= Nymph.

Histological analyses

Mature galls in different conditions (n=5 per condition) were fixed in FAA_{50} (formaldehyde, acetic acid, 50% ethanol, 1:1:18 v/v/v) for 48 h and subsequently transferred to 70% ethanol (Johansen 1940). Then, the samples were embedded in 2-hydroxyethyl methacrylate (Historesin, Leica® Instruments, Heidelberg), sectioned transversely to the long axis of the gall in a rotating microtome (Leica® RM2235) at 5 µm and stained with 0.05% toluidine blue, pH 4.7 (O'Brien *et al.* 1964). The slides were

mounted in Entellan® and photographed under a light microscope (Leica DM750) with an attached digital camera (Leica® ICC50 HD).

Histochemical analyses

Histochemical analyses were carried out on fresh galls recently collected under different conditions (n=5 per condition) from sections obtained freehand and with a razor blade. For protein detection, samples were immersed in Xylidine Ponceau for 15 minutes, followed by washing in 3% acetic acid for 5 minutes (Vidal 1970). For lipids, samples were subjected to saturated Sudan III solution in 70% alcohol for 5 minutes (Sass 1951). Tests for starch were carried out using Lugol's solution (1% iodinated potassium iodide) for 5 minutes (Johansen 1940). Total phenolic compounds were identified by iron III chloride solution (Johansen 1940). Alkaloids were detected by Dragendorff for 15 minutes, with washing in 5% sodium nitrite (Svendsen & Verpoorte 1983). All sections were mounted between slides and coverslips in distilled water or in the reagent itself and were photographed under a light microscope (Leica® DM750) with an attached digital camera (Leica® ICC50 HD).

Micromorphometric analyses

Micromorphometric data were obtained from photomicrographs of cross-sections of five mature galls per condition, with a collection of measurements from five histological sections per gall. The number of cells and the thickness of the cortex were obtained from a straight line drawn from the first layer of the outer region of the cortex to the last one in the inner part, with three measurements per image. The area of cells in the outer, middle, and inner regions of the cortex was collected from five cells per region. Measurements were performed with ImageJ® software and subjected to statistical analysis in RStudio® (R Core Team 2023). The data did not pass the normality tests (Shapiro-Wilk's tests) and, therefore, were compared using the Kruskal-Wallis non-parametric test, followed by Dunn's multiple comparison tests (α= 0.05).

Immunocytochemical analyses

Immunocytochemical analyses were performed on mature galls under different conditions (n=3 per condition), which were fixed, embedded in historesin, and sectioned using a microtome as previously reported. To evaluate hemicelluloses, the samples were initially immersed in 10 ug mL^{-1} of pectate lyase (Sigma-Aldrich) in 2 mM $CaCl_2$ buffer,

50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (Sigma - Aldrich, USA), pH 10 for 2 h. Then, all samples (for pectins, hemicelluloses, and proteins) were incubated in a cross-reaction blocking solution with Molico powdered milk/phosphate-buffered saline (PBS) solution for 30 min. Subsequently, in the dark, the primary monoclonal antibodies LM1, LM2, LM5, LM6, LM11, LM15, LM19, LM20, and LM21 (Centre for Plant Sciences, University of Leeds, UK) (Table 1) were applied to the sections for 2 h. After application of the primary antibodies, the sections were washed in PBS and immersed in the FITC secondary antibody (1:100 in 3% milk/PBS) for 2 h in the dark. The control was carried out by suppressing the application of the primary antibody, which allows the autofluorescence of the samples to be identified and isolated. After the sections were washed in PBS, the slides were mounted in 50% glycerin and analyzed using a Leica DM4000 LED fluorescence microscope coupled with an HD monochromatic camera (DFC3000 G) and analysis software. Fluorescence intensity was defined by "grayscale" methodology, using the ImageJ program version 1.54d (http://rsb.info.nih.gov/ij), where it was established as weak (≤ 10 Gy), moderate (11-19 Gy) and intense (≥20 Gy).

Table 2. List of monoclonal antibodies and their epitopes.

Monoclonal Antibody	**Epitopes**	**References**
LM1	Extensins	Smallwood *et al.* (1995)
LM2	Arabinogalactan glycans (AGP)	Smallwood *et al.* (1996)
LM5	(1→4) β-D-galactans	Willats *et al.* (1999)
LM6	(1→5)-α-L-arabinans	Willats *et al.* (1999)
LM11	(1→4)-β-D-xylans/arabinoxylans	McCartney *et al.* (2005)
LM15	Xyloglucans (XXXG)	Marcus *et al.* (2008)
LM19	Non-methylesterified homogalacturonans (HGs)	Verhertbruggen *et al.* (2009)
LM20	Methylesterified homogalacturonans	Verhertbruggen *et al.* (2009)
LM21	Heteromannans	Marcus *et al.* (2010)

RESULTS

Histological and micromorphometric analyses

The mature galls of *Sapium glandulosum* with the presence of *Neolithus fasciatus* have a single nymphal chamber and uniseriate epidermis, with tabular cells, covered by a thin cuticle and rare stomata (Fig. 2A). The cortex is parenchymatic, with outer layers with large cells, chloroplasts, and intercellular spaces (Fig. 2A). The innermost cells of

the cortex have smaller cells and are compact (Fig. 2A). The vascular bundles are collateral and occur in the middle regions of the cortex and are hypertrophied (Fig. 2B). The innermost cells of the cortex have a voluminous vacuole with evident nuclei (Fig. 2C).

The galls of *S. glandulosum* with inquilines in the larval stage maintain a uniseriate epidermis, with tabular cells and small breakpoints (Fig. 2D). The structure of the cortex remains like the previous stage, but with the inner cells of the cortex having a larger volume and beginning to collapse (Fig. 2D). The vascular bundles are collateral (Fig. 2E). Throughout the cortex, the nuclei are still evident (Fig. 2F). The galls of *S. glandulosum* with inquilines in the pupal stage have a necrotic epidermis (Fig. 2G). The cells of the cortex lose volume and their original shape, with necrosis affecting the innermost cells of the cortex (Fig. 2G). Starch grains are widely distributed in the cortex cells (Fig. 2H). The innermost cells of the cortex show signs of necrosis, with multiple vacuoles (Fig. 2I). The galls of *Sapium glandulosum* with adult-stage inquilines contain collapsed epidermis (Fig. 2J). The volume of cortex cells is smaller compared to galls with *N. fasciatus* and with vascular bundles (Fig. 2J). Starch grains are maintained in different cells of the cortex (Fig. 2K). The innermost layers of the cortex are necrotic and affect more layers compared to the previous stage (Fig. 2J). These cells lose their original shape and have a barely evident protoplast, demonstrating clear necrosis (Fig. 2J).

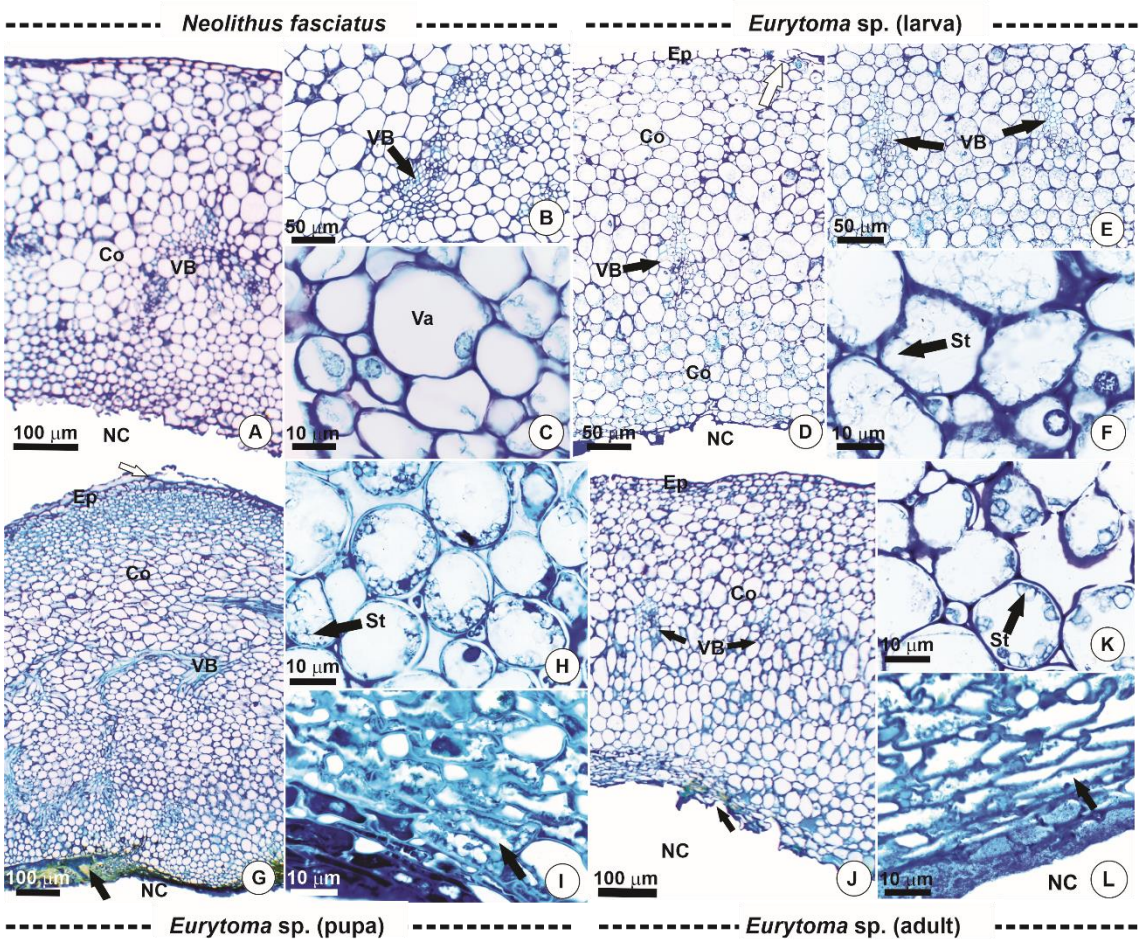

Fig. 2 Cross sections of *Sapium glandulosum* (Euphorbiaceae) galls induced by *Neolithus fasciatus* (Hemiptera), without inquiline (A-C) and with inquiline (D-L) in the larval (D-F), pupal (G-I) and adult (J-L) stages. A- Uniseriate epidermis, parenchymatic cortex and a single nymphal chamber; B- Collateral vascular bundles; C- Inner cortex cells reduced and compact; D- Uniseriate epidermis with points of collapse (white arrow); E- Vascular bundles; F- Starch grains in the cortex; G- Necrotic epidermis (arrow) and cortex with tiny cells; H- Starch grains in the cortex; I- Cells from the innermost layers of the cortex undergoing degradation (arrow); J- Collapsed epidermis, tiny vascular bundles and innermost cells of the necrotic cortex (arrow); K- Starch grains in the cortex; L- Cells from the innermost layers of the cortex undergoing degradation (arrow). Abbreviations: St= Starch; NC= Nymphal chamber; Co= Cortex; Ep= Epidermis; VB= Vascular bundles; Va= Vacuole.

There were cytometric and histometric changes in the galls of *S. glandulosum* under the different conditions analyzed (Fig. 3A-C). The area of cells in the outer region of the cortex was greater in galls with the inducer, followed by galls with adults (Fig. 3A). The galls with larvae and pupa were similar in size (Fig. 3A). The area of cells in the median region of the cortex was greater in galls with the inducer and adult inquilines and smaller in galls with pupa (Fig. 3A). The area of cells in the inner region of the cortex was greater in galls with the inducer and with inquilines in the larval and adult stages (Fig. 3A).

The number of cells was highest in galls with larva of the inquiline, followed by galls with the inducer and with inquiline pupae (Fig. 3B). Cortex thickness was lower in galls with adult inquilines and the same under other conditions (Fig. 3C).

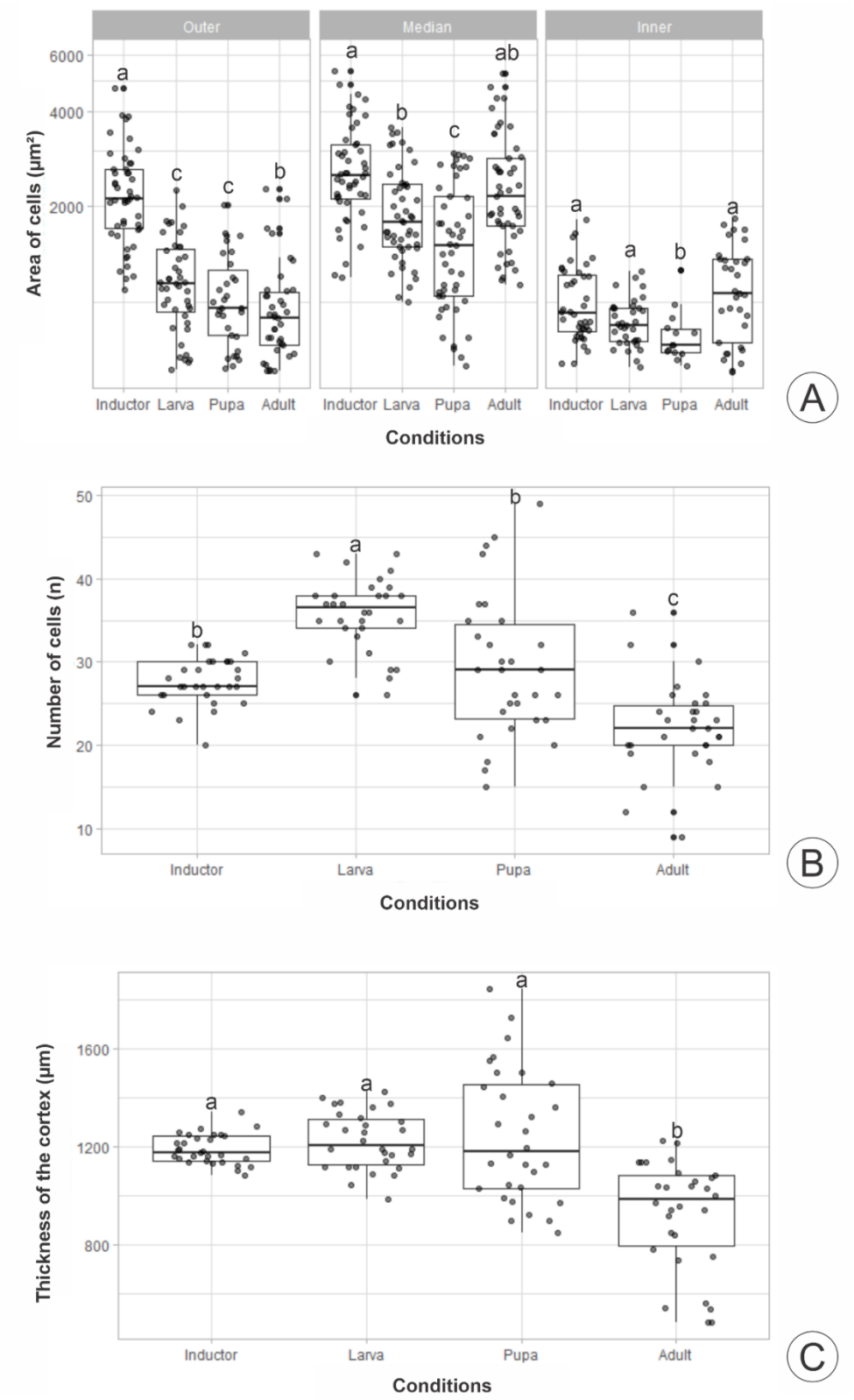

Figura 3 Micromorphometric analyses of *Sapium glandulosum* (Euphorbiaceae) galls induced by *Neolithus fasciatus* (Hemiptera), without inquiline and with inquiline in the larval, pupal and adult stages. A- Area of cells in different regions of the cortex (outer, median, inner); B- Number of cells in the cortex; C- Thickness of the cortex (C). Bars followed by the same letters are not significantly different (Kruskal-Wallis test, followed by Dunn's multiple comparisons test; $\alpha = 0.05$). Boxplots represent medians and corresponding quartiles, and gray dots represent measures.

Histochemical analyses

Starch grains were detected in the gall cortex under all conditions and in the vascular bundles only in galls invaded by the inquiline (Table 2, Fig. 4A-D). These starch grains occurred mainly in the outer and middle layers of the cortex in galls with the inducer (Fig. 4A), with widespread starch marking throughout the cortex in galls with inquiline in the larval stage (Fig. 4B). The reduction of starch grains occurred from the ends of the gall (outer and inner region of the cortex) to the center in galls with inquiline in the pupal (Fig. 4C) and adult stages (Fig. 4D), concentrating in the middle portion of the cortex.

Lipids were found in the same tissues in galls with the inducer and in those with inquiline larvae (Table 2; Fig. 4E, F). In galls with inquiline pupa, lipid droplets occurred throughout the cortex (Table 2; Fig. 4G) and were not detected in the outermost layers of the cortex in galls with adult inquiline (Table 2). Lipids were also detected in the laticifers under all conditions (Fig. 4F, H). The proteins were well detected in all tissues evaluated for the conditions, positioning themselves mainly in the cortex (Table 2; Fig. 4I-L). Negative results occurred only for the epidermis in galls with the inducer and with the inquiline in the adult stage (Table 2). Under all conditions, there was greater protein detection in the inner region of the cortex, close to the nymphal chamber (Fig. 4I-L).

Phenolic compounds were not found in the epidermis under any of the conditions (Table 2, Fig. 5A-D). For galls with the inducer, phenolics were detected throughout the cortex with greater staining in the middle region (Table 2, Fig. 5A) and a negative result for the vascular bundles (Table 2). For galls with larvae (Fig. 5B), pupae (Fig. 5C), and adults (Fig. 5D) of the inquiline, the reaction was positive throughout the cortex and in the vascular bundles (Table 2, Fig. 5B-D). Alkaloids were found throughout the cortex and vascular bundles for the galls with the inducer and with the inquiline larvae (Table 2, Fig. 5E, F). For galls with inquiline pupae, the staining was positive for alkaloids in the median and inner region of the cortex and the vascular bundles (Table 2, Fig. 5G), while for galls with adult inquiline, the alkaloids occurred in the epidermis and the median and inner parts of the cortex (Table 2, Fig. 5H).

Table 2. Histochemical evaluation for primary and secondary metabolites in galls of *Sapium glandulosum* (Euphorbiaceae) induced by *Neolithus fasciatus* (Hemiptera: Triozidae) and with the inquiline *Eurytoma* sp. (Hymenoptera: Eurytomidae) in the larval, pupal and adult stages.

	Inductor (*N. fasciatus*)					**Inquiline (*Eurytoma* sp.)**														
						Larva					**Pupa**					**Adult**				
		Cortex					Cortex					Cortex					Cortex			
	Ep	OR	MR	IR	VB	Ep	OR	MR	IR	VB	Ep	OR	MR	IR	VB	Ep	OR	MR	IR	VB
Starch	-	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+
Lipids	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	-	+	+	-
Protein	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Phenolics	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
Alkaloids	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+

Abbreviations: Ep= Epidermis; VB= Vascular bundles; OR= Outer region, IR= Inner region; MR= Median region; + Positive result; - Negative result.

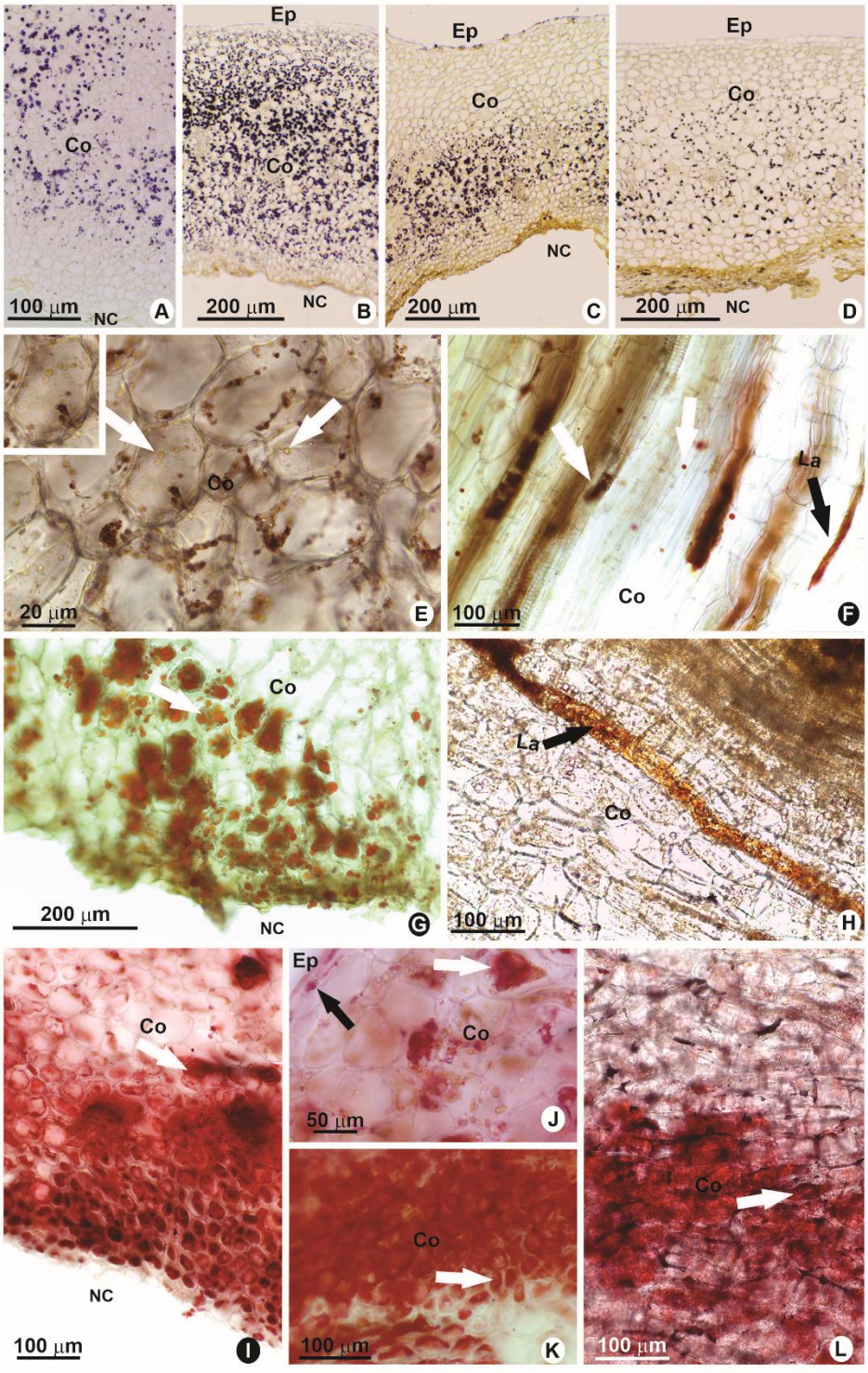

Fig. 4. Histochemical results for starch (A-D), lipids (E-H) and proteins (I-L) in *Sapium glandulosum* galls induced by *Neolithus fasciatus* or with the inquiline (*Eurytoma* sp.). A, E and I- Galls with the inducer only; B, F and J- Galls with the inquiline in the larval stage; C, G and K- Galls with the inquiline in the pupal stage; D, H and L- Galls with the inquiline in the adult stage; A – Starch grains throughout the cortex; B – Starch grains throughout the cortex; C – Starch grains in the middle and inner region of the cortex; D – Starch grains in the middle and inner region of the cortex; E – Lipid droplets (arrows) in the middle region of the cortex in detail; F – Lipid droplets (arrow) in the middle region of the cortex, in the xylem (white arrow) and the laticifers (black arrow); G - Lipid droplets (arrow) in the inner region of the cortex; H – Lipids in laticifers (arrow) in the middle region of the cortex; I - Proteins in the middle (arrow) and inner region of the cortex; J – Proteins in the outer region of the cortex (white arrow) and in the epidermis (black

arrow). K - Proteins in the inner region (arrow) of the cortex. L – Proteins in the middle region (arrow) of the cortex. Ep= Epidermis; Co= Cortex; NC = Nymphal chamber; La= Laticifers.

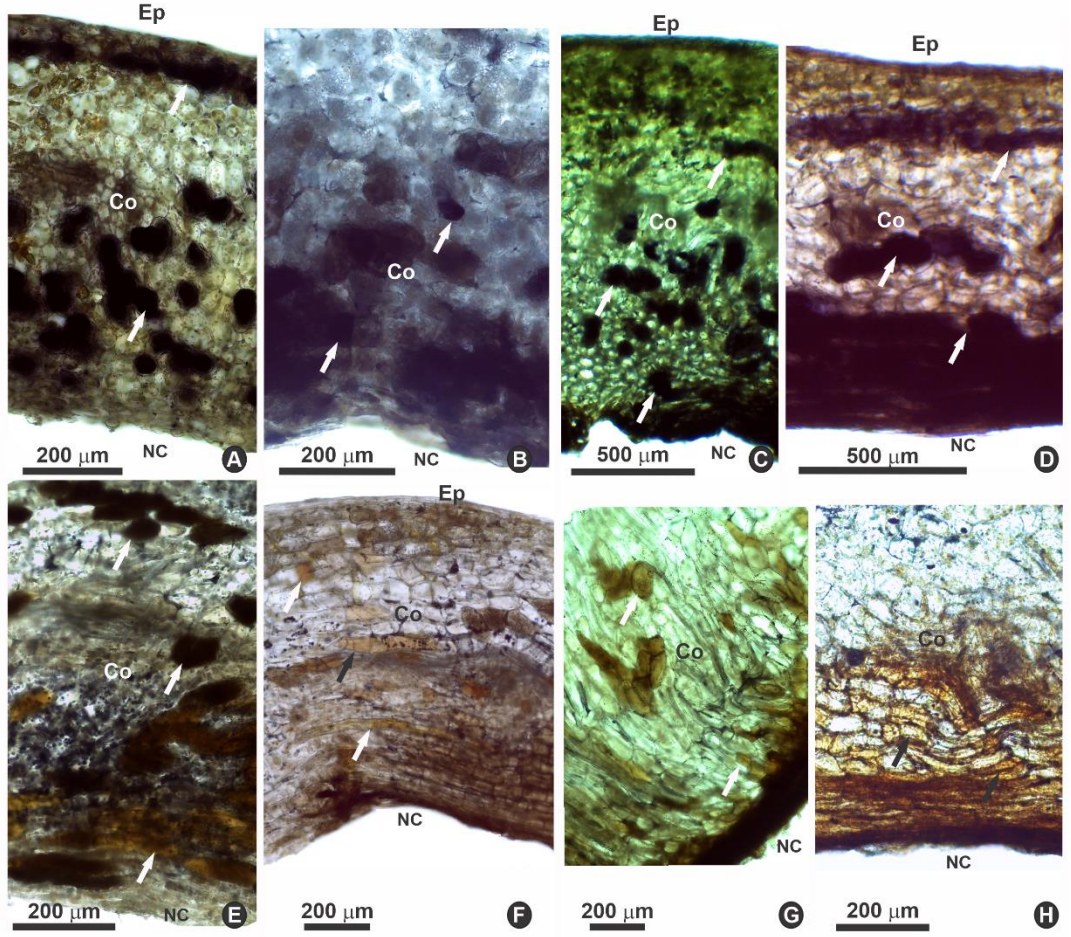

Fig. 5. Histochemical results for phenolic compounds (A-D) and alkaloids (E-H) in *Sapium glandulosum* galls induced by *Neolithus fasciatus* or with the inquiline (*Eurytoma* sp.). A and E- Galls with the inducer only; B and F- Galls with the inquiline in the larval stage; C and G- Galls with the inquiline in the pupal stage; D and H- Galls with the inquiline in the adult stage; A – Phenolic compounds in the outer and middle region of the cortex (arrows); B – Phenolic compounds throughout the cortex (arrows); C – Phenolic compounds throughout the cortex (arrows); D – Positive test for phenolic compounds throughout the cortex (arrows); E – Alkaloids throughout the cortex (arrows); F – Alkaloids throughout the cortex (white arrow) and in the laticifers (black arrow); G – Alkaloids in the middle and inner region of the cortex (arrows); H - Alkaloids in the middle and inner region of the cortex (arrows). Ep= Epidermis; Co= Cortex; NC= Nymphal chamber.

Immunocytochemical analyses

The distribution of pectic, hemicellulosic, and protein epitopes changed in the cell walls of *Sapium glandulosum* galls induced by *Neolithus fasciatus* upon the entry of the inquiline, as well as with the change of their developmental stages (i.e., larva, pupa and adult) (Fig. 6).

Galls induced by Neolithus fasciatus without inquilines

Epitopes of arabinogalactan glycan (AGPs), recognized by LM2, were moderately detected in the cell walls of the inner region of the cortex (Fig. 6A; Fig. 7A). Epitopes of (1→4) β-D-galactans, recognized by LM5, were weakly labeled in the cell walls of the

middle and inner region of the cortex, while epitopes of (1→5)-α-L-arabinans, recognized by LM6, were weakly marked only in the middle region of the gall cortex (Fig. 6A). Epitopes of xyloglucan, recognized by LM15, were intensely labeled in the cell walls of the middle region of the cortex and weakly labeled in the inner region of the cortex (Fig. 6A; Fig. 6B). LM1, LM11, LM19, LM20, and LM21 did not immunolocalize any of their respective epitopes (Fig. 6A).

Galls with larvae of the inquilines inside

Epitopes of extensin were moderately recognized by LM1 in the cell walls of the outer and inner regions of the cortex and weakly recognized in the vascular bundles (Fig. 6B; Fig. 7C). AGPs, recognized by LM2, were weakly labeled in the cell walls of the middle and inner region of the cortex and in the vascular bundles (Fig. 6B; Fig. 7D). Epitopes of (1→4) β-D-galactans, recognized by LM5, were moderately marked in the cell walls of the inner region of the cortex and in the vascular bundles (Fig. 6B; Fig. 7E and F). Epitopes of (1→5)-α-L-arabinans, recognized by LM6, were moderately labeled in the cell walls of the inner region of the cortex and weakly labeled in the vascular bundles (Fig. 6B; Fig. 7G). Epitopes of (1→4)-β-D-xylans, marked by LM11, were weakly labeled in the cell walls of the inner region of the cortex. Non-methylesterified homogalacturonans (HGs), recognized by LM19, were moderately labeled in the cell walls of the middle and inner region of the cortex and in the vascular bundles (Fig. 6B; Fig. 7H). Heteromannan epitopes, marked by LM21, were weakly labeled in the cell walls of the inner region of the cortex (Fig. 6B; Fig. 7I). LM15 and LM20 did not immunolocalize any of their respective epitopes (Fig. 6B).

Galls with pupae of the inquilines inside

Epitopes of AGPs, recognized by LM2, were weakly labeled in cell walls throughout the cortex (Fig. 6C). Epitopes of (1→4) β-D-galactans, recognized by LM5, were intensely labeled in the cell walls of the inner region of the cortex and weakly labeled in the vascular bundles (Fig. 6C; Fig. 8A). Epitopes of (1→5)-α-L-arabinans, recognized by LM6, were weakly labeled in the cell walls of the inner region of the cortex (Fig. 6C). Epitopes of (1→4)-β-D-xylan, recognized by LM11, were weakly marked in the cell walls of the inner region of the cortex and in the vascular bundles (Fig. 6C; Fig. 8B). Epitopes xyloglucan, recognized by LM15, were moderately marked in the intercellular junctions of the inner cortex region (Fig. 6C; Fig. 8C). Non-methylesterified

HGs, recognized by LM19, were weakly labeled in the cell walls of the vascular bundles, while methylesterified HGs, recognized by LM20, were weakly labeled in the cell walls of the inner region of the cortex (Fig. 6C). Heteromannan epitopes, recognized by LM21, were moderately marked in the cell walls of the inner region of the cortex and in the vascular bundles (Fig. 6C; Fig. 8D). LM1 did not immunolocalize its respective epitope (Fig. 6C).

Galls with adults of the inquilines inside

Epitopes of AGPs, recognized by LM2, were weakly marked in the cell walls of the inner region of the cortex and in the vascular bundles (Fig. 5D). (1→4) β-D-galactans, recognized by LM5, were weakly labeled in the cell walls of the inner region of the cortex and in the vascular bundles (Fig. 6D). Epitopes of (1→5)-α-L-arabinans, recognized by LM6, were moderately marked in the cell walls of the inner region of the cortex and in the vascular bundles (Fig. 6D; Fig. 8E). Epitopes of (1→4)-β-D-xylans, recognized by LM11, weakly labeled in the cell walls of the middle and inner region of the cortex and in the vascular bundles (Fig. 6D). Xyloglucans, recognized by LM15, were weakly labeled in the cell walls of the inner region of the cortex (Fig. 6D). Non-methylesterified HGs, recognized by LM19, were moderately labeled in the cell walls of the vascular bundles and weakly labeled in the middle and inner region of the cortex (Fig. 6D; Fig. 8F). Methylesterified HGs, recognized by LM20, were weakly labeled in the cell walls of the middle and inner region of the cortex and weakly labeled in the vascular bundles (Fig. 6D; Fig. 8G). Heteromannans, recognized by LM21, were moderately labeled in the cell walls of the inner region of the cortex and weakly labeled in the middle region of the cortex and in the vascular bundles (Fig. 6D; Fig. 8H).

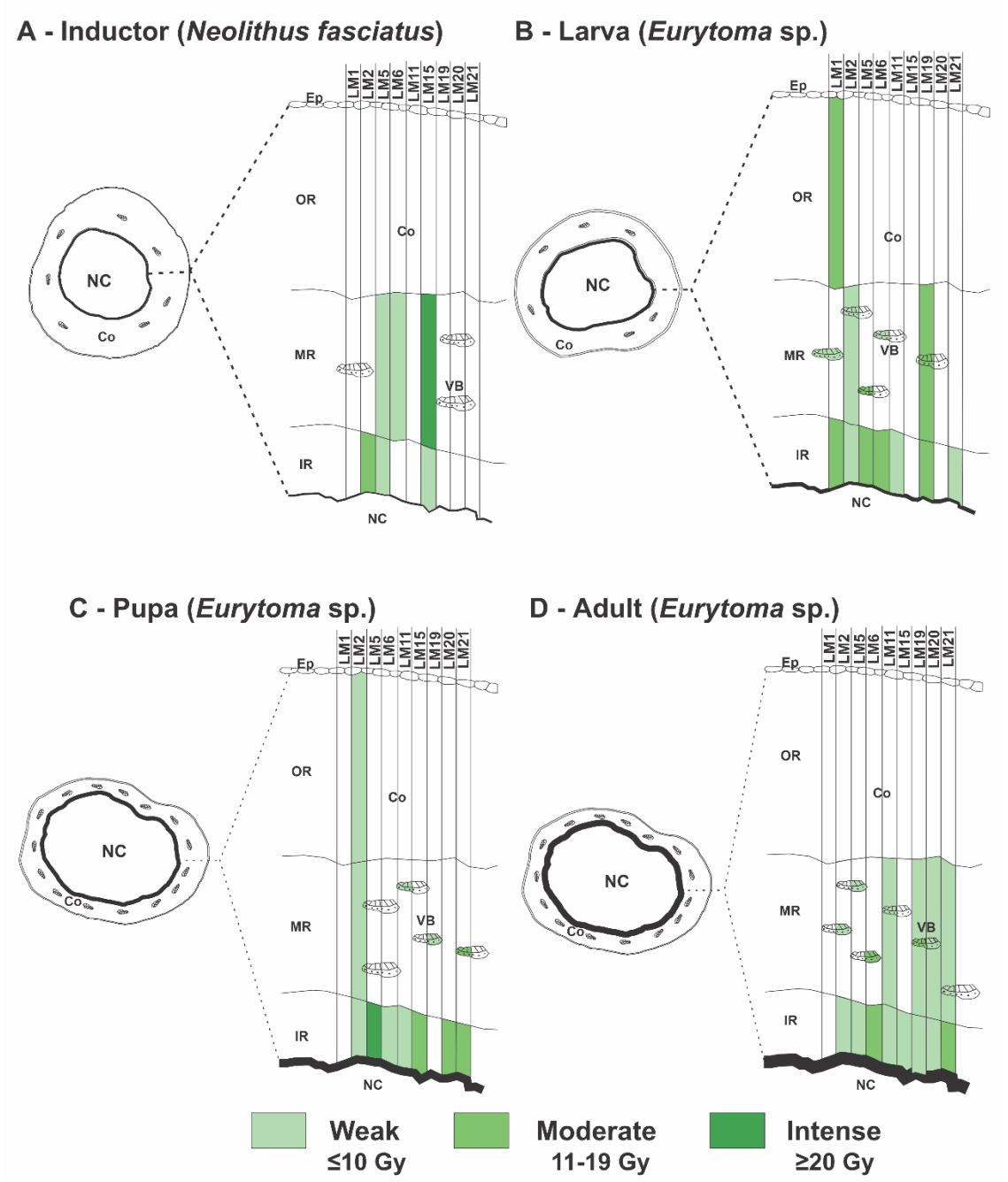

Fig. 6. Distribution and intensity of epitopes of pectins, hemicelluloses, and proteins in the cell walls of *Sapium glandulosum* galls induced by *Neolithus fasciatus* (A) and with the presence of the *Eurytoma* sp. in the following development stages: larva (B), pupa (C) and adult (D). Ep= Epidermis; Co= Cortex; NC= Nymphal chamber; VB= Vascular bundles; OR= Outer region of the cortex; IR= Inner region of the cortex; MR= Middle region of the cortex.

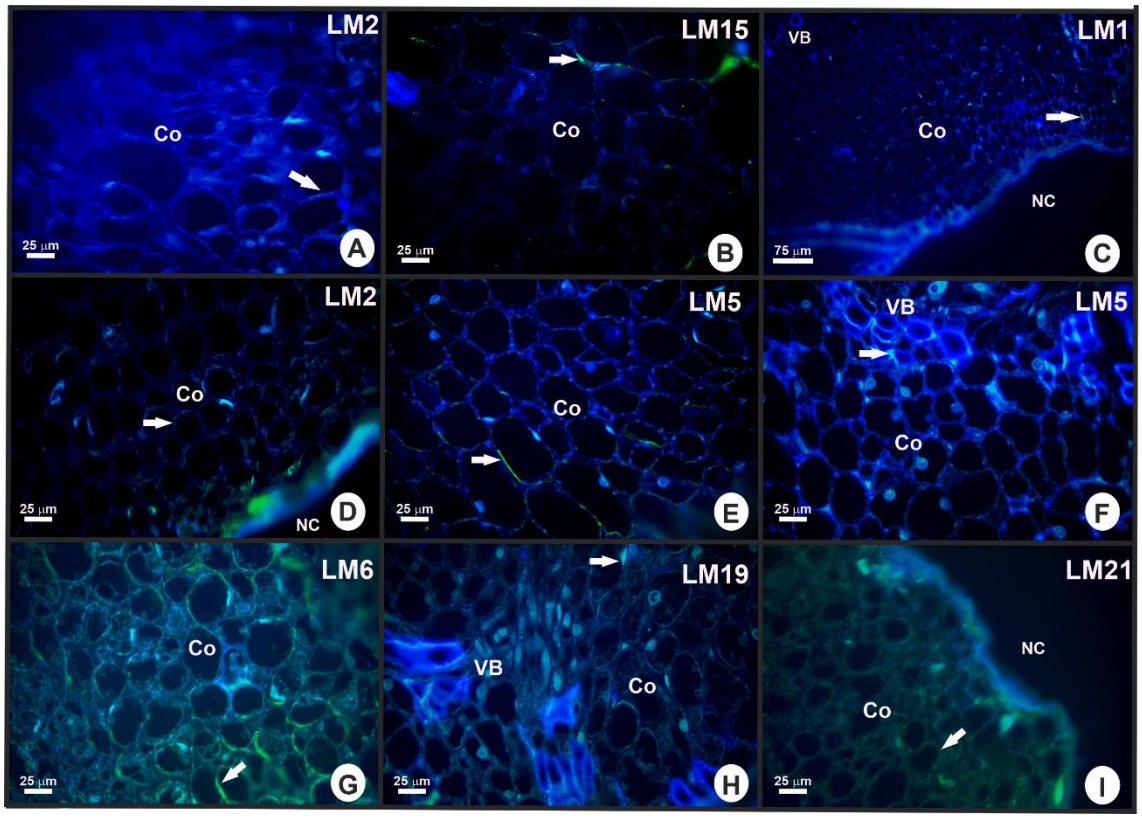

Fig. 7. Cell wall immunocytochemistry results for pectic, hemicellulosic, and protein epitopes in *Sapium glandulosum* galls induced by *Neolithus fasciatus* without inquiline (A-B) and with inquiline larvae (*Eurytoma* sp.) (C-I). A- Arabinogalactan glycans (AGPs) in the inner region of the cortex (arrow); B- Xyloglucans in the middle region of the cortex (arrow); C- Extensins in the inner region of the cortex (arrow); D- AGPs in the inner region of the cortex (arrow); E- (1→4) β-D-galactans in the inner region of the cortex (arrow); F-(1→4) β-D-galactans in vascular bundles (arrow); G- (1→5)-α-L-arabinans in the inner region of the cortex (arrow); H- Non-methylesterified HGs in the middle region of the cortex (arrow); I- Heteromannans in the inner region of the cortex (arrow). Abbreviations: NC= Nymphal chamber; Co= Cortex; VB= Vascular bundles. *The green color indicates epitope labeling by the antibody, while blue indicates cell wall autofluorescence.

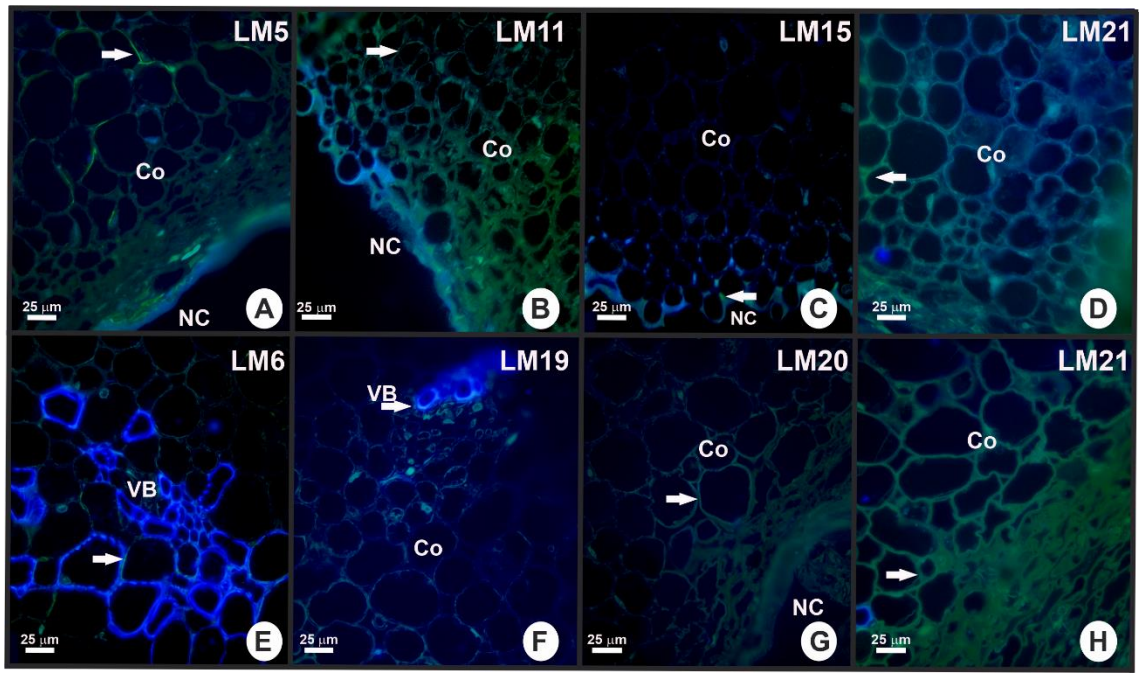

Fig. 8 Immunocytochemistry of the cell wall for pectic, hemicellulosic, and protein epitopes in *Sapium glandulosum* galls induced by *Neolithus fasciatus* with inquiline pupae (A-D) and with inquiline adults (*Eurytoma* sp.) (E-H). A- (1→4) β-D-galactans in the inner region of the cortex (arrow); B-(1→4)-β-D-xylan in the inner region of the cortex (arrow); C- Xyloglucans in intercellular junctions in the inner region of the cortex (arrow); D- Heteromannans in the inner region of the cortex (arrow); E- (1→5)-α-L-arabinans in vascular bundles (arrow); F- Non-methylesterified HGs in vascular bundles (arrow); G- Methylesterified HGs in the inner region of the cortex (arrow); H- Heteromannans in the inner region of the cortex (arrow). Abbreviations: NC= Nymphal chamber; Co= Cortex; VB= Vascular bundles. *The green color indicates epitope labeling by the antibody, while blue indicates cell wall autofluorescence.

DISCUSSION

Gall induction and development depend on the continuous feeding stimuli of the gall inducer (Mani 1964; Bronner *et al.* 1992; Rezende *et al.* 2019). Thus, these organisms are true phenotype manipulators that change the host plant tissues for own benefit, leading to the formation of a huge diversity of morphologies (Stone & Schönrogge 2003; Oliveira *et al.* 2016). However, some structural and chemical traits of the galls can be modified, at different levels, by different guilds that can interact with the gall-inducer and/or with the gall, such as inquilines (Luz & Mendonça-Júnior 2020). Herein, we demonstrate that the death of the galling insect *Neolithus fasciatus* in the galls of *Sapium glandulosum* resulting from its invasion by the inquiline *Eurytoma* sp. led to the onset of necrosis especially in the inner region of the gall cortex. However, there was an increase in the immunolocalization of cell wall compounds and maintenance of most primary and secondary metabolites in the gall cell protoplast with *Eurytoma* sp. at different stages of development, which may indicate that the inquilines maintain certain stimuli for maintenance, even if partial, of the gall structure.

Eurytoma sp. inquiline modifies the histological and histochemical profile of galls

Mature galls of *S. glandulosum* have a parenchymatic and compartmentalized cortex, with cells of reduced size in the inner layers of the cortex and with high metabolic demand (Rosa *et al.* 2024), deviating from the pattern for galls induced by Hemiptera, which generally do not have a compartmentalized cortex (Carneiro *et al.* 2013; Ferreira *et al.* 2019; Oliveira *et al.* 2019). In the inner region of the cortex of the galls on *S. glandulosum*, the process of necrosis occurs with the presence of the larva of the inquiline and increases with the development of these natural enemies. The primary metabolites in internal gall cells of *S. glandulosum* may indicate consumption of these cells by the inquiline. In addition to causing necrosis, the inquiline in the larval stages induces an increase in the number of cell layers in the gall cortex, which may indicate the capacity of the inquiline to stimulate the gall tissue and, a structural reinforcement of the gall (Luz *et al.* 2021) and support the physical pressure imposed by the number of inquilines. Also, the decrease in the number of cortex cell layers in the adult phase of the inquiline may be associated with feeding by *Eurytoma* sp. It was expected that galls with inquiline larvae would have larger cells in the inner region, as in the galls induced in *Schinus polygamus* Cabrera (Anacardiaceae) by *Calophya* aff. *duvauae* Scott (Hemiptera: Calophyidae) and with inquiline larvae (Hymenoptera) (Dias 2010). However, the galls induced by *Diplolepis nodulosa* Beutenmuller, 1909 (Hymenoptera: Cynipidae) on *Rosa blanda* Ait (Rosaceae) showed a reduction of cells in the inner region of the cortex with the presence of the larvae of the inquiline *Periclistus pirata* (Hymenoptera: Cynipidae) (Brooks & Shorthouse 1997). In *S. glandulosum* galls, the inquiline caused a reduction of cell area mostly in the outer and median regions of the cortex, which is related to the structural changes made during its development.

Many galls induced by Hemiptera store starch as a reserve, which are generally associated with cellular metabolic maintenance of the gall (Álvarez *et al.* 2009; Nogal 2011), including the galls of *S. glandulosum* (Rosa *et al.* 2024). Herein, starch was maintained in galls invaded by *Eurytoma* sp.; however, the number of layers with this metabolite was gradually reduced during its developmental cycle. This reduction in starch histolocalization may be due to the remobilization of carbohydrates in the gall towards the host plant or to other regions of the gall, such as the inner region of the cortex. The labeling of starch grains in the vascular bundles only after the presence of the inquiline in the galls of *S. glandulosum* reinforces the remobilization of carbohydrates to the host plant. This remobilization was also related to the senescence process of the galls of *S.*

glandulosum, since there was also a reduction in the reserve of starch grains (Rosa *et al.* 2024). Our results differ from those found for galls induced by *Lopesia* sp. (Diptera: Cecidomyiidae) in *Mimosa gemmulata* Barneby (Fabaceae), as there was a total loss of starch grains after the galls were attacked by an endoparasite and an ectoparasite (Costa *et al.* 2022).

The presence of lipids and proteins around the nymphal chamber of hemipteran-induced galls is common (Rohfritsch 1992; Cornell 1983; Bronner 1992; Gonzalez & Solis 2015; Isaias *et al.* 2018; Aguilera *et al.* 2022), even though it is not the site of gall inducer feeding. Herein, the occurrence of proteins and lipids in the inner region of the cortex may be useful for the *Eurytoma* sp. diet. The highest lipid detection occurred in galls with pupa of the inquiline, mainly in the inner region of the cortex. This greater labeling may be associated with the fact that the pupae do not feed, increasing the lipid reserve (Oliveira *et al.* 2006; Oliveira & Isaias 2010). In galls induced in *Schinus polygamus* (Anacardiaceae) by *Calophya* aff. *duvauae* (Hemiptera: Calophyidae) and with larvae of an inquiline (Hymenoptera), there was a smaller amount of lipid droplets in the outer cortex to the nutritive tissue (Dias 2010), reinforcing their role in the inquiline nutrition.

Phenolic compounds were widely detected throughout the cortex in the galls of *S. glandulosum* with the inquiline, which may be related to the reduction of oxidative stress, since these compounds have an antioxidant role in plants (Dornas *et al.* 2007) and can act in the recovery of homeostasis in gall tissues (Isaias *et al.* 2015; Oliveira *et al.* 2017). This control of oxidative stress may be especially important for the system studied here, since there is always a high number of inquilines in the galls, generally greater than 10 individuals. Phenolic compounds are also promoters of cell division in galls (Formiga *et al.* 2009), as they inhibit the action of AIA oxidases (Hori 1992) and consequently increase auxin levels in tissues (Abrahamson *et al.* 1991). Therefore, the occurrence of phenolics, especially in galls with the inquiline in the larval stage, may be related to the increase in the number of layers that occur at this stage. The alkaloid is a metabolite with a protective function and generally with a certain degree of toxicity (Peeters 2002). This compound was labeled throughout the cortex of *S. glandulosum* galls induced by *N. fasciatus* and maintained with the inquiline in the larval stage, proving to be part of its protective arsenal. Alkaloids are generally reported for the outer cortex in galls attacked by Hymenoptera (Bronner 1992; Aguilera *et al.* 2022); however, in the galls of *S. glandulosum* with the inquiline in the pupal and adult stages there was a loss of this

compound in the outer region of the cortex, which indicates loss of chemical protection in the galls at these stages.

Eurytoma sp. inquiline stimulates the production of cell wall compounds

The invasion of *S. glandulosum* galls by the inquiline *Eurytoma* sp. stimulates the deposition of cell wall components and changes the cell wall structure and dynamics compared with galls with only the inducer of *Neolithus fasciatus* inside, reinforcing the ability of inquilines to modify existing tissues (Brooks & Shorthouse 1997; Van Noort *et al.* 2007). Different cell wall epitopes were recognized especially concentrated in the inner region of the gall cortex and reducing towards the outside, in a centrifugal gradient.

The galls with the gall inducer *Neolithus fasciatus* did not label homogalacturonans (HGs). However, HGs were detected in galls when the inquiline was present. HGs are synthesized in the methylesterified form and deposited in the cell walls of young tissues during the growth and elongation processes (Albersheim *et al.* 1996; Wolf & Greiner 2012). Cell wall development is generally marked by the loss of methyl-ester groups linked to HGs by the activity of the enzyme pectin methylesterases (PMEs), making HGs partially or not methylesterified (Verhertbruggen *et al.* 2009), as reported in galls on *Psidium cattleianum* Sabine (Carneiro *et al.* 2015). The demethylesterification process generally leads to increased cell wall rigidity and is typical for mature cell walls (Albersheim *et al.* 1996). The labeling of non-methylesterified HGs by LM19 in the middle and inner cortex of galls with inquiline larvae may therefore indicate a new stimulus to cell metabolism, increase the structural reinforcement of their cells and support the physical pressure imposed by the number of larvae and pupae (Hongo *et al.* 2012).

The pupa of the inquiline stimulated the deposition of methylesterified HGs, recognized by LM20, in the inner region of the cortex, where the cells are already necrotic. On the other hand, there is a similar balance between methylesterified and non-methylesterified HGs in the galls with adult inquiline, which may indicate that the walls of the cells in the middle and inner region of the cortex are in a balance between rigidity and flexibility (Albersheim *et al.* 1996; Willats *et al.* 2001). The pattern found here differs from that reported for gall development, where two main patterns were reported: i) continuous process of demethylesterification of HGs throughout gall development, as reported for *Psidium cattleianum* (Carneiro *et al.* 2015); and ii) pairing of the methylesterification process of HGs and maintenance of cell walls with youthful

characteristics, as reported for three morphotypes of galls induced in *Baccharis reticularia* (Formiga *et al.* 2013), in *Baccharis dracunculifolia* DC. (Oliveira *et al.* 2014) and in *Croton floribundus* (Teixeira *et al.* 2018).

The dynamics of the side chains of rhamnogalacturonans I (RG-I), i.e., (1 → 4)-β-D-galactans and (1 → 5) α-L-arabinans imply functional characteristics of the cell wall, mainly related to flexibility and/or adherence (O'Donoghue & Sutherland 2012). These side chains were moderately recognized by LM5 and LM6 in the middle and inner region of the gall cortex with the inducer, intensifying and/or appearing in the inner cortex and vascular bundles in galls with inquiline larvae. The highlight here is the intense labeling of (1 → 4)-β-D-galactan and moderate labeling of (1 → 5) α-L-arabinan epitopes in the vascular bundles. The (1 → 4)-β-D-galactans, recognized by LM5, maintain some extensibility of the cell wall, and are marked in young to mature tissues in galls, such as in galls of *Croton floribundus* (Texeira *et al.* 2018). In *Matayba guianensis* galls, marking of this epitope was associated with greater flexibility of the cell wall and the growth of the vascular system (Silva *et al.* 2021). In contrast, (1 → 5) and α-L-arabinans promote greater cell adhesion (Brummell *et al.* 2004). Together, both epitopes can allow flexibility and adhesion of the cell walls of vascular bundles, characteristics that are necessary to support the high flux of metabolites given by the remobilization of reserves. In galls with adult inquilines, immunolocalization is reversed, with intense labeling of the (1 → 5) α-L-arabinan epitope in the inner region of the cortex and vascular bundles. This epitope is marked predominantly in mature or senescent galls, such as in galls induced by Triozidae on *Psidium myrtoides* O. Berg leaves (Carneiro *et al.* 2014), and indicates the end of cellular development (Oliveira *et al.* 2014; Silva *et al.* 2021).

Extensins are associated with the cell wall and act to reinforce it, especially in mature tissues (Sabba & Lulai 2005; Castilleux *et al.* 2018). Extensins were not recognized by LM1 in the galls with only the gall-inducer, but they were intensely marked in the outer and inner regions of the cortex in the galls with the larvae of inquiline. Extensins associated with non-methylesterified HGs seem to reinforce the cell wall to withstand mechanical pressure caused by the high number of larvae inside the gall (see Niebel *et al.* 1993). The labeling of extensins in vascular bundles, together with RG-I, can demonstrate a reinforcement of cell walls and a high flux of metabolites. Conversely, arabinogalactan glycoproteins (AGPs) appear to be involved in cell elongation, proliferation, adhesion, growth, and nutrition (Pennell & Roberts 1990; Majewska-Sawka & Nothnagel 2000; Seifert & Roberts 2007). AGPs were intensely recognized by LM2 in

the inner region of the cortex in the galls of *S. glandulosum* with the gall inducer, which may indicate proliferative features of these cells. The invasion of the galls of *S. glandulosum* by the inquiline led to weak labeling of this epitope by the cortex throughout its developmental stage, indicating that mainly the inner region of the cortex reduced its proliferation capacity.

Hemicelluloses are polysaccharides that regulate cell expansion (Cosgrove 2016; Chen *et al.* 2019) and are a source of storage, like heteromannans (Scheller & Ulvskov 2010). Hemicelluloses were not recognized by any of the antibodies in the galls of *S. glandulosum* with the gall inducer, however, they were detected in the galls with inquiline, mainly in the inner region of the cortex and more widely marked in galls with inquiline adults. This extensive labeling of hemicelluloses in galls with inquiline adults can be associated with the end of the gall's life, since the next step is the exit of the inquiline from the gall. Hemicelluloses are frequently reported for senescent galls, as reported for fusiform galls of *Inga ingoides* (Rich) Willd. (Bragança *et al.* 2020), where xyloglucans were only detected at this stage.

LM11 recognized (1→4)-β-D-xylans in galls with the inquiline, at different stages of development, in the cell walls of the inner region of the cortex, indicating the end of the cell cycle of these cells (McCartney et al. 2005). This labeling is atypical, since xylans are found especially in cells with a secondary wall (McCartney et al. 2005). LM21 weakly recognized heteromannans only in the galls of *S. glandulosum* with the inquiline, at different stages of development, especially in the inner region of the cortex. Heteromannans are hemicelluloses involved in cell expansion or rigidity (Voiniciuc *et al.* 2019), but may also be associated with carbohydrate reserves in seeds (Santos *et al.* 2004). Heteromannans were associated with inducer feeding when they were immunolocalized in the cell walls of the nutritive tissue in four galls induced on *Mimosa gemmulata* (Fabaceae) by *Lopesia* sp. (Diptera: Cecidomyiidae) (Costa *et al.* 2021). Therefore, we believe that the presence of heteromannans in the inner region of the cortex may be related to the inquiline feeding in the galls of *S. glandulosum*. LM15 recognized xyloglucans moderately only in the wall of cells in the inner region of the gall cortex with pupa and adult inquiline. Xyloglucans have been implicated in preventing the sliding of cellulose microfibrils, therefore limiting cell expansion (Park & Cosgrove 2015; Cosgrove 2016). Herein, this immunodetection may indicate cellular maturity that is associated with more advanced stages of inquiline development.

FINAL CONSIDERATIONS

The inquiline *Eurytoma* sp. induces structural and metabolic changes in the gall necessary for its development, showing great ability to modify existing tissues. The presence of inquiline leads to subtle metabolically and histochemical changes in the galls, for instance the apparent remobilization of some primary metabolites, such as starch, and loss of secondary metabolites, such as alkaloids. The inquiline *Eurytoma* sp. managed to stimulate the deposition of cell wall compounds in the galls of *S. glandulosum*, forming a centrifugal labeling gradient. Thus, the inner region of the gall cortex showed intense labeling for different epitopes of cell wall components, a clear indication of inquiline's capacity to stimulate gall tissues. In addition, these cell wall components seem to be associated with its feeding, reduced proliferative capacity, and stimulation of necrosis. The labeling of non-methylesterified HGs and extensins in the cortex of galls with larvae and pupae of the inquiline ensured greater structural reinforcement in the cell walls, which may be related to the physical pressure caused by the number of inquilines in the gall.

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