UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL

MARIANA DE SOUSA COSTA FREITAS

GALHAS INDUZIDAS POR *ERIOSOMA LANIGERUM* HAUSMANN (HEMIPTERA: APHIDIDAE) EM *MALUS DOMESTICA* BORKH. (ROSACEAE)

BELO HORIZONTE – MG
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Mariana de Sousa Costa Freitas

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Orientador: Prof. Drª. Rosy Mary dos Santos Isaias
Universidade Federal de Minas Gerais

Coorientador: Prof. Dr. Edgard Augusto de Toledo Picoli
Universidade Federal de Viçosa

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Comissão Examinadora

Dra. Rosy Mary dos Santos Isaias (Universidade Federal de Minas Gerais)

Dr. Edgard Augusto de Toledo Picoli (Universidade Federal de Viçosa)

Dr. Bruno Garcia Ferreira (Universidade Federal do Rio de Janeiro)

Dra. Lubia María Guedes (Universidad de Concepción -Chile)

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RESUMO

As árvores de *Malus domestica* (Rosaceae) cv. 'Eva' cultivadas em porta-enxerto 'M9' são susceptíveis ao pulgão *Eriosoma lanigerum* (Hemiptera: Aphididae). Este pulgão é considerado uma praga global, induzindo galhas nas raízes e nos caules das macieiras, nos quais assume uma posição externa aos tecidos vegetais. Essa posição é incomum em galhas induzidas por insetos que, geralmente, habitam uma câmara larval inserida em meio as células e tecidos vegetais. Embora a maioria dos pulgões se alimente diretamente do conteúdo das células do floema, o pulgão lanígero pode se alimentar no xilema. A alimentação do *E. lanigerum* induz uma atividade cambial incomum, resultando em um campo cecidogenético assimétrico no sistema radial do xilema secundário. Quanto mais próximas do sítio de alimentação das colônias de pulgões, maiores são as alterações na estrutura do xilema, com diferenciação de elementos vasculares anormais e maior diferenciação de células parenquimáticas. O perfil histoquímico das raízes e caules de *M. domestica* nos locais de desenvolvimento de galhas também é alterado pela atividade do *E. lanigerum* e está associado, principalmente, a manutenção do metabolismo da galha com acúmulo de amido e compostos fenólicos. O campo cecidogenético é menos evidente no sistema axial do xilema secundário, pois os elementos de vaso nas porções do caule abaixo e acima das galhas são semelhantes às porções de caule não galhado, contradizendo a hipótese de "constrição da galha". Assumimos, portanto, que o dano da indução de galhas às culturas está relacionado ao perfil do xilema secundário nas galhas de *E. lanigerum* e sua influência na condutividade da água em *M. domestica*. Essa influência prioriza o suprimento e o acúmulo de água no local de desenvolvimento da galha, devido aos elementos de vaso maiores e anormais e ao aumento da área de parênquima. Tais características anatômicas favorecem o estado hídrico dos tecidos ao longo do desenvolvimento da galha e a hidratação do pulgão lanígero.

Palavras-chave: atividade cambial, elementos de vaso, macieiras, pulgão lanígero, xilema secundário

ABSTRACT

The trees of *Malus domestica* (Rosaceae) 'Eva' cultivar on 'M9' rootstock are susceptible to high infestation by the galling aphid *Eriosoma lanigerum* (Hemiptera: Aphididae). This aphid is considered a global pest, which induces galls on roots and stems of the apple trees, where it has an external position regarding plant tissues. Such a position is uncommon in galling insects, which usually inhabit a larval chamber within plant cells and tissues. Although most aphids feed directly on phloem cells, the woolly-apple-aphid can also ingest xylem content. The *E. lanigerum* feeding activity induces an unusual cambial activity, causing an asymmetrical cecidogenetic field in the secondary xylem radial system. The closer the aphid colonies are, the higher are the alterations in xylem structure, where abnormal vascular elements differentiate and vascular parenchyma cells are overproduced. The histochemical profile of the roots and stems of *M. domestica* in gall developmental sites are also altered by the *E. lanigerum* galling activity and is mainly associated with the maintenance of gall metabolism with greater accumulation of starch and phenolic compounds. The cecidogenetic field is less evident in the secondary xylem axial system, for the vessel elements in the stem portions below and above the galls are similar to those of the non-galled stem portions, contradicting the "gall constriction" hypothesis. We assume that the damage of gall induction to the crops relates to the secondary xylem profile of *E. lanigerum* galls and its influence on the water conductivity in *M. domestica*. This influence prioritizes water supply and accumulation to the gall developmental site, due to the large abnormal vessel elements and increased differentiation of parenchyma cells. Such peculiar anatomical traits favor the water status to gall development and the woolly-aphid hydration.

Keywords: apple trees, cambial activity, secondary xylem, vessel elements, wolly-apple-aphid.

Sumário

1. INTRODUÇÃO GERAL

Malus domestica Borkh. (Rosaceae) é a terceira "fruta" mais produzida no mundo (FAO 2021) com cultivares adequados para diferentes climas ao redor do mundo. O cultivar 'Eva', nosso modelo de estudo, foi desenvolvido pelo Instituto Agronômico do Paraná (IAPAR) para plantio em regiões com inverno ameno e vem sendo cultivado com sucesso no estado de Minas Gerais, Brasil (Oliveira et al. 2011, 2014). As maçãs 'Eva' têm ganhando espaço no mercado brasileiro pelo seu sabor, cor, pela colheita precoce dos frutos (Oliveira et al. 2014) e por serem resistentes a altas temperaturas. A possibilidade de cultivo em clima tropical é recente e promissora, devido a perspectiva de um aumento na produção total de maçãs no país. No Brasil, o cultivar 'Eva' é comumente enxertado sobre o porta-enxerto 'M9', o qual é susceptível ao ataque de colônias de *Eriosoma lanigerum* (Hemiptera: Aphididae) Hausmann, 1802 (Denardi et al. 2015).

O pulgão lanígero (*E. lanigerum*) é uma praga global considerada crítica para a economia da maçã no hemisfério sul. De origem da América do Norte, o pulgão é introduzido inequivocamente junto com as macieiras nas culturas em todo o mundo, afetando sua economia (Denardi e Spengler 2001). O ataque por *E. lanigerum* nas raízes e caules de *M. domestica* resulta em galhas, estruturas definidas como produto de uma interação entre a planta hospedeira e um organismo galhador que, além dos insetos, podem também ser vírus, bactérias, fungos, algas, ácaros, nematódeos ou mesmo plantas. Como resposta à interação, as células vegetais passam por processos de hipertrofia (aumento de volume) enquanto os tecidos tornam-se hiperplásicos (passam por intensa proliferação celular) levando à formação das galhas (Mani 1964, Shorthouse et al. 2005, Raman 2007). Os indivíduos de *E. lanigerum* formam colônias nas partes externas de raízes e caules, situação não usual em galhas de insetos, que em geral se instalam numa câmara larval em meio às células e tecidos vegetais (Mani 1964). O *E. lanigerum*, como todos os insetos sugadores, perfura os tecidos

vegetais com seus estiletes para se alimentar, estimulando o desenvolvimento das galhas desde as primeiras etapas. O estímulo alimentar, além de alterar os padrões ontogenéticos, induz alterações no metabolismo primário e secundário das plantas hospedeiras (Bronner 1992, Oliveira et al. 2011, Kuster et al. 2020). Ademais, as galhas funcionam como drenos de fotoassimilados e de água e, portanto, seu desenvolvimento nas macieiras reduz a condutividade tanto via floema quanto xilema (Brown et al. 1995, Ateyyat e Al-Antary 2009), reduzindo o vigor das plantas infestadas (Danielsson 1979, Brown e Schmitt 1990, Asante 1994, Rinallo et al. 1995, Ateyyat e Al-Antary 2009, Mandalon et al. 2020). Tendo em vista que o desenvolvimento de muitas galhas envolve o estímulo a células meristemáticas neoformadas ou pré-existentes (Carneiro et al. 2017; Ferreira et al. 2017), hipotetizamos que o pulgão lanígero, ao inserir seus estiletes nos tecidos vegetais para se alimentar, modifica a atividade cambial, alterando as características dos tecidos vasculares secundários.

O câmbio vascular é um meristema lateral responsável pelo crescimento secundário nos órgãos vegetativos, como raiz e caule. Este meristema é formado por células iniciais fusiformes, que se diferenciam em elementos traqueais, fibras, parênquima do xilema e do floema e em elementos de tubo crivado, e por células iniciais radiais, que se diferenciam em células parenquimáticas dos raios vasculares (Fahn 1990). O câmbio produz o xilema secundário centrípeta e o floema secundário centrifugamente. O xilema secundário é responsável pela translocação de água e sais minerais na planta, no sentido raiz – folha, enquanto o floema secundário é responsável pela translocação de fotoassimilados das folhas para o restante da planta (Fahn 1990). O câmbio vascular pode ter seu desenvolvimento e atividade alterado por fatores como desbalanço de fitormônios, alteração de temperatura, chuvas e fotoperíodo (Begum et al. 2013). Os fitormônios, principalmente auxinas, giberelinas, citocininas e etileno, são de grande importância para a atividade cambial (Wang

2020, Aloni 2021), atuando, também, na formação e desenvolvimento de galhas (Bedetti et al. 2014, Ferreira et al. 2019a, Bragança et al. 2021).

O estudo do sistema *M. domestica*-*E. lanigerum* visa identificar as alterações anatômicas e histoquímicas induzidas no xilema secundário e como essas alterações influenciam as relações hídricas da planta. A presente dissertação está organizada em dois capítulos, dos quais o primeiro trata das principais alterações anatômicas e histoquímicas induzidas pela alimentação do *E. lanigerum* no xilema secundário de raízes e caules. Apesar do principal sítio de alimentação dos afídeos ser o floema (Wool 2005, Álvarez et al. 2009, Ferreira et al. 2019b), estudos realizados usando a técnica de Gráfico de Penetração Elétrica (GPE) (McLean e Kinsky 1964) identificaram a ingestão ativa do conteúdo de xilema por *E. lanigerum* (Sandanayaka e Hale 2003, Sandanayaka et al. 2003, Hao et al. 2020, Zhou et al. 2021). Assim, hipotetizamos que a atividade alimentar do *E. lanigerum* induz alterações na atividade cambial tanto de raízes quanto de caules de *M. domestica*. Tais alterações refletem na estrutura do xilema secundário com peculiaridades relativas ao ambiente aéreo e subterrâneo sobre a fisiologia dos órgãos da planta hospedeira, galhas de caule no cultivar ‘Eva” e de raiz no porta-enxerto ‘M9’. Buscamos testar esta hipótese por meio de análises anatômicas e histoquímicas de forma comparativa nas galhas caulinares e radiculares em relação aos órgãos não galhados.

O segundo capítulo trata das peculiaridades do xilema secundário, em galhas caulinares e nos ramos portadores de galhas, e da caracterização dos elementos de vaso. Neste capitulo, as alterações teciduais e celulares no sistema vascular que resultam na priorização do fluxo hídrico para as galhas foram investigadas, conforme postulado pela hipótese de constrição da galha (Aloni et al. 1995) e observado em galhas de *Ricinus communis* induzidas por *Agrobacterium tumefaciens* (Aloni et al. 1995). Essa hipótese prevê que a região do caule abaixo da galha tem maior diferenciação de xilema com raios e

elementos de vasos de tamanho similar ao ramo caulinar não galhado, enquanto a região do caule acima da galha tem elementos de vasos mais estreitos, raios aumentados e ausência de fibras. Estas alterações no tecido vascular resultam em uma limitação do transporte de água para a parte aérea da planta (Aloni et al. 1995), ou seja, o ramo caulinar acima do sítio de desenvolvimento da galha. Assim, buscamos, por meio das alterações no xilema secundário, estimar o efeito da atividade alimentar do pulgão em termos de restrição hídrica nos ramos caulinares afetados. Nossos resultados contribuem para o entendimento de como esta praga afeta os cultivares suscetíveis e compromete a produtividade das culturas de macieira.

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2. Capítulo 1

Anatomical and histochemical responses of *Malus domestica* to *Eriosoma lanigerum* galling activity

Anatomical and histochemical responses of *Malus domestica* to *Eriosoma lanigerum* galling activity

Mariana de Sousa Costa Freitas[1], Ravena Malheiros Nogueira[1], Gener Augusto Penso[2], Edgard Augusto de Toledo Picoli[2], Rosy Mary dos Santos Isaias[1]*

1 Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Laboratório de Anatomia Vegetal, Departamento de Botânica, Belo Horizonte, Minas Gerais, Brazil.

2 Universidade Federal de Viçosa, Departamento de Biologia Vegetal, Centro de Ciências Biológicas II, Viçosa, Minas Gerais, Brazil.

*Corresponding author: rosy@icb.ufmg.br

2.1. Abstract

Eriosoma lanigerum is a global pest that induces galls on roots and stems of *Malus domestica* trees. The external position of *E. lanigerum* is an unusual situation described for galling insects, and despite the fact that most aphids feed direct from phloem cells, *E. lanigerum* can also ingest xylem content. Accordingly, we consider that the aphid feeding on xylem cells influences water conductivity and the vigor of host apple plants, which may occasionally cause their death. The mapping of anatomical and histochemical alterations reveals an asymmetrical cecidogenetic field, which is expressed in the histochemical profile of host roots and stems. The feeding activity of *E. lanigerum* causes abnormal vascular cambium activity, with the high proliferation and reorganization of parenchyma cells, and neoformation of vessel elements, both in roots and stems. The metabolites accumulated in gall developmental site are associated with the maintenance of gall metabolism. Although

the host plant tissue responsive processes to the galling exophytophagous aphid is similar to that of galling endophytophagous aphids, the over-differentiation of parenchyma cells, the redifferentiation of vessel elements, and the formation of a potential alternative feeding site to the aphid (the secondary tissue islets) seem peculiar to the *M. domestica* - *E. lanigerum* system.

Keywords: apple trees, endophytophagous, plant anatomy, vascular cambium activity, wolly-apple-aphid, xylem

2.2. Introduction

Malus domestica Borkh (Rosaceae) cultures are susceptible to the attack of *Eriosoma lanigerum* (Hemiptera: Aphididae) Hausmann, 1802, popularly known as woolly-apple-aphid, a global pest, which has been considered critical to the apple economy in the Southern hemisphere. This aphid occurs worldwide and affects the economy of the third most produced fruit around the globe (Denardi and Spengler 2001, FAO 2021), with approximately 130 million tons per year. In North America, *E. lanigerum* life cycle alternates generations, reproducing sexually in the American elm (*Ulmus americana*), and parthenogenetically in the apple trees (Danielsson 1979). In Brazil, more than 1.2 million tons of apple fruits are produced per year (FAO 2021), but several commercial varieties are susceptible to the attack of *E. lanigerum.* This aphid is parthenogenetic and restricted to the apple trees in Brazil, where the apterous forms induce galls on roots and stems (Danielsson 1979, Brown and Schmitt 1990, Asante 1994, Rinallo et al. 1995, Ateyyat and Al-Antary 2009, Stokwe and Malan 2016, Mandalon et al. 2020).

The development of *E. lanigerum* galls reduces the water conductivity and nutrient flow in the host stems and roots (Ateyyat and Al-Antary 2009), and impair the vigor of the trees, as the aphid feeding activity on phloem cells removes carbohydrates, occasionally causing the death of the trees (Madsen and Bailey 1958, Brown et al. 1995, Ateyyat and Al-Antary 2009, Zhou et al. 2013). Even though, the main feeding site for most aphids is the phloem (Wool 2005, Álvarez et al. 2009, Ferreira et al. 2019a), studies carried out using the Electric Penetration Graph (EPG) technique (McLean and Kinsky 1964) identified that the nymphs and adults of *E. lanigerum* perform the passive ingestion of phloemic sap, and the active ingestion of xylem content (Sandanayaka and Hale 2003, Sandanayaka et al. 2003, Hao et al. 2020, Zhou et al. 2021).

Malus domestica cv. 'Eva', our model of study, was developed by the Instituto Agronômico do Paraná (IAPAR) for cultivation in regions with mild winter, and has been successfully cultivated in Minas Gerais State, Brazil (Oliveira et al. 2011, 2014). The 'Eva' cultivar is resistant to high temperatures and has gained space in Brazilian market due to its flavor, color, and the early harvest of the fruits (Oliveira et al. 2014). The cultivar 'Eva' has been grafted on 'M9' a rootstock susceptible to the wooly-apple-aphid, but one of the most used in Brazil (Denardi et al. 2015). The *E. lanigerum* infestation has caused the precocious decline of the crops, with symptoms related to a 'dieback' of stem branches.

Most of gall inducers are endophytophagous organisms, that is, they are sheltered within plant tissues (Wool et al. 1999, Álvarez et al. 2009, Isaias et al. 2011, Liu et al. 2014, Richardson et al. 2017), but the woolly-aphid maintains the external position in its host roots and stems. From the host organ surface, the aphid feed on phloem sap causing the hypertrophy of phloem cells, and the establishment of a gradient of carbohydrate storage toward the gall chamber, as reported for other galling Hemiptera (Carneiro et al. 2014, Carneiro and Isaias 2015, Álvarez et al. 2021). The feeding habit on xylem, a mix of dead and live cells, and the establishment of a cecidogenetic field may imply in alterations in cambial activity, and in the differentiation of vessel elements and vascular parenchyma cells regarding structural and histochemical features.

We map the anatomical and histochemical alterations induced by *E. lanigerum* feeding activity on *M. domestica* stems of 'Eva' cultivar and roots of rootstock 'M.9' to evaluate the effects of the exophytophagy on both host organs. We assume that due to the peculiarities of the aerial and the subterraneous environment over the physiology of the host organs, stem and root galls may differ in structural and histochemical profiles. Alternatively, the impact of the wooly-aphid feeding activity may induce convergent structural and histochemical responses. The following questions address our discussion: (1) what are the xylem responses

of *M. domestica* roots and stems to the galling activity of *E. lanigerum*? And (2) do the histochemical profiles differ between the root and the stem galls? The elucidation of these questions may lead to a better understanding of the tolerance/susceptibility of *M. domestica* to *E. lanigerum* and may help crop management activities.

2.3. Material and methods

2.3.1. Sampling

Samples (1 cm² of the middle portion) of non-galled roots and stems, and of root and stem galls were collected from *M. domestica* individuals (cultivar 'Eva' stems and rootstock 'M.9' roots) ($n \geq 10$, per sample) in a private orchard in the municipality of Ervália, Minas Gerais, Brazil (20°52'02''S, 42°38'41''W). The local climate is Cwa of Köppen (Alvares et al. 2014) (humid subtropical zone, with dry winter and hot summer) with an annual rainfall of about 1,319 mm and a mean temperature of 22 °C (INMET 2021). The samples were fixed in Karnovsky's solution (2.5% glutaraldehyde and 4.5% formaldehyde) (Karnovsky 1965, modified to 0.1 M phosphate buffer, pH 7.2) for 48 h.

2.3.2. Anatomical and histochemical analyses

For anatomical analyses, the fixed samples were dehydrated in an ethanol series, followed by isoamyl acetate, and embedded in Paraplast X-TRA® at 60°C (Kraus and Arduin 1997, Álvarez et al. 2009). These samples were prepared to be sectioned in three anatomical planes (transverse, radial longitudinal, and tangential longitudinal; $n = 5$ per anatomical plane). The sections (18-20 μm) were obtained in a rotatory microtome (Leica® 2035 BIOCUT) and affixed to the slides using Bissing's adhesive (Bissing 1974, Kraus and Arduin 1997). The sections were deparaffinized in butyl acetate, rehydrated in an ethanol series and stained in Astra blue and safranin 9:1, v/v (Bukatsch 1972, modified to 0.5%, Kraus and Arduin 1997).

104 The sections were dehydrated in an ethanol series followed and butyl acetate and mounted
105 using colorless varnish Acrilex® (Paiva et al. 2006).

106 For histochemical analyses, the rehydrated sections were subjected to reagents for the
107 detection of starch, proteins, lipids, lignins, reducing sugars, and phenolic compounds (Table
108 1). The anatomical and histochemical sections were photographed with a Leica® ICC50HP
109 digital camera coupled to a Leica® DM500 light microscope.

110 **2.4. Results**

111 *2.4.1. Structural traits*

112 *Malus domestica* cv. 'Eva' is a deciduous tree with alternate leaves (Fig. 1A), white
113 pentamerous flowers (Fig. 1B), and pink to red pseudofruits (Fig. 1C). The *E. lanigerum*
114 attacks the roots (Fig. 1D), and the vegetative and reproductive shoots (Fig. 1E).

115 *2.4.2. Anatomical traits of non-galled roots and stems*

116 The *E. lanigerum* has been observed on the adventitious roots of *M. domestica* in secondary
117 growth, whose dermal system is a periderm with compacted suberized cells. The ground
118 system is constituted of 8-12 cell-layered cortical parenchyma, which is limited by small
119 strands of phloem fibers. The vascular cambium produces a continuous cylinder of
120 secondary phloem outward and secondary xylem inward with parenchyma rays, fibers, and
121 vessel elements. The central portion of the root is occupied by a parenchyma pith.

122 The dermal system of the stems in secondary growth is constituted by a periderm with
123 compacted suberized cells, and lenticels. The cortical parenchyma has 8-12 cell layers, and
124 phloem fibers arranged in arcs limit the inner vascular system. The vascular cambium
125 produces a continuous cylinder of secondary phloem outward and secondary xylem inward,
126 with parenchyma rays and fibers. The central portion is occupied by a parenchyma pith.

127 *2.4.3. Anatomical traits of root and stem galls*

128 The root and stem galls are divided in three regions regarding the position of *E. lanigerum*
129 colonies on the host organ surface: proximal (PR), median (MR), and distal regions (DR),
130 which are evident in root galls (Fig. 2A). The PR has semi-organized islets of secondary
131 vascular cells originated from the activity of the cambium-like cells, with the over
132 differentiation of larger parenchyma cells and vessel elements (VE) (Fig. 2B). In the
133 longitudinal radial section, we can observe VE surrounded by large amount of parenchyma
134 cells, whose traits are similar to those of the gall PR in transverse section (Fig. 2C). In the
135 longitudinal tangential section of the gall PR, the VE and parenchyma cells assume traits
136 similar to those of the other planes (Fig. 2D). The axiality of the vascular parenchyma cells
137 is lost in the PR of root galls. The MR has some redifferentiated cells and remnants of the
138 conserved vascular system, with reoriented VE and parenchyma cells (Fig. 2E). In
139 longitudinal radial sections, irregular vascular rays are observed (Fig. 2F). In longitudinal
140 tangential sections, the gall MR has parenchyma rays with irregular series (Fig. 2G). The
141 vascular cambium produces a continuous cylinder of secondary phloem outward and
142 secondary xylem inward in the gall DR with parenchyma rays, fibers, and isolated, small,
143 and regularly distributed VE (Fig. 2H). The central portion of the root is occupied by a
144 parenchyma pith. The vascular rays are homogenous (Fig. 2I), and uniseriate (Fig. 2J). The
145 DR anatomical organization is similar to that of the non-galled roots, and is located at the
146 opposite site of the wooly-aphid-apple colonies. The radial pattern is absent in the PR and
147 MR root gall.

148 The development of the stem gall disrupts the standard pattern of the stem secondary growth,
149 as observed in the proximal (PR'), the median (MR'), and the distal (DR') gall regions (Fig
150 3A). The gall PR' in transverse section has semi-organized islets of secondary vascular cells
151 originated from the activity of cambium-like cells originating parenchyma cells with vessel

elements interspersed (Fig. 3B). In the longitudinal radial section, we can observe that the radial pattern is disrupted and the VE and parenchyma cells assume similar traits of the gall PR' in transverse section (Fig 3C). In the longitudinal tangential section of the gall PR', the tangential pattern is not observed, and the VE and parenchyma cells assume traits similar to those of the other planes (Fig. 3D). The gall MR' has an irregular distribution of VE, rays, and parenchyma cells, as observed in transverse sections (Fig. 3E). In longitudinal radial sections, irregular vascular rays are observed together with parenchyma cells and VE (Fig. 3F). In longitudinal tangential sections, the gall MR has parenchyma rays with up to two irregular series (Fig. 3G). The secondary xylem of the gall DR' has isolated, small, and regularly distributed VE (Fig 3H). Grouped vessels may occur. The vascular rays are homogeneous (Fig. 3I) and uni or biseriate (3J). The gall DR' traits are similar to those of the non-galled stem portions.

2.4.4. Histochemical traits of non-galled roots and root galls

In the non-galled roots, the cells of the cortical parenchyma, parenchyma rays, and pith accumulate starch (Fig. 4A), reducing sugars (Fig. 4B), proteins, and phenolics (Fig. 4C). Lipids are detected as suberin in the periderm (Fig. 4D). The cells of parenchyma rays and pith accumulate starch, reducing sugars, and phenolics. Lignins are detected in the walls of fibers, radial parenchyma cells, and vessel elements (Fig. 4E). There is no detection of these metabolites in non-galled root cambial cells.

In root galls, starch was detected in the parenchyma cells of the PR (Fig. 4F) and MR (Fig. 4G), and in the cortical parenchyma, parenchyma rays, and pith of the DR. Reducing sugars are detected in the vascular cambium and parenchyma cells of the PR (Fig. 4H), parenchyma cells of the MR (Fig. 4I), and parenchyma cells, parenchyma rays, and pith of the DR. Phenolics are detected in parenchyma cells of the PR (Fig. 4J), cortical parenchyma, parenchyma rays, parenchyma cells of the MR (Fig. 4K), and phloem of the three regions.

Proteins are detected in the vascular cambium and parenchyma cells in the PR. Lipids are detected as suberin in cell walls of periderm. Lignins are detected in the parenchyma cells (Fig. 4L), and vessel elements of the PR and of the MR (Fig. 4M), and fibers, parenchyma rays, and vessel elements of the DR.

2.4.5. Histochemical traits of non-galled stems and stem galls

In the non-galled stems, starch grains are detected in cells of the cortical parenchyma, parenchyma rays, and pith (Fig. 5A). Reducing sugars are detected in parenchyma cells (Fig. 5B), parenchyma rays, and pith. Lipids are detected as suberin in the periderm (Fig. 5C), and proteins are not detected. Phenolic compounds are detected in parenchyma rays (Fig. 5D). Lignins are detected in cell walls of parenchyma ray, and vessel elements (Fig. 5E). These metabolites are absent in non-galled stem cambial cells.

In the stem galls, starch grains are detected in the cortical parenchyma cells (Fig. 5F), and in the parenchyma rays, and in the parenchyma cells of the MR' (Fig. 5G) and the DR'. Reducing sugars are detected in the parenchyma cells of the PR' (Fig. 5H) and MR', and cortical parenchyma, parenchyma rays, and pith of the DR', and in the phloem of the three regions. Lipids are detected in parenchyma cells of the PR' (Fig. 5I), and as suberin in periderm cell walls, and parenchyma rays of the DR' (Fig. 5J). Proteins are not detected. Phenolic compounds are detected in vascular cambium, parenchyma cells and parenchyma cells of the PR' (Fig. 5K) and parenchyma rays of the MR' (Fig. 5L), and parenchyma cells of the DR'. Lignins are detected in the walls of xylem cells of the PR' (Fig. 5M), parenchyma cells of the MR' (Fig. 5N), and fibers and vessel elements of the DR'.

2.5. Discussion

The feeding activity of *E. lanigerum* triggers the development of *M. domestica* root and stem galls by the insertion of its stylets inter- and intracellularly (Staniland 1924, Tjallinggi 2006).

New cell differentiation is promoted, but remaining conserved areas occur in the galls at the DR and DR', i.e., opposite to the feeding site of the wooly-aphid-apple colonies. The stylets reach the phloem, vascular cambium, and xylem cells (Sandanayaka and Hale 2003, Sandanayaka et al. 2003, Hao et al. 2020), probably causing the abnormal cambial activity observed in *E. lanigerum* galls, due to the cecidogenetic field generated by the salivary secretion (Hori 1992, Ferreira et al. 2019a). The outcomes of a localized and abnormal cambial activity is confirmed by our histological observations. Despite the structural changes of roots and stems of *M. domestica* toward the gall developmental sites, the histochemical profiles of the non-galled host organs and of the galls have differences just in the intensity of the labeling on some metabolites.

2.5.1. Main responses of host root and stem tissues

As expected, the main tissue responses occur in the region proximal to the feeding site of *E. lanigerum* (PR and PR') and the most significant and visible alterations are observed in the gall xylem. The *E. lanigerum* galls result from the rupture of the vascular system developmental patterns because of rapid and successive cell divisions (Brown et al. 1991), and to the stimulation of an abnormal cambial activity. The abnormal cambial activity has been reported for hemipteran galls (Crystal 1926, Smith 1967), and in the case of *E. lanigerum* galls results in the overproduction of thin-walled parenchyma cells and the differentiation of abnormal vessel elements. These responses may be related to the ingestion of xylem sap by the aphids, an alternative and efficient method to restore and maintain their water balance (Cull & Van Emden 1977, Spiller 1990, Sandanayaka and Hale 2003), and to promote the reduction of water deficit in gall developmental sites. Also, the hypertrophy of parenchyma cells in galls has been related to the improvement of water storage (Oliveira et al. 2006). Indeed, the islets of secondary parenchyma cells in galls is an output of the altered cambial activity that are expected to benefit the aphids, which is yet to be tested.

The benefit of water storage to the galling aphid counteracts with the effect of the anomalous xylem, which reduce the growth of the apple trees, impacting the water flow (Brown et al 1991) from the host organs toward the *E. lanigerum* galls. The new organization of the vascular system in gall developmental site guarantees high water resources, mainly, in the PR, where the islet of secondary tissues originated from the abnormal cambial activity confers a specialized feeding site for *E. lanigerum*. Furthermore, the xylem alterations result in an asymmetrical cecidogenetic field, as the *E. lanigerum* colony is usually in contact only with one side of the organ, where the anatomical alterations are concentrated. It must be highlighted that the regions opposite to the observed galls remain with the expected non-alterated cambial activity.

2.5.2. The histochemical determination of gall impact on roots and stems

The accumulation of starch and reducing sugars in roots and stems is crucial to the maintenance of plant metabolism and growth (Tromp 1983, Breen et al. 2020), while in galls, these carbohydrates can support the maintenance of gall tissue metabolism, as well as the nutrition of the galling insect (Oliveira et al 2006; Álvarez 2012, Castro et al. 2013, Ferreira et al. 2019b), resulting in loss of potential plant growth. The reducing sugars may also support respiration and wall synthesis in dividing cells (Koch 2004, Ferreira et al. 2019b).

The slight detection of proteins only in non-galled roots and in root galls can be related to the reallocation of nitrogen reserves to other plant parts, as reported during leaf senescence (Tromp 1983, Paungfoo-Lonhienne et al. 2008, Castro et al. 2013), and for galls at the end of their life cycles (Oliveira and Isaias 2010). The lipids were detected as suberin impregnation in cell walls of the periderm of the non-galled organs and galls indicating that this class of metabolites work out only as a structural component of *E. lanigerum* galls on *M. domestica*. The low lignification process in the PR of the galls may facilitate the *E.*

250 *lanigerum* feeding, since the excess of lignins can interfere in the insertion of the stylets in
251 plant tissues (Staniland 1924).

252 The reallocation of phenolics to the proximal, median, and distal regions of the *E. lanigerum*
253 gall tissues may be a protection against the oxidative stress generated by the galling organism
254 respiration and gall cytological metabolism, as previously proposed for other galls (Isaias et
255 al. 2015). The phenolic derivatives can also act on signaling and stimulation of gall growth,
256 when associated with indol-acetic acid (IAA) (Bedetti et al. 2014; 2017). The phenols-IAA
257 association in cambial cells modulate cell expansion and division, as the polyphenols may
258 act as IAA oxidase inhibitors (Bedetti et al. 2014; 2017). So, the accumulation of phenolics
259 in the parenchyma cells of the three regions of *E. lanigerum* root and stem galls is indicative
260 of their involvement in gall tissue growth rather than in an antiherbivore defense.

261 *2.5.3. The exophytophagy implications on **E. lanigerum** galls*

262 The root and the stem galls induced by *E. lanigerum* have characteristic sites of parenchyma
263 cell hypertrophy and hyperplasia, an increase in the area of vascular tissues, and the non-
264 development of a nutritive-like tissue, similarly to plant responses to endophytophagous
265 hemipteran galls (Isaias et al. 2011, Álvarez 2012, Carneiro et al. 2014, Guedes et al. 2018,
266 Ferreira et al. 2019a, Silva et al. 2019). However, some anatomical features observed in
267 some hemipteran stem galls are distinct from those of *M. domestica-E. lanigerum* system.
268 The galls induced by the first instar of *Bystracoccus mataybae* (Eriococcidae) has alterations
269 restricted to the periderm development (Silva et al. 2019). In galls induced on *Populus*
270 *angustifolia* (Salicaceae) leaves by *Pemphigus betae* (Aphididae), the xylem was absent
271 (Richardson et al. 2017), and in galls induced by *Euphalerus ostreoides* (Psyllidae) on
272 leaflets of *Lonchocarpus muhelbergianus* (Fabaceae), the cambial activity results in higher
273 differentiation of phloem than xylem cells (Oliveira et al. 2006). Such features differ from

the over-differentiation of xylem parenchyma cells and phellogen activation observed on *E. lanigerum* galls on *M. domestica*.

Comparing the galls of *E. lanigerum* on *M. domestica* with other systems involving exophytophagous taxa, such as the *Ditylenchus gallaeformans* (Nematoda) on species of *Miconia* (Melastomataceae) (Ferreira et al. 2017a, 2017b, Arriola and Isaias 2021), we can see that the indeterminate growth induced by enzymes, such as cellulases and pectinases of the nematode's saliva (Arriola and Isaias 2021), and supported by the promeristematic activity of nutritive cells, is absent in the aphid galls. In fact, the manipulation of cambial activity is crucial for the establishment of the galls on *M. domestica*. Cambial activity is regulated by auxins, cytokinins, and ethylene (Matsumoto-Kitano et al. 2008, Wang 2020), being auxins linked to the positive regulation of the differentiation of vessel elements (Smetana et al. 2019, Wang 2020, Bragança et al. 2021). The low number of vessel elements in relation to the parenchyma cells observed in the gall PR can indicate a reduction in auxin activity and a high activity of cytokinins, which regulate hyperplasia in gall developmental sites (Ferreira 2017b, 2019b, Bragança et al. 2021). The cytokinins can reduce the auxin transport in the vascular cambium, leading to a slow differentiation of vessel elements (Bishopp et al. 2011, Bragança et al. 2021), while the over-production of xylem parenchyma (Junghans et al. 2004, Wang 2020) may be influenced by ethylene produced under stressful conditions (Yang and Hoffman 1984).

2.6. Final considerations

The analyses of the galls induced by *E. lanigerum* on *M. domestica* roots and stems reveal novelties on the knowledge about aphid-induced galls. The interruption of cambium continuity and enhanced parenchyma cell differentiation in the gall MR mark the transition zone of the gall DR and PR. The islets of cambium-like cells differentiating parenchyma cells with interspersed vessel elements in the gall PR account for a new fate for fusiform

299 cambial initials, which also lose their axiality. The slight differences on root and stem gall
300 anatomical and histochemical profiles presents a convergent response and do not support the
301 hypothesis of environmental constraints over gall development. The alteration in cambial
302 activity is restricted to PR and do not extend to cambial regions before, after and neither
303 opposite to the *E. lanigerum* gall site.

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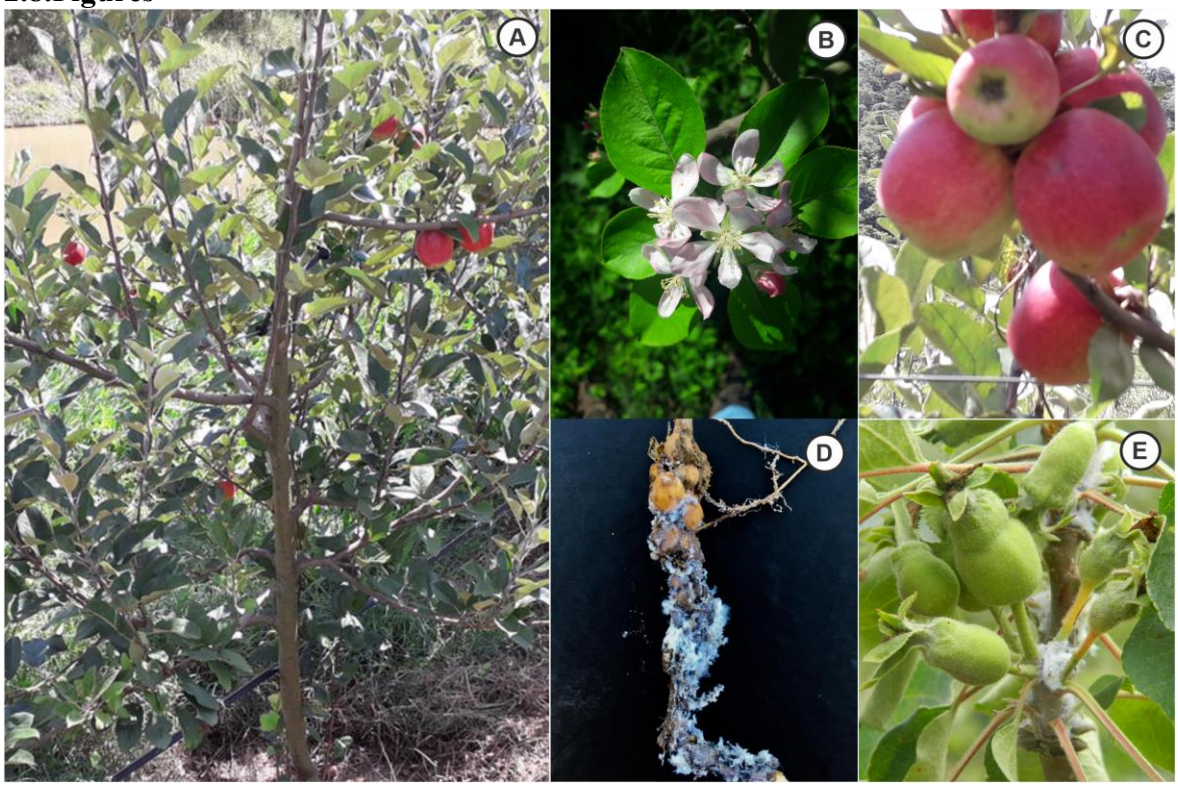
527 **2.8.Figures**



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529 **Fig. 1** Habitus of *Malus domestica* 'EVA' cultivar. (A) Apple tree. (B) Flowers. (C) Apple
530 fruits. (D) Root galls. (E) Stem branch with galls.

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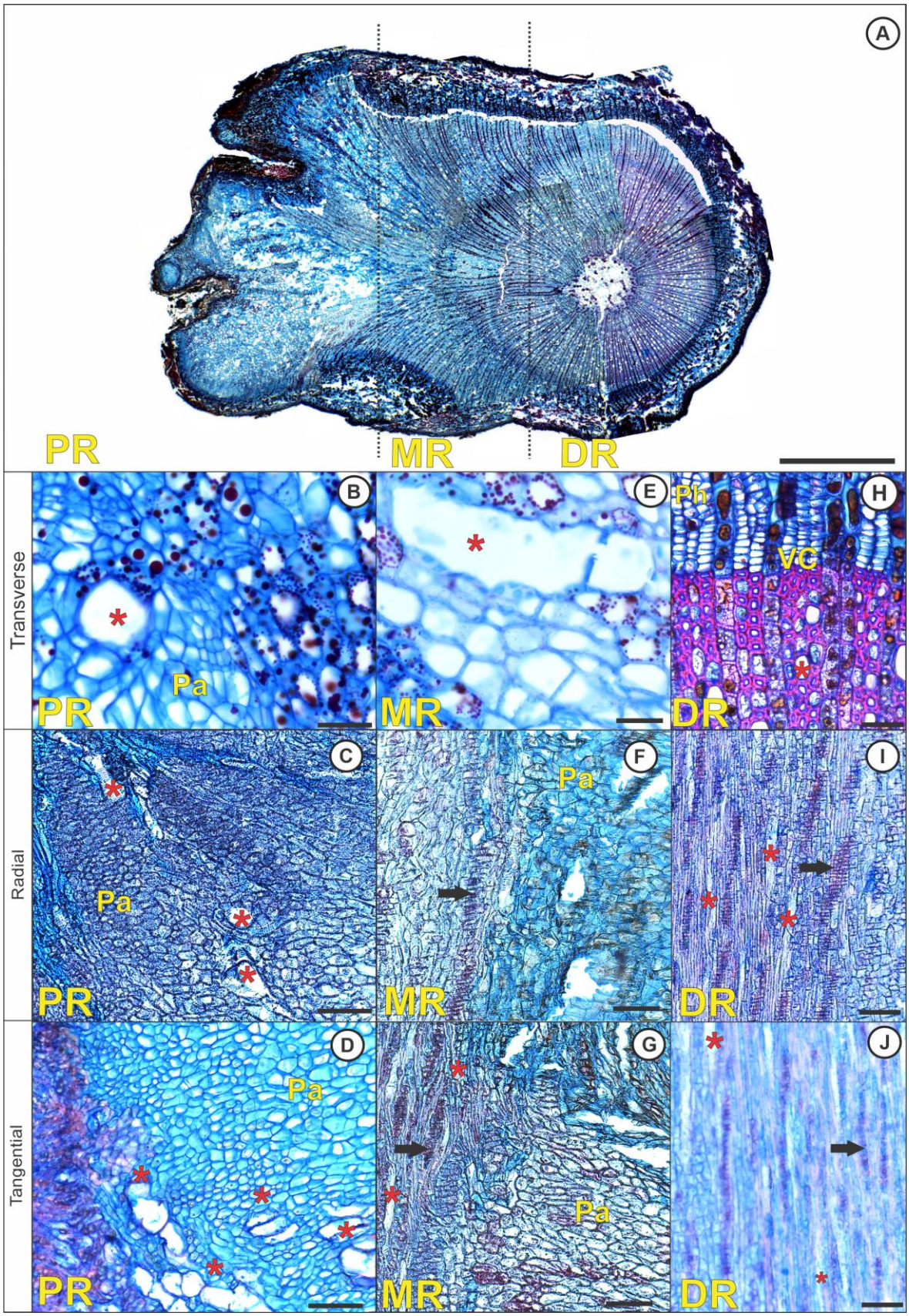

Fig. 2 Anatomy of root galls induced by *Eriosoma lanigerum* on *Malus domestica* 'M.9' cultivar. (A) The dotted lines indicate gall regions regarding the aphid position in root surface: (PR) proximal region, (MR) median region, and (DR) distal region. (B) Transverse section of an islet of cambium-like cells in the PR with reoriented parenchyma cells and

538 vessel elements. (C) Longitudinal radial section of the PR with large amount of parenchyma
539 cells and vessel elements without longitudinal radial pattern. (D) Longitudinal tangential
540 section of the PR with parenchyma cells and vessel elements without longitudinal tangential
541 pattern. (E) Transverse section of the MR with reoriented parenchyma cells and irregular
542 distribution of vessel elements. (F) Longitudinal radial section of the MR with irregular
543 vascular rays (arrow) along with parenchyma cells. (G) Longitudinal tangential section of
544 the MR with uni-biseriate irregular rays (arrow) (H) Isolated vessel elements of the DR. The
545 phloem and cortical parenchyma are similar to those of the non-galled roots. (I) Homogenous
546 rays (arrow) and vessel elements in the DR are similar to the non-galled roots. (J) Uniseriate
547 rays (arrow) and vessel elements in the DR, whose arrangement is similar to that of the non-
548 galled roots. Asterisks (vessel elements). Pa – parenchyma, Ph – Phloem, Vc – vascular
549 cambium. Bars: 500 μm (A), 200 μm (C, D, F, G, I, J), 50 μm (B, E, H).

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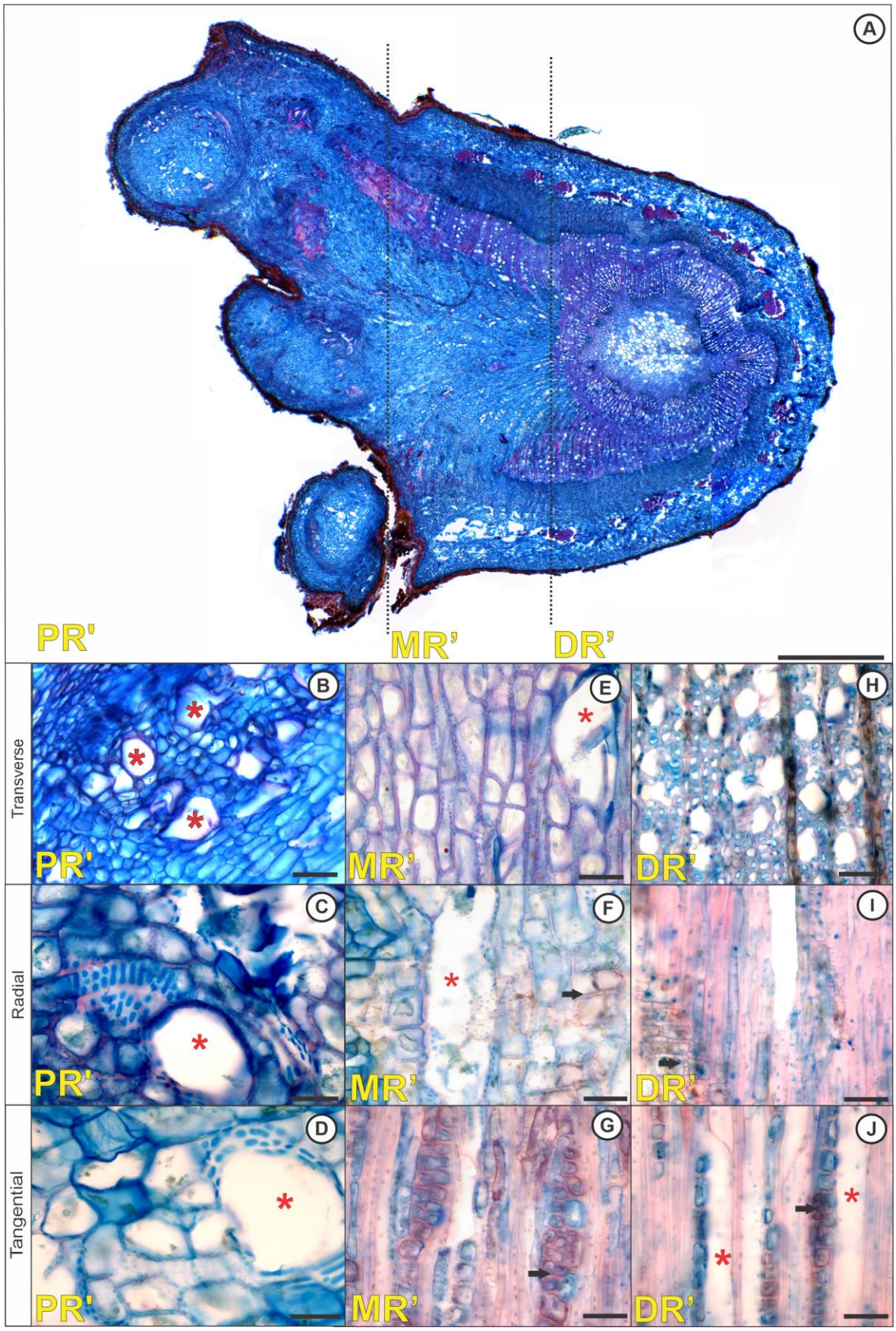

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553 **Fig. 3** Anatomy of stem galls induced by *Eriosoma lanigerum* on *Malus domestica* 'Eva'
554 cultivar. (A) The dotted lines indicate gall regions regarding the aphid position in stem
555 surface: (PR') proximal region, (MR') median region, and (DR') distal region. (B)

Transverse section of an islet of cambium-like cells in the PR' with reoriented parenchyma cells and vessel elements. (C) Longitudinal radial section of the PR' with parenchyma cells and vessel elements without longitudinal radial pattern. (D) Longitudinal tangential section of the PR' with parenchyma cells and vessel elements without longitudinal tangential pattern. (E) Transverse section of the MR' with reoriented rays, parenchyma cells, and irregular distribution of vessel elements. (F) Longitudinal radial section of the MR' with irregular vascular rays (arrow) along with parenchyma cells and vessel elements. (G) Longitudinal tangential section of the MR' with uni-biseriate irregular rays (arrow). (H) Isolated vessel elements in the DR' similarly to the non-galled stems. (I) Homogenous rays (arrow) and vessel elements in the DR', whose arrangement is similar that of the non-galled stems. (J) Uniseriate rays (arrow) and vessel elements in the DR, whose arrangement is similar to the non-galled stems. Asterisks (vessel elements). Bars: 500µm (A), 50µm (B-J).

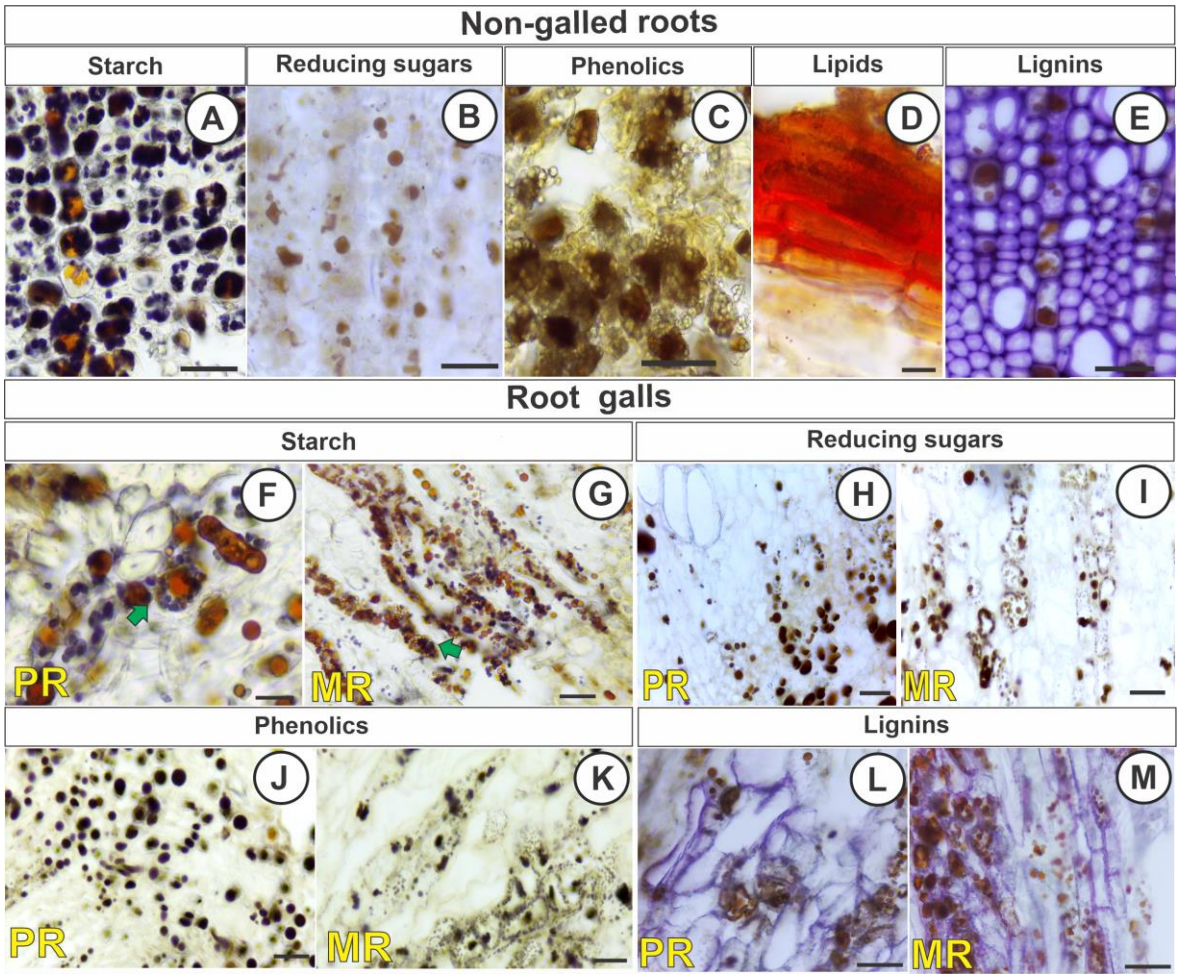
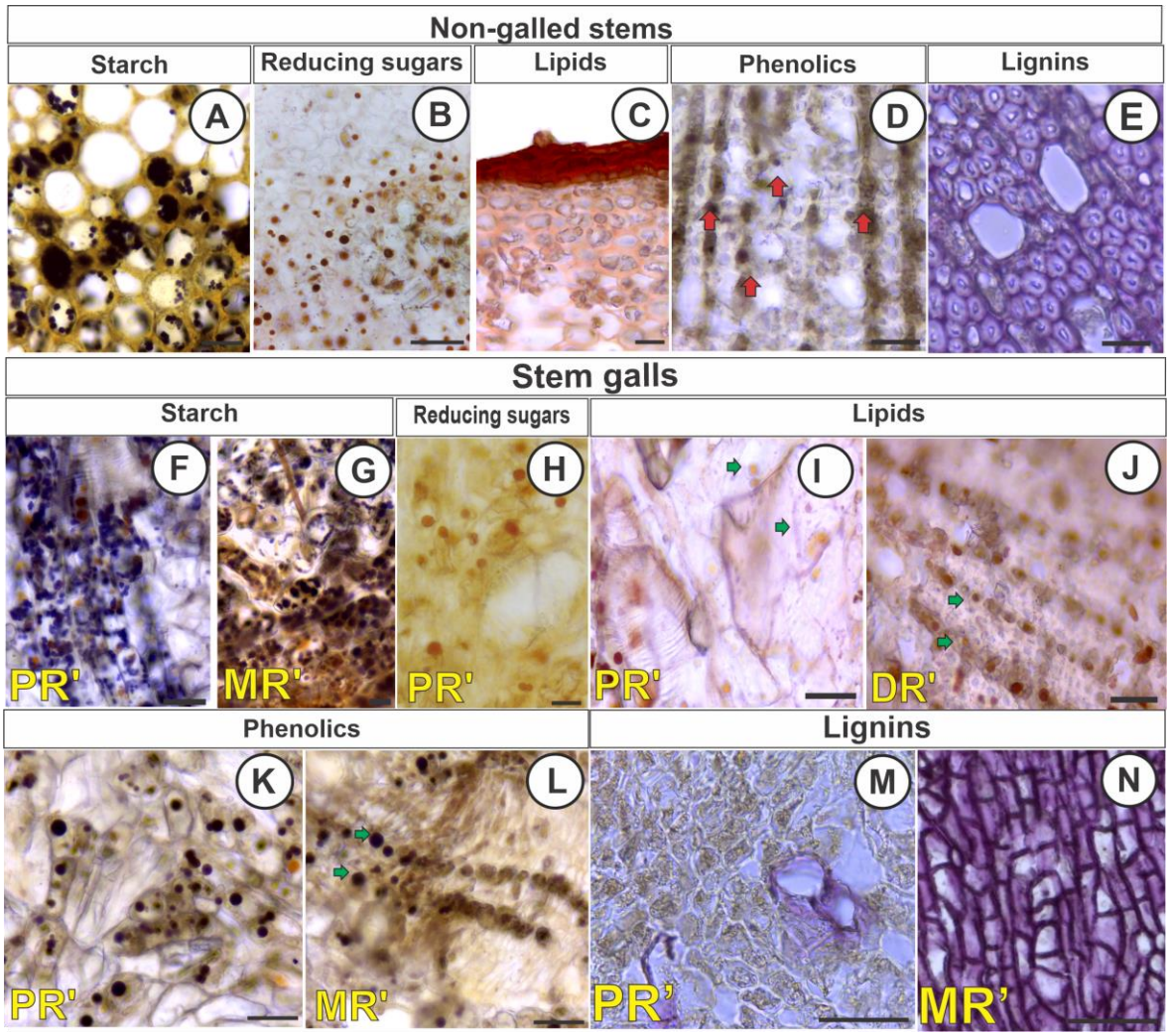


Fig. 4 Histochemical profiles of non-galled roots and root galls induced by *Eriosoma lanigerum* on *Malus domestica* cv. 'Eva'. (A-E) Non-galled roots. (A) Starch grains in parenchyma rays. (B) Reducing sugars in parenchyma cells. (C) Phenolics in parenchyma cells. (D) Suberin in periderm. (E) Lignin in xylem. (F-M) Root galls. (F) Starch grains in parenchyma cells of the PR and (G) MR (green arrow) (H) Reducing sugars in parenchyma cells of the PR and (I) MR. (J) Phenolics in parenchyma cells of the PR and (K) MR. (L) Lignins in parenchyma cell walls of the PR and (M) MR. PR- proximal region, MR- median region, DR- distal region. Bars: 50 µm (A-B; D-N), 20 µm (C).



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586 **Fig. 5** Histochemical profiles of non-galled stems and stem galls induced by *Eriosoma*
587 *lanigerum* on *Malus domestica* cv. 'Eva'. (A-E) Non-galled stems. (A) Starch grains in pith
588 cells. (B) Reducing sugars in parenchyma cells. (C) Suberin in the periderm. (D) Phenolics
589 in parenchyma rays (red arrows). (E) Lignins in xylem cell walls. (F-N) Stem galls. (F)
590 Starch grains in parenchyma cells of the PR' and (G) MR'. (H) Reducing sugars in
591 parenchyma cells of the PR'. (I) Lipids in parenchyma cells of the PR' and (J) parenchyma
592 rays of the DR'. (K) Phenolics in parenchyma cells of the PR' and, (L) parenchyma rays of
593 the MR'. (M) Lignins in xylem of the PR', and (N) parenchyma cells of the MR'. PR'-
594 proximal region, MR'- median region, DR'- distal region. Bars: 50 µm (A-O)

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599 **2.9. Table 1. Reagents used for histochemical analyses in sections of *Malus domestica***
600 **non-galled roots and stems, and in root and stem galls.**

Metabolite	Histochemical tests	Positive reaction	Reference
Lipids	Sudan III (saturated solution in 70°GL ethanol for 5 min)	Red / Orange droplets	Feucht et al. (1986)
Starch	Lugol´s reagent (1% potassium iodine–iodide solution for 5 min)	Black grains	Johansen (1940)
Proteins	Mercuric bromophenol blue solution (mercury chloride 10% and bromophenol blue 0.1%) for 15 min, wash in 5% Acetic Acid for 20 min and wash in distilled water for 15min	Blue precipitates	Mazia et al (1953)
Reducing sugars	Solution A (II copper sulfate 6.93% w: v) and solution B (potassium sodium tartrate 34.6% and sodium hydroxide 12%, m: m: v), 1:1, heated until pre-boiling	Bright red	Sass (1951)
Phenolic compounds	Ferric chloride (10% solution for 5 min)	Black or brown precipitates	Johansen (1940)
Lignins	Acidified phloroglucinol (phloroglucinol 2% in hydrochloric acid 25%) for 5 min	Pink	Johansen (1940)

601

3. Capítulo 2

Xylem responses to the feeding activity of *Eriosoma lanigerum* in *Malus domestica* stems under the light of the "gall constriction" hypothesis

Xylem responses to the feeding activity of Eriosoma lanigerum in Malus domestica stems under the light of the "gall constriction" hypothesis

Mariana de Sousa Costa Freitas[1], Ravena Malheiros Nogueira[1], Gener Augusto Penso[2], Edgard Augusto de Toledo Picoli[2], Rosy Mary dos Santos Isaias[1]

1 Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Laboratório de Anatomia Vegetal, Departamento de Botânica, Belo Horizonte, Minas Gerais, Brazil.

2 Universidade Federal de Viçosa, Departamento de Biologia Vegetal, Centro de Ciências Biológicas II, Viçosa, Minas Gerais, Brazil.

*Corresponding author: rosy@icb.ufmg.br

3.1. Abstract

Malus domestica (Rosaceae) stems are susceptible to the infestation of the aphid *Eriosoma lanigerum* (Hemiptera: Aphididae). The feeding activity of the aphid alters the plant vascular system in such a degree that may prioritize the water flow to the galls, as postulated by the "gall constriction" hypothesis. Accordingly, the *E. lanigerum* feeding activity on *M. domestica* stems causes an asymmetrical cecidogenetic field regarding the aphid location on stem axis, whose symptoms may be evaluated through the influence on the differentiation of xylem cells. In the anatomical perspective, *M. domestica* stem galls have three regions regarding the position of the aphid colonies, a proximal (PR), a median (MR), and a distal region (DR). We investigate the secondary xylem aspects of the galls on the PR+MR and on the DR comparatively to the non-galled stems. We also investigate xylem cells in three galled stem portions: below the gall, the gall, and above the gall, to test the "gall constriction"

hypothesis. In *M. domestica – E. lanigerum* system, the similarity in the dimensions of the vessel elements in the portions above and below the gall contradicts the "gall constriction" hypothesis, however the over-differentiation of parenchyma cells, redifferentiation of abnormal vessel elements, and reorientation of cell axis may promote a higher water supply to the gall than to the non-galled stem portions, which functions as compensatory mechanism for the maintenance of the water status in gall developmental site. Such symptoms may be related to the decline of the crops by dehydration of apical buds.

Keywords: apple trees, secondary xylem, vessel elements, wooly-apple-aphid

3.2. Introduction

The apple tree (*Malus domestica*) crops are susceptible to the infestation of the aphid *Eriosoma lanigerum* (Hemiptera: Aphididae), which result in gall induction both on roots and stems. Our model of study, *M. domestica* cv. 'Eva', was developed by the Instituto Agronômico do Paraná (IAPAR) for cultivation in regions with mild winter, such as in Minas Gerais state, Brazil (Oliveira et al. 2011, 2014). The cultivation in these areas is recent and promising, allowing an increase in the area and in the total production of apples in the country. Despite the success of the cultivation, the plants were infested by *E. lanigerum*, which has caused the precocious decline of the crops. The development of the galls reduces the water conductivity in the affected plants, impacting the vigor of the trees. In addition, the feeding of the aphid in phloem cells removes carbohydrates, which can cause the individual's death (Madsen and Bailey 1958, Brown et al. 1995, Ateyyat and Al-Antary 2009).

Gall establishment on stems may cause alterations in all plant tissue systems, which may be more conspicuous in secondary vascular tissues (Aloni et al. 1995, Best et al. 2004) due to the influence of gall induction on phellogen and vascular cambium. Vascular alterations are independent of the taxa of the organisms involved in the interaction as they have been reported in *Ricinus communis* galls induced by *Agrobacterium tumefaciens* (Aloni et al. 1995) and in *Eremanthus erythropappus* (Asteraceae) galls induced by *Neolasioptera* sp. (Diptera: Cecidomyiidae) (Jorge et al. 2021). Furthermore, changes in vascular tissues have been reported in galls induced by phloem sucking-insects, such as *E. lanigerum*, on *Populus angustifolia* (Salicaceae) leaves by *Pemphigus betae* (Aphididae) (Richardson et al. 2017) and on *Lonchocarpus muhelbergianus* (Fabaceae) leaflets by *Euphalerus ostreoides* (Psyllidae) (Oliveira et al. 2006). The alterations generated by gall induction in plant vascular system may lead to a prioritization of water flow to the galls as postulated by the

"gall constriction" hypothesis (Aloni et al. 1995). This hypothesis predicts that the stem regions below the gall have rays and vessel elements of regular size, while the gall and the region above the gall has narrower vessel elements, increased rays, and absence of fibers, which relates to a limited water transport to the aerial plant portions (Aloni et al. 1995; Ulrich and Aloni 2000). Such anatomical profile in galled stems may explain the dieback of *M. domestica* branches and the decline of the crops.

Due to the asymmetrical cecidogenetic field generated by *E. lanigerum* feeding activity on *M. domestica* stems, its galls have three anatomical regions regarding the position of the aphid colonies, a proximal (PR), a median (MR), and a distal region (DR). Such regions have a decreasing degree of alterations the more distant the feeding site is (*cf*. Freitas et al. 2021). To evaluate this gradient of effects of *E. lanigerum* galls on the xylem of *M. domestica*, we investigate the aspects of the gall PR+MR and of the gall DR comparatively to the non-galled stems. We also investigate isolated vessel elements in three stem portions, below the gall, the gall itself, and above the gall, to verify if the impairment to the growth of *M. domestica* trees may be consequence of "gall constriction" (Aloni et al. 1995). Our discussion follows the functional implications of xylem alterations to the water supply of *M. domestica* stem branches under the attack of *E. lanigerum*.

3.3. Material and methods

3.3.1. Sampling

Samples of non-galled stems and stem galls were collected from four-year old individuals (n = 5) of *M. domestica* cv. Eva grafted on 'M9' rootstock in a commercial orchard in the municipality of Ervália, Minas Gerais State, Brazil (20°52'02''S, 42°38'41''W), and were fixed in FAA (formalin, acetic acid, 50% ethanol, 1:1:18) (Johansen 1940).

3.3.2. Dissociation of vessel elements

Fragments (0.25 cm²) of the non-galled stems and of the stem portions below and above the galls, and of the galls in PR+MR and DR (n = 5 per sample, total of 25 samples) were submitted to cell dissociation. The samples were washed three times in tap water and immersed in 50% sodium hypochlorite, which was changed several times for approximately three days; the samples were washed in tap water, stained in 0.5% safranin for 24 h, submitted to cell manual dissociation, and washed in tap water. The slides (n = 3 per sample) were mounted with Kaiser's jelly glycerin (Kraus and Arduin 1997). These slides were used for measuring the length and width of vessel elements (n = 10 vessel elements per slide, total of 300 measurements) using the AxioVision 7.4 software (Carl Zeiss® Microscopy GmbH, Jena, Germany).

The number of vessel elements in an image area of 8 mm² were counted in transverse sections of the non-galled stems and of the stem galls (n = 5 individuals, 10 images per individual, total of 50 vessel elements per individual). The density of vessel elements per xylem area were obtained by dividing the average number of vessel elements per the image of xylem area (8 mm^2), and adjusted to vessels per mm^2.

3.3.3. Image capture

The anatomical, cytometrical, and histometric analyses, were performed in images obtained with a Leica® ICC50HP digital camera coupled to a Leica® DM500 light microscope.

3.3.4. Statistical analyses

Parametric data were compared using the Student's T test (for two categories) or one-way ANOVA (for three or more categories) followed by Tukey's test. Non-parametric data were compared with the Mann-Whitney' test (for two categories) and Kruskal-Wallis test (for three or more categories) followed by Dunn's test. The tests were performed with the SigmaStat® (Systat Software, Inc., Chicago, Illinois) and the graphs were made in GraphPad

Prism 8.0® software. All tests used $\alpha = 0.05$.

3.4. Results

3.4.1. Analyses of vessel elements

The analysis of the galled stem portions: above the gall, gall, and below the gall (Fig. 1A) evidenced similarities and differences regarding the vessel elements (VE) dimensions. The VE differ in length ($p < 0.001$) and width ($p = 0.009$) between the stem portion below the gall and in the gall, and they also differ in length ($p < 0.001$) and width ($p = 0.005$) between the gall and the stem portions above the gall (Fig. 1B-C).

The average density of VE in the non-galled stems (3.5 mm-²) is different from that of the galled stems (4.9 mm-²) ($p = 0.008$) (Fig. 2A). The VE in the non-galled stems are significantly longer ($p \leq 0.001$) and narrower ($p = 0.008$) than the VE in the gall PR+MR, but they are similar to those of the gall DR in length ($p = 0.171$) and width ($p > 0.05$). The VE in gall DR are narrower than those of the gall PR+MR in length ($p < 0.001$) and width ($p < 0.05$) (Fig. 2B). The average dimensions of the VE in non-galled stems are 142.9 ± 13.7 µm x 11.03 ± 0.65 µm, while the average dimensions of the VE in the gall DR are 126.3 ± 11.2 µm x 10.8 ±12 µm (Fig 2C). The VE of the non-galled stems and of the gall DR have various sized appendices in one or both extremities, the perforation plates are simple and the pits are opposite (Fig. 2C). The VE observed in the gall PR+MR presents simple displaced perforation plates and the appendices are absent in most of these VE, and its average dimensions are 52.5 ± 3.2 µm x 16.12 ± 3.2 µm (Fig. 2C).

3.5. Discussion

The galls induced on *M. domestica* by the colonies of *E. lanigerum* result from abnormal divisions in the initial cells of the vascular cambium with pronounced effects observed in secondary xylem organization, which are the focus of current investigation. The main

131 diagnostical features observed in the secondary xylem are an increment in the differentiation
132 of abnormal vessel elements, the reorientation of cell axis, and the over-differentiation of
133 parenchyma cells (*cf*. Freitas et al. 2021).

134 The similarity of the dimensions of the vessel elements in the portions below and above the
135 stem galls indicates a restrict amplitude for the cecidogenetic field, the limitation of xylem
136 cells to increase the water supply to the galls on *M. domestica*. Distinctly, variations in
137 vascular tissues in other host plant-gall inducer systems, such as *Ricinus communis*-
138 *Agrobacterium tumefaciens*, have been observed in the stem portions above and below the
139 gall, which supported the gall constriction hypothesis (Aloni et al 1995, Ulrich and Aloni
140 2000). In *E. lanigerum*- *M. domestica* system, the vessel elements in the gall PR+MR are
141 different from those observed above and below the gall. Such neo-formed cells potentially
142 increase the storage of water in the gall developmental site. The over differentiation of xylem
143 parenchyma cells is also accompanied by a higher average number of vessel elements in the
144 stem galls than in the non-galled stems on *M. domestica*, which is like the observations on
145 the *Agrobacterium*-induced galls (Aloni et al. 1995, 1989, Aloni 2013). The higher number
146 of both parenchyma cells and vessel elements is restricted to the gall PR+MR, and configure
147 not only the storage but an increment in the potential translocation of water inside the gall.
148 This water increment is related to the microenvironment hypothesis (Price et al. 1987, Stone
149 & Schonrögge 2003), since the can gall protect the galling insect from water stress (Cull &
150 Van Emden 1977, Spiller 1990, Stone & Schonrögge 2003).

151 The apple trees attacked by *E. lanigerum* have impairment in vigor, sometimes resulting in
152 plant death (Brown et al. 1995, Ateyyat & Al- Antary 2009). As the *E. lanigerum* gall
153 induction and development on *M. domestica* affects the differentiation of xylem cells, it can
154 be related to the reduction in water conduction in affected plants reported in the literature
155 (Ateyyat & Al-Antary 2009). The effects of this reduction in water conduction occurs in two

fronts, roots and stems of *M. domestica* are affected. The over-differentiation of parenchyma cells in insect galls has been associated with processes of hyperplasia and hypertrophy (Oliveira & Isaias 2010), which can be influenced by ethylene action over vascular cambium cells (Junghans et al 2004). Ethylene is a phytohormone produced under stressful situations (Yang and Hoffman 1984), such as gall induction (Aloni et al. 1988). The more parenchyma cells differentiate in stem galls, the higher is the water supply to the galled stems than to the non-galled stem portions (Jorge et al. 2021), corroborating the harmful effect of the galls in *M. domestica* plants.

The abnormal vessel elements in *E. lanigerum* galls, whose size and shape are distinct from those of the vessel elements of the non-galled stem portions, do not seem to differentiate directly from the vascular cambium initials. Instead, they seem to originate from the redifferentiation of xylem parenchyma cells. The abnormal vessel elements alter the fluid dynamics favoring the water supply to the galls. This redifferentiation relates to hormone transport and concentration, mainly to auxins (Aloni et al. 1995, Best et al. 2004, Aloni 2013, Dolzblasz et al. 2018, Bragança et al. 2021), which lead us to infer that the feeding activity of *E. lanigerum* is modulating the auxin transport in the gall developmental site. The auxin transport, the activity of receptors, and its influx in xylem parenchyma cells seem to orchestrate the differentiation of vessel elements and parenchyma cells in the gall PR+MR. Further, the reorientation of vessel elements observed in the gall PR+MR may imply in a high water supply to the galled stem portions, an assumption that aligns with the gall constriction hypothesis (Aloni et al 1995).

In the *M. domestica – E. lanigerum* system, the main alterations in the secondary xylem are the over-differentiation of parenchyma cells, which also contributes to the redifferentiation of vessel elements. The lack of differences in the dimensions of the vessel elements in the portions above and below the galled stems does not support the “gall constriction”

hypothesis proposed by Aloni et al (1995). The alterations restricted to the gall PR+MR, and the maintenance of the conserved vascular cylinder in the DR, indicate adaptability toward maintaining the water status in the gall developmental site, which also favor the aphid water balance.

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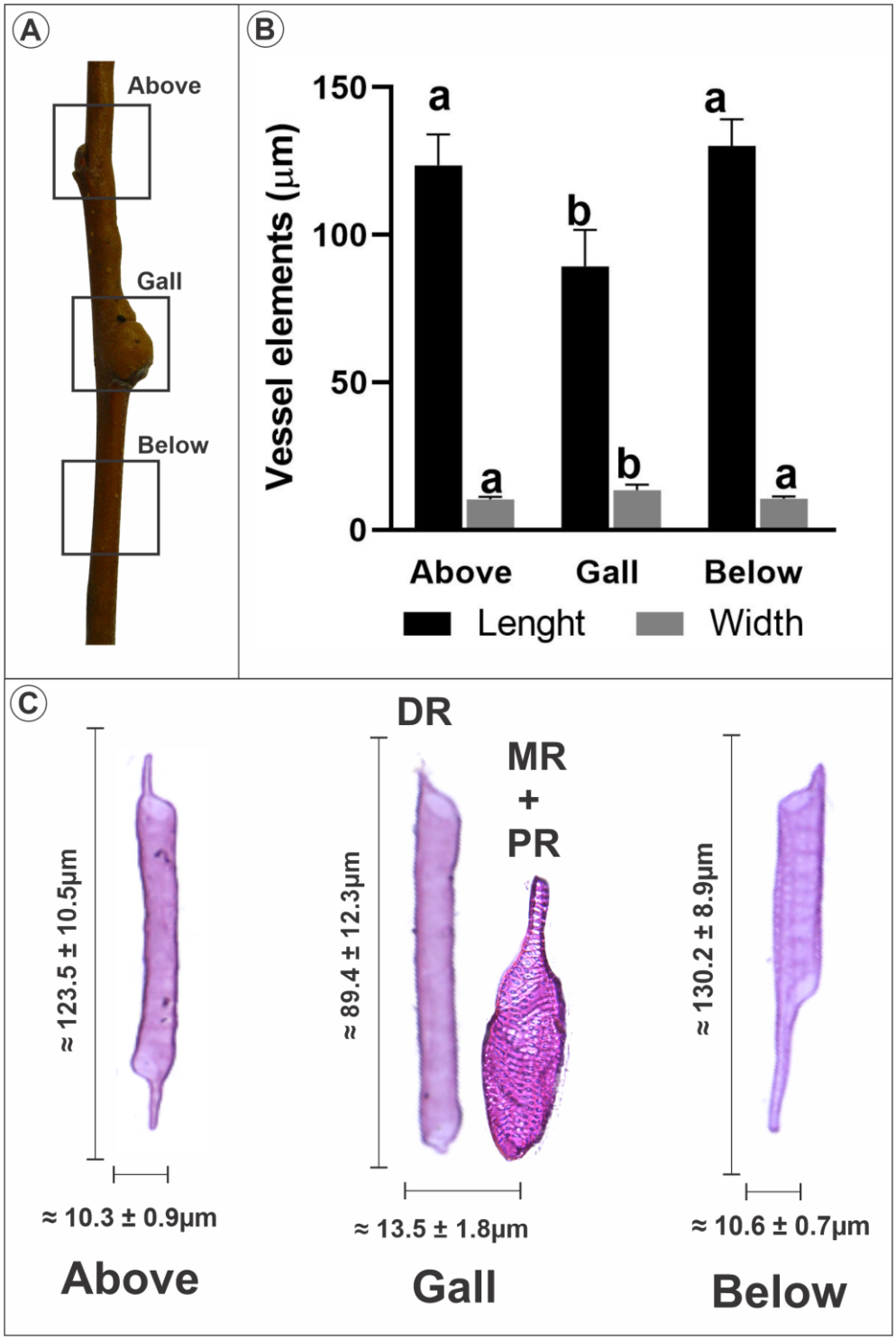
271 **3.7. Figures**



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273 **Figure 1.** Vessel elements features of *Malus domestica-E. lanigerum* system. (A) Galled
274 stem fragment indicating the three analyzed regions. (B) Vessel element (VE) dimensions in
275 the three stem portions. (C) Mean length and width of VE in the three regions of galled stems
276 (one-way ANOVA and Tukey's test). The gall VE may have simple perforation plates in DR
277 and abnormal VE with displaced perforation plates and appendices in the PR+MR. PR =
278 proximal region. MR = median region. DR = distal region.

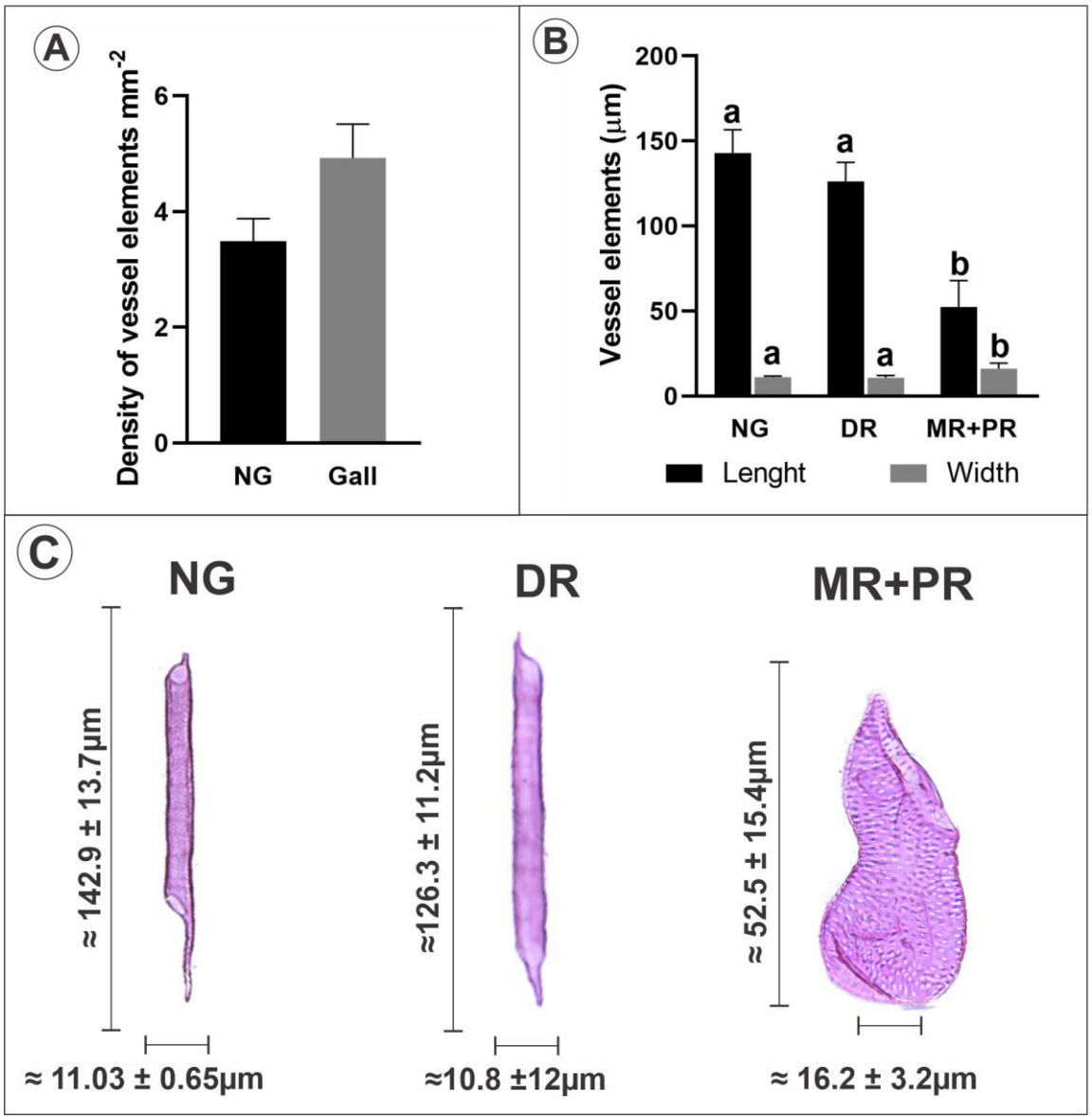

Figure 2. Xylem features of *Malus domestica-E. lanigerum* system. (A) Density of VE per area (mm^{-2}) in non-galled stems and stem galls. (B) Mean length and width of VE in non-galled stems, and in gall DR, and PR+MR (one-way ANOVA and Tukey's test; Kruskal-Wallis and Dunn's test). (C) Mean length and width of VE in non-galled stems, and in gall DR, and PR+MR. VE in NGS and gall DR have with simple perforation plate, while the VE in gall PR+MR have large and displaced perforation plate. PR = proximal region. MR = median region. DR = distal region.

4. CONSIDERAÇÕES FINAIS

A atividade alimentar das colônias de *E. lanigerum* nas raízes e caules de *M. domestica* estimula a proliferação e reorganização do parênquima vascular e a neoformação de elementos traqueais de formatos alterados. Essas peculiaridades anatômicas observadas no xilema secundário, em regiões proximais ao sítio de alimentação do galhador, resultam no desenvolvimento de um campo cecidogenético assimétrico.

As semelhanças observadas nos perfis estruturais e histoquímicos das galhas radiculares e caulinares não permitiram o diagnóstico de graus diferenciais de impacto entre os órgãos hospedeiros, portanto, a hipótese de que o ambiente aéreo e subterrâneo determinaria diferenças no desenvolvimento das galhas não foi corroborada. Contudo, em ambos os órgãos, os atributos anatômicos indicam estratégias para manutenção de um estado hídrico adequado tanto para o desenvolvimento do *E. lanigerum* quanto para o desenvolvimento dos tecidos da galha.

A hipótese de constrição hídrica da galha proposta por Aloni et al. (1995) não é corroborada no sistema *M. domestica*-*E. lanigerum*, uma vez que os elementos de vasos apresentaram dimensões semelhantes nas porções acima e abaixo da galha em ramos caulinares infestados pelo pulgão lanígero. No entanto, a reorientação dos elementos de vasos do eixo axial para o radial parece indicar o redirecionamento de água para a galha em detrimento dos ramos apicais o que parece implicar na redução da longevidade dos indivíduos afetados pelo *E. lanigerum*.

Os caracteres citológicos do xilema indicam um maior aporte de água redirecionado para o sítio de desenvolvimento da galha em detrimento dos ramos não galhados e reprodutivos de *M. domestica*. O perfil histológico e histoquímico das galhas caulinares e radiculares de *E. lanigerum* em *M. domestica* revela peculiaridades típicas deste sistema planta hospedeira-galhador. Ademais, a interferência do pulgão lanígero no xilema parece

ser crucial para sua sobrevivência em detrimento das plantas hospedeiras, representando uma via de interesse pela qual o controle da praga pode ser explorado.