UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOLOGIA

INVESTIMENTO DIFERENCIAL EM COMPOSTOS QUIMICOS NOS TECIDOS DE GALHAS INDUZIDAS POR *PALAEOMYSTELLA OLYGOPHAGA* (LEPIDOPTERA) EM *MACAIREA RADULA* (MELASTOMATACEAE)

GUILHERME DE FARIA SILVA NAVES

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Dissertação apresentada à Universidade Federal de Uberlândia, como parte das exigências para obtenção do título de Mestre em "Ecologia e Conservação de Recursos Naturais".

Orientador

Prof. Dr. Denis Coelho de Oliveira

Coorientador

Prof. Dr. Vinícius Coelho Kuster

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Reuniu-se no Anfiteatro do Bloco 4K, Campus Umuarama, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ecologia e Conservação de Recursos Naturais, assim composta: Professores Doutores: Jamir Afonso do Prado Júnior - INBIO/UFU; Thiago Alves Magalhães - UFLA; Denis Coelho de Oliveira - INBIO/UFU, orientador do candidato.

Iniciando os trabalhos o presidente da mesa, Dr. Denis Coelho de Oliveira, apresentou a Comissão Examinadora e o candidato, agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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Resumo

O hábito endofítico evoluiu e se especializou de várias formas. Uma delas foi por meio do hábito galhador, que fornece ao seu agente indutor, principalmente insetos, proteção, nutrição e abrigo. Insetos galhadores alteram e manipulam os tecidos e o desenvolvimento ontogenético da planta hospedeira em benefício próprio, favorecendo a amplificação da especificidade entre as interações de insetos com plantas. Com isso, o constante estímulo do galhador, somado à várias interações que podem ocorrer de outras espécies com as galhas, pode resultar em alterações, principalmente químicas, nessas estruturas. Nosso sistema de estudo são galhas induzidas po *Palaeomystella oligophaga* (Lepidoptera) em *Macairea radula* (Melastomataceae), onde o metabolismo da galha depende do constante estímulo alimentar do galhador. Estas galhas, além de serem grandes, apresentam uma coloração que varia do verde claro ao vermelho escuro e possuem parênquima espesso, com projeções de tricoma que as conferem um aspecto ainda mais conspícuo. Além disso, é uma galha comumente atacada por uma série de inimigos naturais, como cecidófagos e parasitoides. Neste contexto, investigar a associação da coloração dessas galhas com o aporte nutritivo, e quantificar os compostos presentes em seus tecidos, pode revelar como se dá o investimento em compostos, tanto nutritivos quanto de defesa, dessas galhas. Este estudo, portanto, teve como objetivo quantificar (I) compostos nutritivos como açúcares, polissacarídeos, amido, proteínas e lipídios, (II) compostos de defesa e sinalizadores como fenólicos, (III) estresse oxidativo e (IV) associar possíveis investimentos nesses compostos, com a coloração dessas galhas. Nos compostos analisados, esperava-se encontrar aqueles associados à nutrição, nos tecidos que circundam a câmara larval. Por este mesmo motivo, esperava-se encontrar também um maior nível de estresse

oxidativo. No entanto, polissacarídeos, MDA (medidor para estresse oxidativo), e compostos fenólicos foram encontrados em maior quantidade na porção mais externa do córtex.

Introdução geral

Tecidos vegetais têm a capacidade de responder a diferentes estímulos, abióticos ou bióticos, dentre os quais se destacam os agentes indutores de galhas. Neste grupo, os principais representantes são os insetos, que induzem estruturas compostas por tecidos vegetais denominadas galhas, que são usadas como nutrição, proteção e abrigo (Mani 1964, Shorthouse & Rohfristch 1992). Este processo é caracterizado pela rediferenciação celular na planta hospedeira, a ponto de originarem estruturas com funções que caracterizam um novo órgão, a galha (Mani 1964, Lev-Yadun 2003, Shorhouse et al 2005, Oliveira & Isaias 2010).

Insetos galhadores, ainda, são capazes de modificar metabolicamente os tecidos da hospedeira ao seu redor (Isaias et al 2015, Oliveira et al 2017), fazendo com que o desenvolvimento da estrutura seja dependente de seu estímulo constante. Por serem consideradas micro-habitat, estas estruturas possibilitam ao inseto viver em um ambiente com condições mais estáveis (Lill & Marquis 2007) e obter nutrição de maneira mais fácil e rápida. Assim, o hábito de vida galhador é mais vantajoso quando comparado com outros herbivoros de vida livre (Mani 1964). Ainda, este tipo de interação pode ser visto como uma adaptação contra a ação de inimigos naturais, e a pressão seletiva destes inimigos pode

explicar a grande variedade e diversidade morfológica de galhas (Price et al. 1987, Stone and Schönrogge 2003).

De fato, a galha com suas características morfofisiológicas específicas confere vantagem adaptativa aos galhadores em relação aos seus ancestrais de vida livre (Price et al 1986). Em termos gerais, as características morfológicas e químicas das galhas são discutidas com base em três hipóteses adaptativas: a hipótese do microambiente; a nutricional; e a hipótese do inimigo (Stone, Shonrogge 2003).

A hipótese do microambiente prediz que características morfológicas e químicas das galhas conferem proteção contra fatores abióticos desfavoráveis, em especial dessecação e altas temperaturas (Stone, Shonrogge 2003). A hipótese nutricional propõe que os tecidos da galha oferecem uma dieta adequado ao organismo galhador, muitas vezes com o desenvolvimento de um tecido nutritivo típico (Mani 1964, Palct & Hassler 1967, Malyshev 1968, Braun 1969, Shannon & Brewer 1980, Bronner 1992). Entretanto, o investimento em recursos nutricionais pode levar a uma queda no potencial de defesa químico, especialmente na concentração dos compostos fenólicos, durante a formação da galha (Meyer 1957), favorecendo o ataque de organismos externos. Por fim, a hipótese do inimigo propõe que características estruturais e químicas da galha protegem o galhador de seus inimigos naturais. (Price et al 1986).

De fato, existe uma grande diversidade de organismos que atacam galhas (Price, Fernandes, Waring 1987), sendo que a maior taxa de mortalidade de galhadores é atribuída a parasitoides e cecidófagos (Washburn, Cornell 1981, Rezende 2017). Assim, a grande diversidade morfológica de galhas é atribuída a pressão seletiva imposta pelos inimigos naturais (Askew 1961, Price 1980, Cornell 1983). Por se tratar de uma interação parasítica

onde os insetos são os maiores agentes indutores, as plantas necessitam de mecanismos de defesa que torne possível amenizar o estresse biótico gerado pelos insetos galhadores. Estes mecanismos de resposta podem ser químicos, como substâncias toxicas ou compostos que dificulte a digestão do tecido vegetal, ou físicos, como por exemplo tricomas e cutículas na superfície foliar (Melo & Silva-Filho 2002).

Quimicamente, os tecidos das galhas diferem da planta hospedeira por conter menor concentração nitrogênio e maiores níveis de compostos fenólicos (Hartley 1998). Além disso, fitormônios como etileno, ácido jasmônico, ácido salicílico e ácido abcísico também podem desempenhar importante papel na defesa contra a herbivoria (Davies 2004; Erb et al 2012; Howe & Jander 2008; Walling 2000; Wasternack & Hause 2013). Estudos com afídeos mostraram que o conteúdo fenólico da planta hospedeira está correlacionado com a resistência a insetos galhadores, sugerindo que, em alguns casos, altos níveis de compostos fenólicos na planta hospedeira pode ser uma defesa contra insetos galhadores (Tjia & Houston 1975; Westphal et al. 1981).

O tecido da galha frequentemente apresenta altos níveis de compostos fenólicos e taninos, e foi proposto que o agente indutor se beneficia, de alguma forma, destes compostos químicos (Askew 1984; Abrahamson et al. 1991; Hartley 1992). Com isso, estes taninos, nas galhas, podem causar uma redução na taxa de mortalidade do inseto causada por infecção gerada por fungos (Taper et al. 1986), ou protegendo os insetos galhadores contra parasitoides ou de animais que se alimentam de folhas (Cornell 1983; Taper & Case 1987; Schultz 1992). O tecido da galha produzido pela mesma planta em resposta a diferentes agentes indutores pode diferir quanto a composição química. O tecido da galha é geralmente rico em nutrientes, e com baixa quantidade de compostos secundários, sendo,

portanto, uma vantagem desta interação, favorecendo a hipótese nutritiva (Price et al. 1986, 1987).

O inseto galhador altera a fisiologia da planta, principalmente nas células do tecido nutritivo que envolvem a câmara larval (Bronner 1977; Shorthouse 1986), mantendo um equilíbrio metabólico em seu benefício. O tecido nutritivo possui altos níveis de aminoácidos e açúcares, e os níveis de compostos como nitrogênio, por exemplo, podem ser maiores nos tecidos da galha quando comparados com os tecidos da folha sadia (Bronner 1992, Birch et al. 1992). De fato, o estímulo alimentar dos insetos galhadores fazem com que as galhas atuem como um dreno de fotoassimilados de outras partes da planta hospedeira (Stone & Schonrogge 2003). Assim, galhas tendem a acumular grandes quantidades de citocininas. Este fitohormônio é responsável pela divisão celular e crescimento desempenham um papel biológico no metabolismo da planta (Mok & Mok 2001; Sakakibara 2006), além de retardar a senescência de folhas, mobilizando nutrientes (Mothes & Engelbrecht 1963; Gan & Amasino 1995), desenvolvimento de flores e frutos entre outros. Além disso, o aumento na concentração de citocinina pode levar ao aumento na biossíntese de um importante pigmento vegetal, a antocianina. Flavonoides como taninos condensados e compostos fenilpropanoides como ligninas, também são acumuladas em resposta a luz, açúcar e citocinina (Guo et al 2005). Isso pode explicar a observação de que insetos indutores de galhas contem altas concentrações de taninos (Cornell 1983; Hartley 1998; Nyman and Julkunen-Tiitto 2000; Motta et al. 2005; Ikai and Hijii 2007). O ataque de um inseto ou algum patógeno, resulta num aumento de citocinina no local de infecção, podendo levar a uma reconfiguração do metabolismo primário e secundário da planta como forma de defesa (Giron et.al 2013). Quando um recurso é

realocado, pode ocorrer um decréscimo de fonte de energia para o crescimento da planta. Por outro lado, pode haver um maior investimento no sistema defesa química.

Em um efeito antagônico, os efeitos da citocinina podem aumentar a defesa da planta contra invasores, mas também a torna mais vulnerável a crescimento e estabelecimento de micróbios e insetos (em razão de seu efeito de retardar a senescencia da folha), resultando num fornecimento de nutrientes, ou estruturas tidas como proteção contra fatores externos, como galhas (Giron et.al 2013). Citocininas e outros hormônios já foram encontrados na saliva de agentes indutores, o que pode indicar um potencial estímulo ao crescimento de galhas (Hori 1992). Em galhas, os insetos indutores também podem promover a multiplicação de citocinina (Connor et al 2012), e esta indução leva a uma cascata de efeitos que incluem a regulação e síntese de antocianina, causando a coloração vermelha nas galhas.

Concentrações maiores de fitormônios, tanto nos tecidos que circundam a galha quanto no agente indutor sugerem que o inseto pode estar elevando e até mesmo fornecendo estes fitormônios (Tokuda et al 2013; Straka et al 2010; Tooker & De Moraes 2009, 2011a; Kaiser et al 2010; Ollerstam et al 2002; Tooker & De Moraes 2011b; Zhu et al 2011; De Bruyn et al 1998; Mapes & Davies 2001a, 2001b; Tooker et al 2008; Wood & Payne 1988; Dorchin et al 2009; Yamaguchi et al 2012). Algumas larvas de insetos indutores apresentaram concentrações de citocinina significativamente altas no tecido da galha circundante. Larvas de *Eurosta solidaginis* por exemplo, apresentaram concentração 50 vezes maior de citocinina nos tecidos da galha que induziram em *Solidago altíssima* (Mapes & Davies 2001a, 2001b; Tooker et al 2008). Já larvas de *Trichilogaster acaciaelongifoliae* apresentaram concentração 62 vezes maior de citocinina nos tecidos da

galha de seu hospedeiro, *Acaciae longifólia* (Dorchin et al 2009). Este padrão se repete na interação entre o psilídeo *Pachypsylla celtidismamma* e *Celtis occidentalis*. As ninfas do inseto apresentaram valores de citocinina 40 vezes maior em tecidos não galhados, e 200 vezes maior em tecidos galhados (Straka et al 2010). O estímulo na produção de açúcares e citocininas regulam as vias flavonoides, levando a um acumulo da antocianina localizada (Connor et al 2012). Na presença de luz, a citocinina induz um acumulo de antocianina (Diekman & Hammer 1995; Piazza et al 2002; Guo et al 2005; Chen et al 2006; Carvalho et al 2010), e quando estimulada por um agente indutor, promove o acumulo de antocianinas em folhas em desenvolvimento, reagindo à luz e alongando o pecíolo. Assim, espera-se que o acúmulo de açúcares em algumas galhas possa estar relacionado a coloração vermelha.

No sistema *Palaeomystella oligophaga* (Lepidoptera) - *Macairea radula* (Melastomataceae) as galhas globulares apresentam projeções pilosas, além de apresentarem colocação que varia do verde claro ao vermelho (Rezende 2017). Entretanto, o que define ao certo essa variação de cores no sistema ainda é incerta. Baseado nisso, o presente estudo busca investigar o investimento a partir da quantificação de metabólitos primários e secundários nos tecidos compartimentalizados da galha (tecido nutritivo e de reserva) desse sistema, partindo da hipótese de que o tecido nutritivo armazena mais carboidratos, lipídios e protepinas, e também sofre mais estresse oxidativo, uma vez que este tecido é o sítio direto de alimentação do inseto galhador. Ainda, carboidratos mais complexos (i.e., amido) e compostos de defesa se acumulariam mais no tecido de reserva, sendo este último, agiria na defesa da galha. Por fim, espera-se encontrar mais compostos nutritivos em galhas avermelhadas, uma vez que o composto relacionado ao dreno se

encontra na mesma rota metabólica do hormônio responsável pela pigmentação.

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Differential investment in chemical compounds in the gall tissues induced by *Palaeomystella Olygophaga* (Lepidoptera) on *Macairea Radula* (Melastomataceae)

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Abbreviations

WSP = Water Solube Polyssacharides

TSS = Total Soluble Sugars

ST = Starch

MDA = Malonaldehyde

PCA= Principal Component Analysis

ST = Storage Tissue

NT = Nutritive Tissue

PR = Proteins

LI = Lipids

PHN = Phenolics

Keywords: Gall; Nutritive Tissue; Storage Tissue; Cytokinin; Anthocyanin; Oxidative Stress

ABSTRACT

Galling insects manipulate the host plant tissues into own benefit. Because of galling feeding activity both structural and metabolical occurs leading to chances in the associated fauna. Herein, galls induced by the *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* (Melastomataceae) dependes on the constant larval feeding stimulus for the gall establishment and development. These galls, besides being large, have a coloration ranging from light green to dark red and have thick parenchyma, with projections and trichomes that give them an even more conspicuous appearance. The gall showed two tissue compartments, the storage and nutritive tissue and here, we look for investigate the levels of oxidative stress and if there is a chemical differential investment on these compartments. In addition, we look for investigate the relationship between the gall coloration and the concentration of carbohydrates. So, we aimed to quantify (I) nutritive compounds such as sugars, polysaccharides, starch, proteins and lipids, (II) defensive and signaling compounds as phenolics, (III) oxidative stress and (IV) associate possible investments in these compounds with the gall coloration. Polysaccharides, MDA (oxidative stress meter), and phenolic compounds were found in high concentration in the outermost portion of the gall cortex (storage tissue). However, the total soluble sugar occurred in high concentration in

the nutritive tissue. The red galls may be associated with high concentration of water-soluble polysaccharides.

INTRODUCTION

As phenotype manipulators, galling insects change the structure and metabolism of the host plant tissues to develop a new plant organ, the gall (Mani 1964, Oliveira et al. 2014, Oliveira et al. 2016, Martini et al 2019). The gall develops from cell hypertrophy and tissue hyperplasia (Carneiro et al. 2014, Oliveira and Isaias 2010, Oliveira et al. 2016), as well as from cellular redifferentiation of host plant tissues (Oliveira & Isaias 2010). Thus, the gall provides a safe place against natural enemies (enemy hypothesis) and unfavorable environmental conditions (microenvironment hypothesis) (Mani 1964, Shorthouse & Rohfristch 1982 and 1992, Price et al. 1987, Shorthouse et al. 2005). Additionally, the gall provides to the galling insect the nutritional resources from the host plant (nutritional hypothesis), which helps for their development and feed (Bronner 1992, Mani 1964, Price et al 1987).

The nutritional resources are stored in a specialized tissue surrounded the larval chamber, named as nutritive tissue. This nutritive tissue is induced by some taxa of galling insects (Ferreira et al. 2017), and dependents on the constant larval feeding activity (Rezende et al. 2019). Carbohydrates, proteins and lipids are substances commonly found in the nutritive tissue of different gall systems, and the presence of each substance are related to feeding behavior and taxa of galling insects (Bronner 1992; Róstas et al. 2013, Ferreira et al. 2017). Conventionally, the galling larvae of Diptera (Cecidomyiidae) induce carbohydrate accumulation, whereas Hymenoptera and Lepidoptera-induced galls

accumulate lipids in the nutritive tissue (Motta et al 2005; Macêdo Vieira & Kraus 2007). Besides that, some galling insects do not induce nutritive tissue, feeding directly from the phloem cells, taking carbohydrates with phloem-sucking feeding apparatus (Bronner 1992; Oliveira et al. 2016; Ferreira et al. 2017), as reported for some Hemiptera galls. However, some nutritive cells has been reported occurring around the vascular bundles in phloem-sucking galling insects, as showed for *Nothotrioza cattleianum* (Triozidae) on *Psidium cattleiani* (Myrtaceae), which presented protein resource in its perivascular parenchyma cells (Carneiro & Isaias 2015).

The primary metabolites have been revealed in some galls also in the outer gall tissues, being considered as a storage tissue (Rezende et al. 2018), which can present some complex carbohydrates, as starches (Bronner 1991). However, the storage of secondary metabolites is more common in the outer cortex of galls (Kuster et al. 2019), normally associated with gall defense and regulation of homeostasis in the gall tissues (Isaias et al. 2015). In general, non-galled tissues have less concentration of secondary metabolites when compared with gall tissue (Motta et al. 2005). The occurrence of the secondary metabolites in gall tissues corroborates the enemy hypothesis, which predicts that natural enemies act as selective pressure on gall-inducing insects (Fernandes 2009). Therefore, the investment in protective morphological and chemical traits in galls seen to be a tool to keep galling insects safe (Stone et al. 2002, Weis 1982, Weis et al. 1985, Rossi et al. 1992, Kuster et al. 2019).

Morphological and structural features as well as conspicuous colors can often be interpreted as traits that can promote gall defenses against natural enemies (Stone & Schönrogge 2003). Galls can present several colors (e.g. yellow, pink and purple) that are

normally associated with the effect of the gall development stage (Lev -Yadun 2016) as well as to the local light incidence influence (Bomfim et al. 2019). In some cases, the gall with red coloration is attributed to “fabricational noise” of gall development (Connor et al. 2012). This hypothesis argue that the galling insect produce or stimulate exogenous or endogenous cytokinins, leading to the sink establishment in gall tissues and promoting anthocyanin synthesis. In this sense, the same metabolic pathway between nutrition and pigmentation compounds may suggest that red galls may storage more nutritional substances (especially carbohydrates) that non red galls.

One of the challenges of the galling insect during gall inducing and establishment is to control the oxidative stress and maintain the gall tissue homeostasis (Isaias et al. 2015). This control may be associates with the gall structure and metabolism profile and is dependent of galling taxa (Kuster et al 2020 *in press*). Recently, the oxidative stress has been accessed by malonaldehyde approach, indicating an oxidative damage by lipid peroxidation (Khattab & Khattab 2005; Minotti & Aust 1987; Kmiec et al. 2018). The assessment of oxidative stress using malonaldehyde approach was reported in galls of *Tetraneura ulmi* (Hemiptera: Eriosomatinae) on elm leaves, as well as the antioxidant enzyme activities (Kmiec et al. 2018). In addition, the production of secondary metabolites, especially phenolic derivatives, seems to be related to oxidative stress scavengers in the gall tissues (Isaias et al. 2015, Oliveira et al. 2017). Lastly, although malonaldehyde results have showing increase of oxidative stress in some galls, no reports were found in literature about its production between different tissue compartments (i.e. storage and nutritive tissues) in the gall.

Some galls induced by Diptera, Hymenoptera and Lepidoptera can be divided into

113 an outer cortex, generally associated with storage tissue, and an inner cortex normally with
114 nutritive tissue (Bronner et al 1991). Based on that, we investigate the differential
115 investment in primary and secondary metabolites in the outer (storage tissue) and inner
116 cortex (nutritive tissue) of galls induced by *Palaeomystella oligophaga* (Lepidoptera) on
117 *Macairea radula* (Melastomataceae). We hypothesize that the nutritive tissue may present
118 high accumulation of less complex carbohydrates, proteins and lipids, as well as high
119 oxidative stress due to the direct feeding activity from galling larva on this site. On the
120 other hand, more complex sugars (e.g. starch) and phenolic compounds may occur in the
121 storage tissue. Lastly, it was expected to find more nutritive compounds in slightly reddish
122 galls (based on fabricational noise hypothesis).

123

124 **MATERIALS AND METHODS**

125 *Study site and galling insect-host plant system*

126 The study was carried out at Cachoeira das Irmãs (19° 02' S, 49° 10' O), in
127 Araguari municipality, Minas Gerais state, Brazil; and at Fazenda Douradinho (48° 21'
128 00.42" O), in Uberlândia municipality, Minas Gerais state, Brazil. The *Macairea radula*
129 (Melastomataceae) species occurs in an ecotone area named 'vereda' (wetland) in 'Cerrado
130 *sensu strictu*'. *M. radula* is a perennial and shrub species, can reach 2 meters high and hosts
131 the galling insect *Palaeomystella oligophaga* (Lepidoptera).

132 The galls are globoid-shaped *(sensu* Isaias et al. 2014*)* (Figure 1a, b) and occur*s*
133 on the axillary stem bud. They are covered by densely and long projections, which varying
134 in color and diameter (10-15mm) (Figure 1a). The galling insect showed a bivoltine life

cycle and the gall and galling insect can be attacked by a diversity of natural enemies, including parasitoids and cecidophages (Rezende et al. 2019). The galls can be red (Figure 1a), green (Figure 1b) or a mixture of these colors and, the color variations were not associated with gall development stage or presence of natural enemies (Rezende et al 2020, in preparation) The gall cortex can be divided into (i) an inner nutritive tissue that surrounds the larval chamber and (ii) an outer storage tissue (Figure 1c). The galling larva (Figure 1d) feed directly on the nutritive tissue.

Sampling procedures

For the quantification of the gall resources, the mature galls were collected both at the end of the second infestation period (August-September, n =30), and the beginning of the first infestation (March-April, n = 30) using liquid nitrogen, followed by its storage in an ultra-freezer at -81ºC. After that, the cortex of galls was manually separated into nutritive tissue and storage tissue, as well as the insect was removed. In this process, two procedures were adopted: (i) only the insect was removed, keeping all cortex; and (ii) the insect and the nutritive tissue were removed, keeping just the storage tissue. Therefore, we measured malonaldehyde levels, water-soluble polysaccharides, total soluble sugars, starch, phenolics, lipids and proteins levels. The results were obtained by the difference between the two treatments mentioned above. In addition, all protocols were also applied between reddish and green galls, for coloration analysis. In this case, however, the treatment used was the insect removal only.

Lipid peroxidation assays

158 The malondialdehyde (MDA) concentration were calculated in order to measure lipid
159 peroxidation on nutritive (n=5) and storage (n=5) tissue samples (Minotti & Aust, 1987).
160 The MDA content was determined by thiobarbituric acid (TBA) method, where samples
161 (0,05g) were macerated in liquid nitrogen with 0,005g of polyvinylpolypyrrolidone
162 (PVPP). One mL of 1% trichloroacetic acid solution (TCA) (m/v) was added per sample,
163 then centrifuged at 8,700 rpm for 20 min at 4° C; 500 μL of the samples were added in
164 solution with 1.5 mL of thiobarbituric acid (TBA) and trichloroacetic acid (0.5% TBA,
165 20% TCA, m/v) and kept in a water bath at 90° C for 20 min. The reaction was ceased on
166 ice and the samples were centrifuged at 3000 g for 4 min. Lastly, readings were performed
167 in triplicate with a spectrophotometer (Thermo Spectronic Mod. Genesys 10UV) at
168 wavelengths of 440, 532 and 600 nm.
169
170 *Quantification of carbohydrates*
171 For carbohydrate dosage method we based on Dubois et al. (1956) phenolic-
172 sulfuric protocol, adapted by Chow & Landhauser (2004), taking glucose as standard. All
173 samples were dried a 50 °C in a kiln for 24 hours, followed by macerated with mortar and
174 pestle. The determination of total soluble sugars (TSS) was based on 10 samples of 20 g of
175 fresh galls for nutritive and storage tissues. In the current procedure, 5 ml of MCA
176 (Methanol, chloroform and water in a 12: 5: 3 ratio) were added to each sample and then
177 centrifuged at 2500 rpm for 10 minutes. After many centrifugation protocols, two phases
178 were originated: (i) the chloroform phase, which was discarded and (ii) the volume of the
179 methanol-water phase, which was used for the reaction with sulfuric phenol. For water-

soluble polysaccharides (WSP) and starches (ST), the pellet obtained from TSS determination was dried at 50° C overnight to remove the solvent.

For water soluble polysaccharides (WSP) extraction (Dubois et al. 1956, adapted by Chow and Landhauser 2004), the same dried sample was resuspended in 5ml of 10% ethanol, and centrifuged for 6 minutes, until the soluble and insoluble phases be completely separated. After that, the volume of the soluble phase was used for the phenol sulfuric reaction. For starch (ST) quantification (Dubois et al. 1956, adapted by Chow and Landhauser 2004), the samples were resuspended in 5ml of 30% perchloric acid and centrifuged until the soluble and insoluble phases be separated. The soluble phase was used for the reaction with sulfuric phenol protocol.

The standard 1% glucose curve was adopted for all carbohydrate determination, and all supernatant absorbances were read at 490 nm using a spectrophotometer (Thermo Spectronic Mod. Genesys 10UV).

Total lipid and protein quantification assays

To quantify total lipids (Ranjan et al. 2010), 400 g of the plant material were weighed on a precision analytical scale, followed by 2 mL of 8M hydrochloric acid addition and subsequently heated into a 70 ° C water bath for 30 minutes. Then, (i) 2.5 mL of ethyl ether, (ii) 2 mL of absolute ethyl alcohol and (iii) 2.5 mL of petroleum ether (Ranjan et al. 2010) were added with vortex stirring for about 1 minute. The material was then centrifuged for 5 minutes at 10.000 rpm and the supernatant extracted and directed to the water bath at 60 ° C for solvent evaporation. Thereafter, further extraction via ethyl ether-petroleum ether and oven drying at 50-60° C was performed until the solvent could be

extracted completely. At the end of the process, the material was successively weighed on a precision analytical scale.

For total proteins quantification (Swaun & Hillis 1959; Wettasingile & Shadidi 1999), after ethanolic extraction, 0.02 g of pellet was used. The pellet was then washed in 1 ml of 99% ethanol until the samples were homogenized, followed by centrifugation for 10 minutes at 10.000 rpm. The supernatant was then discarded, 400 µl of 0.2M KOH added and incubated in a thermostatic bath at 90 ° C for 1 hour. The reaction was stopped on ice for 5 minutes, with 70 µl of 1M acetic acid added and vortexed for about 1 minute. Then, the supernatant was collected and 0.2 M KOH and Bradford reagent (Bradford 1976) added. The supernatant absorbance was read at 595 nm using a spectrophotometer (Thermo Spectronic Mod. Genesys 10UV).

Determination of total phenolics

The total phenolics content of nutritive and storage tissues were determined by the spectrophotometric method using the Folin-Ciocalteau reagent (Swaun & Hillis, 1959; Wettasingle & Shahidi, 1999) and standard tannic acid curve. Results were expressed as percentage of total phenolics in each tissue analyzed.

Statistical analysis

A t-test we applied for all protocols, as well as Shapiro test for each variable. The R statistical environment version 3.5.1 (R Core Team 2018) were adopted, and after verification of data normality, we assumed as statistically different *p* values lower than 0,05, The graphs were made using the ggplot2 and dplyr (Wickham et al 2019) packages.

The effect of nutrition and defense related compounds on nutritive and reserve tissues were evaluated by principal component analysis (PCA). Thus, PCA reduced the seven parameters (i.e. total soluble sugars, water soluble polysaccharides, starch, lipids, proteins, MDA and phenolics) to two major components.

RESULTS

The malondialdehyde (MDA) levels were higher in storage tissue (ST) (Average / Av = 0,50, standard deviation / sd = 0,35) than nutritive tissue (NT) (Av = 0,06, sd = 0,15 - Figure 2a). Phenolics content followed malonaldehyde results, showing low concentration in NT comparing with ST (ST- Av= 0,013, sd = 0,008; NT- Av = 0,00025, sd =0,0006 - Figure 2b).

The total soluble sugars (TSS) were more concentrated in NT (Av = 152,5, sd = 23,30) than in ST (Av= 78,89, sd= 27,49 -Figure 3a). Conversely, water-soluble polysaccharide (WSP) and starch (ST) levels were higher in ST (Av = 19,27, sd = 5,02, and Av = 45,51, sd = 15,42, respectively) than in NT (Av = 10,28, sd = 2,02, and Av = 19,40, sd = 6,62, respectively) (Figure 3b, c). The data indicate an increase of 2-fold of these types of carbohydrates in storage tissue.

In relation to proteins (Pr) and total lipids (Li), all of this primary substances were higher in ST (Pr- Av = 41,36, sd = 14,30; Li- Av = 49,38, sd = 17,96) than in NT (Pr- Av = 19,39, sd = 6,62; Li- Av = 0,00, sd = 0,00), with about 50% more protein and lipid in the storage tissue (Figure 4a, b).

Highlighting carbohydrate differences in relation to gall pigmentation, only the water-soluble polysaccharide levels presented significant results, with more accumulation in red galls (Av = 21,56, sd = 6,76) than green galls (Av = 7,31, sd= 7,09) (Figure 5a). The another carbohydrates (i.e. total soluble sugars and starch) did not change between red (WSP- Av = 71,14, sd= 38,52; ST- Av = 38,04, sd= 24,19) and green (WSP- Av = 148,06, sd= 21,73; ST- Av = 22,87, sd= 11,32) galls (Figure 5b, c).

The principal components analysis (PCA) had two first axes that explained 77,6% of the variation (Figure 6). The nutritive and storage tissues were separated by the correlation of the compounds analyzed. Only the total soluble sugars (TSS) data correlates negatively with the axis 1, while just protein and malondialdehyde levels correlate negatively with the axis 2 (Table 1). The TSS help to cluster the group nutritive tissue, while the other parameters allowed the storage tissue grouping (Figure 6).

Discussion

The metabolism of both primary and secondary compounds is a result of the metabolic demand on each gall tissue compartment and, for instance, the capacity of host plant tissue controls the oxidative stress (Isaias et al 2015). These compounds can act as a galling source nutrition and/or protecting against natural enemies. The gall induced by *Palaeomystella oligophaga* on *Macairea radula* present two functional tissue compartments, the nutritive and storage tissues (Rezende et al. 2019) which invest differently in the concentration of chemical substances. Our analyzes showed more accumulating of simple carbohydrates (e.g. sucrose and fructose) in the nutritive tissue, and more complex carbohydrates (e.g. starch, pectins and structural carbohydrates), phenolics, proteins and lipids in the storage tissue. These results reinforce the idea of

functionally distinct compartments in galls, as proposed by Bragrança et al. (2017). In addition, the high concentration of Water-Soluble Polysaccharides (WSP) in red galls may corroborate the "fabricational noise" hypothesis.

Stress-related parameters and nutritional resources between gall tissues

The feeding behaviors of the galling insect are related to their mouth apparatus and insect taxa and, consequently, can determinate the structural and physiological profile of the gall (Oliveira et al 2016). In the phloem-sucking insects, the larvae feed from phloem (Champan & de Boer 1995) and they do not have nutritive tissue around the chamber. In galls induced by lepidopteran, as *P. oligophaga,* there are a nutritive tissue where the larva feeds (Rezende et al 2019), when the galling alternate between quick feeding and resting in order to conserve energy (Chapman & de Boer 1995). The nutritive tissue is metabolically maintained by the galling inducer activity (Bronner 1992) that herein showed high levels of soluble compounds and some complex polysaccharides. In this sense, galling insects also can produce enzymes capable to convert complex carbohydrate (i.e. starch) into simple sugar, resulting in the accumulation of these compounds (Joshi et al. 2009, Oliveira and Isaias 2010) in the nutritive tissue itself or in the outermost compartment of the gall cortex. On galls of *Macairea radula*, the large accumulation of total soluble sugars (TSS) in the nutritive tissue indicate that this soluble carbohydrate intake by the galling insect, as seen in the horn shaped galls of *Copaifera langsdorfii* (Castro et al. 2012, Oliveira et al 2011). The water-soluble polysaccharides and starch accumulation in the storage tissue (outer cortex) may be a consequence of physiological drain in the gall imposed by the galling insect activity.

Starch may be use as an important resource in the galling feeding dynamics, since this sugar break down in other sugars such as fructose, glucose and sucrose in the gall development sites (Oliveira & Isaias 2010b). As well, the starch accumulation may increase in gall cortex when the galling stops feeding and reduces gall tissue metabolism (Bronner 1992). For *M. radula* galls, therefore, carbohydrates were drained during their development and accumulated as starch grains in the storage tissue. Tracking the starch results, the water-soluble polysaccharides (WSP), represented by pectins and structural carbohydrates, was found in outer cortex on *M. radula* galls. The WSP may be related to an increase in cell wall polysaccharides, which are responsible for characteristics such as cell shape, adhesion, mechanical properties, and signaling (Willats et al. 2001). These characteristics, in turn, act on the structural development of gall and can support cell hypertrophy and tissue hyperplasia, common structural features in gall (Formiga et al., 2013; Carneiro et al., 2014a; Oliveira et al., 2014b),

The gall provides to the galling insects nutrition and protection against natural enemies, however, the gall development increases the oxidative stress in the host plant (Hartley, 1998; Isaias et al., 2011; Oliveira and Isaias, 2010b; Oliveira et al., 2011a). The galling organism induces the production of reactive oxygen species (ROS) molecules that can damage the cellular constituents in the host plant tissues, as well as can trigger gall development (Pasqualini et al., 2003; Oliveira et al., 2010; Oliveira and Isaias, 2010). Many stress situations, such as low temperatures and drought, can lead to the accumulation of soluble sugars (Roitsch 1999). Based on that, we may associate the contents of soluble sugars as defense mechanisms against oxidative stress (Coueé et al. 2006). Since the accumulation of these sugars is a well-known feature of many galls (e.g. Bronner 1992,

305 Isaias et al. 2015), the storage of these compounds on the cells of nutritive and storage
306 tissues of *Macairea radula* galls can be taken as a local response production of oxidative
307 stress in galls. Once the feeding activity of *P. oligophaga* occur directly on the nutritive
308 tissue, we hypothesized that this tissue would have the highest oxidative impact (measure
309 by the concentration of MDA), which was not corroborated. Thus, we can suppose that the
310 stress molecules have been produced on a larger scale in storage tissues due to the intrinsic
311 metabolism of the cells (Harir & Mittler 2009), especially because of intense cell
312 hypertrophy in the storage tissue.

313 The plant tissues have an arsenal of ways to deal with excess of stress molecules,
314 however when dealing with galls, phenolic compounds are the most associated in studies
315 with the uptake of stress molecules (Gottlieb & Kaplan 1993, Kunkler et al. 2013, Nyman
316 & Julkunen-Titto 2000). Moreover, there are a clear relation between phenolics and defense
317 function in plants, as reported for Zucker (1982) and Tjia & Houston (1975), being
318 considered a low palatability component of plants. Phenolic derivatives can be simple or
319 complex, and actively participate in lignification, anti-auxin activities, pathogen resistance
320 (Sgherri et al. 2004) and can act against herbivory (Askew 1984, Abrahamson et al. 1991,
321 Hartley 1992). The galls normally present a high level of phenolic production, acting both
322 in protection against the effects of free radicals (stress molecules) and natural enemies of
323 the galling organism (Gottlieb & Kaplan 1993, Kunkler et al. 2013, Nyman & Julkunen-
324 Titto 2000). In addition to chemical protection, phenolic compounds may be involved in
325 stimulating or inhibiting enzymes that produce plant hormones, playing a key role in plant
326 growth (Hartley 1999). In *Macairea radula* galls, the occurrence of phenolics fit with the
327 malondialdehyde place on outer cortex, showing balance between stress generation and

328 dissipation in this compartment. Moreover, the low presence of phenolics in nutritive tissue
329 may be related to their low palatability role just in the site where the galling feed.

330 *Gall coloration x carbohydrate accumulation*

331 Plant galls can show different colors depending on the host plant and taxa of the
332 galling insect. However, the green and red coloration are the most frequent and generally
333 related to changes during gall development (Russo 2007, Redfern 2011, Sáiz & Núñez
334 1997; White 2010; Lev -Yadun 2016), and/or be a result of the anthocyanin and chloroplast
335 ratio alteration (Dias et al. 2013). In addition to that, red coloration in galls can occur by
336 the stimulation of anthocyanin production by light exposure, as occur in galls induced by
337 Cecidomyiidae on *Qualea parviflora* (Vochysiaceae) (Bomfim et al 2019). Red gall seems
338 to be a signal of warning because of unpalatability and the presence of antagonist-host
339 secondary metabolites, in a classical aposematic hypothesis (Inbar et al. 2010). In *Macairea*
340 *radula* galls, the coloration varies from light green to dark red. Despite of the hypotheses
341 of defensive roles of gall pigmentation (Lev-Yadun 2016) and the influence of changes in
342 color by the interference of natural enemies (Dias et al 2013), no evidence was found in
343 galls induced on *M. radula* (Rezende 2018).

344 In the "fabricational noise" proposal, the galling insect should produce exogenous
345 cytokinis, leading to increase and establishment of the gall as a sink and, as consequence,
346 this cascade of events lead to anthocyanin and red coloration (Connor et al 2012).
347 Citokinins are classical phytohormones related to the sink establishment, that is, plant
348 organs with high concentration of this hormone can accumulate more carbohydrates (Taiz
349 et al 2016). However, the correlation between anthocyanin synthesis and citokinins is not
350 mandatory and the content of this pigment and the hormone has been found to increase

under many conditions (Gerchman et al 2013). Fruit at younger stages are considered to be powerful drains, containnig high levels of cytokinin, but are typically green, while many red fruits, with their growth are already completed, have low levels of cytokine even though they are highly pigmented (Gillaspy et al 2003).

The anthocyanin accumulation can be the most responsible pigment for the red coloration in galls (Sáiz & Núñez 1997; White 2010; Lev -Yadun 2016), and this characteristic is just a consequence of the mechanism of gall induction by the insect (Connor et al. 2012). Guo et al (2005) showed that cytokinins and sugars stimulate anthocyanin both individually and more so combined, acting directly on its accumulation, having therefore a positive correlation between these two compounds. Based on that, the red galls on *Macairea radula* were expected to accumulate more carbohydrates, compared to green galls. However, our results showed that only starch and WSP levels were higher in red galls while green galls accumulated more TSS. Starch is a common complex polysaccharide associated with reserve organs in plants that, depends of high content of citokinins to drain establishment (Taiz et al 2016). Yet, WSP are structural polysaccharides associated to cell wall growth, common process during gall development (Taiz et al 2016; Oliveira et al 2016).

Final consideration

The accumulation of starch and water-soluble polysaccharides in the external cortex indicates that this tissue acts as a reserve for these carbohydrates, which eventually will travel to the tissue surrounding the larva. The high level of oxidative stress, on the external cortex suggests a relationship with the high attack rates of natural enemies suffered

by these galls (Rezende 2019). Also, a possible correlation between the nutritional and pigmentation compounds was analyzed. It was concluded that the starch accumulated in red gall acts as a storage in the outer cortex compartment of the gall, which also stores sugars. Despite of interference of the biotic and abiotic agents, red galls may be associated with high concentration of water-soluble polysaccharides.

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Figure legends

Fig 1. Morphological traits of galls induced by *Palaeomystella oligophaga* on *Macairea radula* leafs. (**A)**- Branch with a red gall; (**B)**- Branch with a green gall; (**C)-** Cross section of the gall showing the nutritive and storage tissues. (**D)-** Enlarged cross section showing the galling insect on its feeding site, surrounded by the nutritive tissue. The storage tissue occurs in outermost part of the cortex. Abbreviation: LC- Larval chamber; ST – Storage tissue, NT - Nutritive tissue, IN – galling insect.

Fig 2. Quantification of stress compound and phenolics on nutritive and storages tissues on galls induced by *Palaeomystella oligophaga* on *Macairea radula* leafs. **a -** Level of

799 malonaldehyde (MDA) on both nutritive and storage tissues. The nutritive tissue
800 demonstrated more lipid peroxidation than storage tissues (t = 3,695, df = 18, p = 0,019).
801 **b-** Phenolic compound levels, showing a higher accumulation in storage tissue (t = 4,575,
802 df = 18, p = 0,0002)
803
804 **Fig 3**. Carbohydrate quantification in both storage and nutritive tissues on galls induced by
805 *Palaeomystella oligophaga* on *Macairea radula* leafs. **a-** Total soluble sugar levels,
806 showing more accumulation in the nutritive tissue (t = 5.7735, df = 9, p < 0,05); **b-** Level
807 of water-soluble polysaccharides, demonstrating more scores in storage tissue (t = -5,5436,
808 df = 9, p-value = 0.0003594); **c-** Starch quantified, revealing difference between tissues,
809 which was higher in the storage tissue (t = -4.654, df = 9, p< 0,05).
810
811 **Fig 4**. Quantification of **a-** Proteins and **b-** Lipids in both storage and nutritive tissues on
812 galls induced by *Palaeomystella oligophaga* on *Macairea radula* leafs. Both substances
813 were higher in ST (Pr- Av = 41,36, sd = 14,30; Li- Av = 49,38, sd = 17,96) than in NT (Pr-
814 Av = 19,39, sd = 6,62; Li- Av = 0,00, sd = 0,00), and showed 50% more Pr and Li in the
815 storage tissue. Abbreviation: Pr – Protein; Li - Lipids
816
817 **Fig 5**. Level of water-soluble polysaccharide, total soluble sugars, and starch levels in
818 reddish and green galls induced by *Palaeomystella oligophaga* on *Macairea radula* leafs.
819 **a-** Reddish galls accumulated more polysaccharide than the green ones (t = -3.30; df = 18;
820 p = 0.003); **b-** There were no difference between reddish and green galls (t = 5.1158, df =

18, p = >0,05); **c-** There were no difference between reddish and green galls (t = 1.8034, df = 18, p = 0.088).

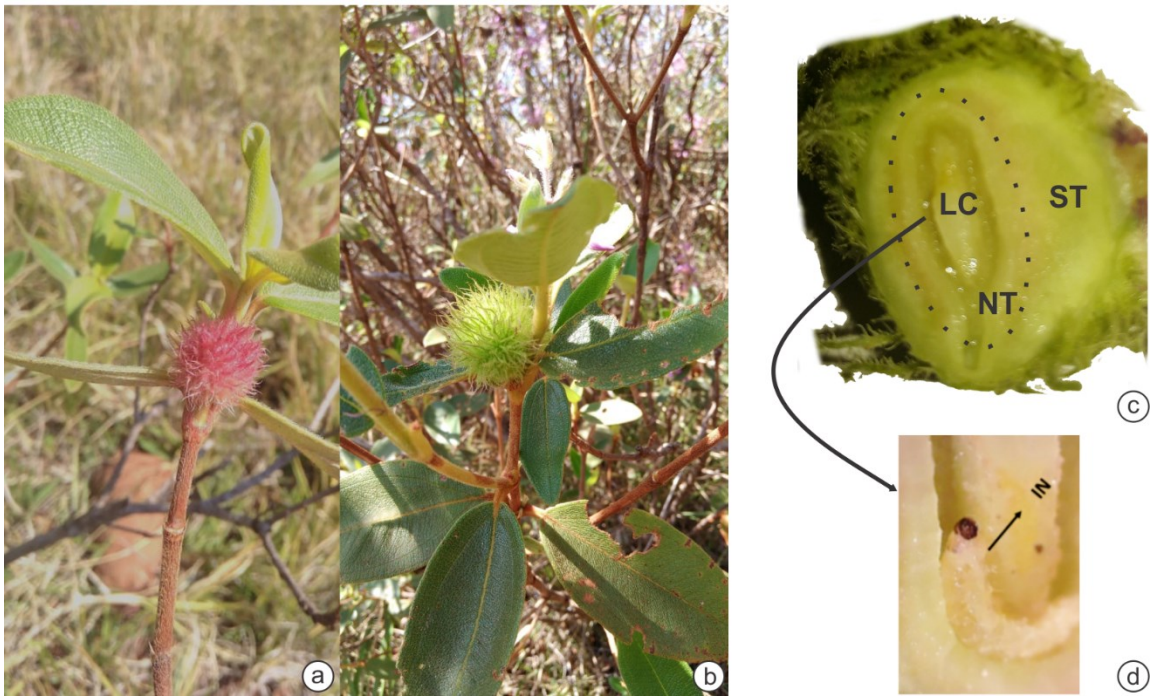
Fig 6. Scores of the two first axes of the principal component analysis (PCA) obtained from resource traits in both storage (circles) and nutritive (triangles) tissues in galls induced by *Palaeomystella oligophaga* on *Macairea radula* leafs. Abbreviations: TSS – Total Soluble Sugars; WSP – Water Soluble Polisaccharydes; ST – Starch; PHN – Phenolics; PR – Proteins; LI – Lipids; MDA – Malonaldehyde

Table 1. Principal components 1 (PC1) and 2 (PC2) from the correlation of the compounds on NT and ST in P. oligophaga galls. The two first axes of the principal components analysis (PCA) explained 77,6% of the variation. Only the total soluble sugars (TSS) data correlates negatively with the axis 1, while just protein and malondialdehyde levels correlate negatively with the axis 2. Abbreviations: TSS – Total Soluble Sugars; WSP – Water Soluble Polisaccharydes; ST – Starch; PHN – Phenolics; PR – Proteins; LI – Lipids; MDA – Malonaldehyde

Compounds	PC1	PC2
TSS	-0.99758	0.06951
WSP	0.98801	0.15439
ST	0.98550	0.16970
PHN	0.81088	0.58522
PR	0.76511	-0.64391
LI	0.99792	0.06454
MDA	0.96346	-0.26784

842

843



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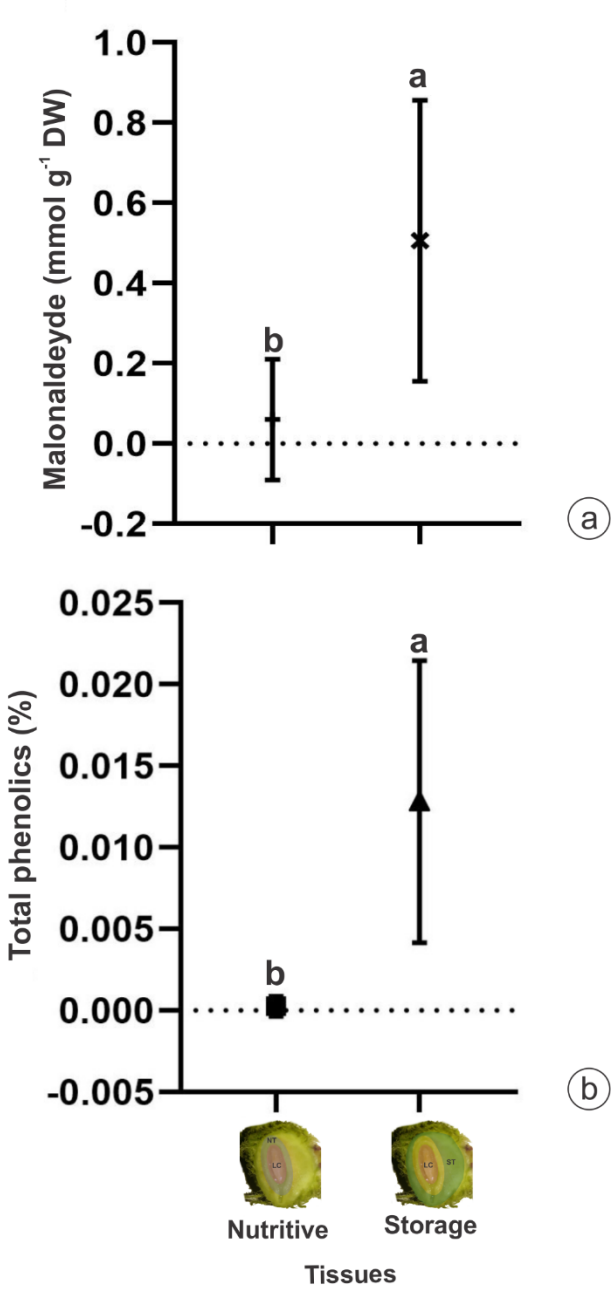
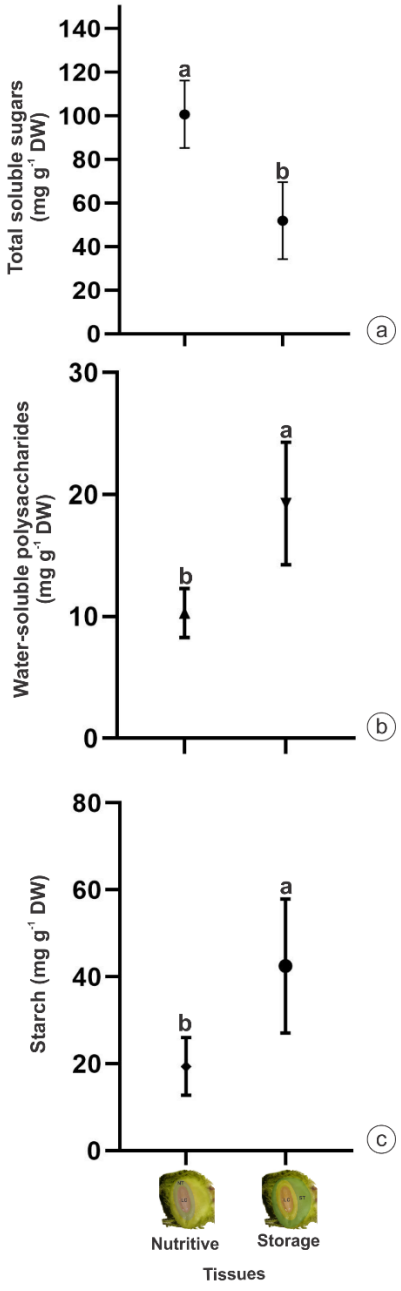
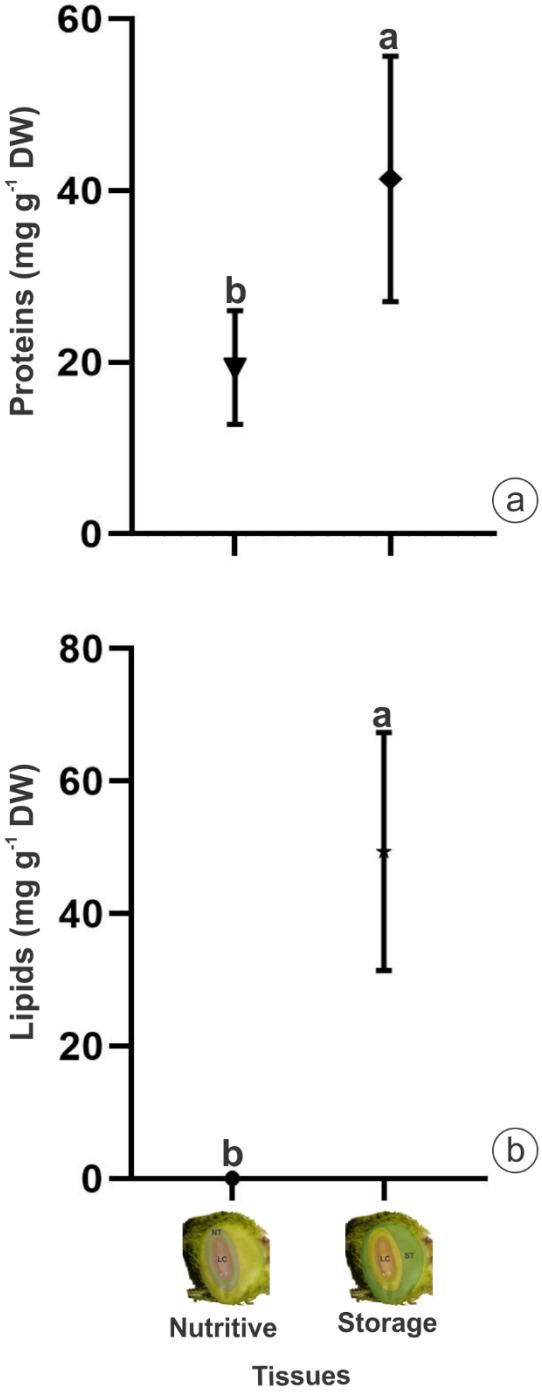
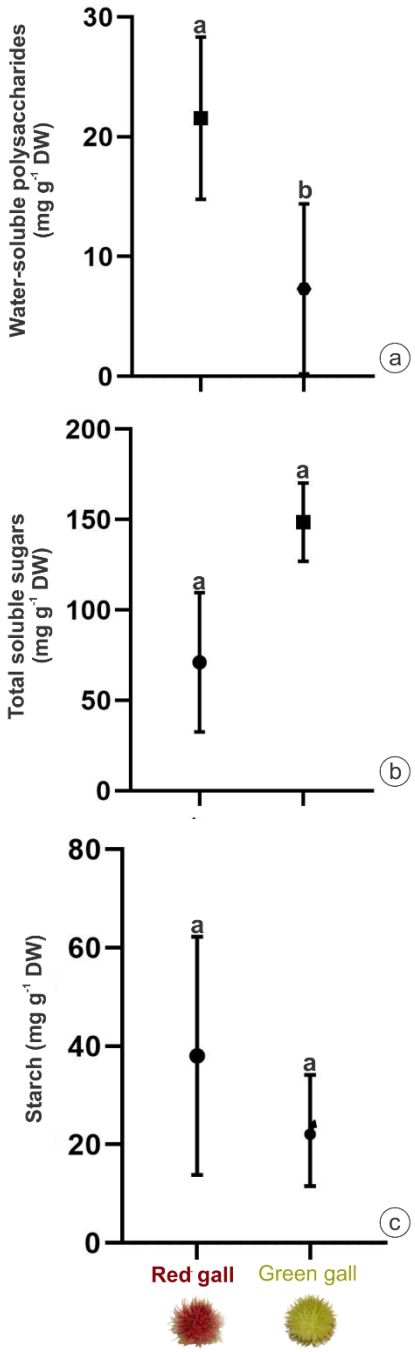

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Malonaldeyde (mmol g-1 DW)
a
b
a
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
Total phenolics (%)
a
b
b
NT
LC
LC
ST
Nutritive
Storage
Tissues

847


Total soluble sugars (mg g-1 DW)
140
120
100
80
60
40
20
0
a
b
a
Water-soluble polysaccharides (mg g-1 DW)
30
20
10
0
b
a
b
Starch (mg g-1 DW)
80
60
40
20
0
b
a
c
Nutritive
Storage
Tissues

848

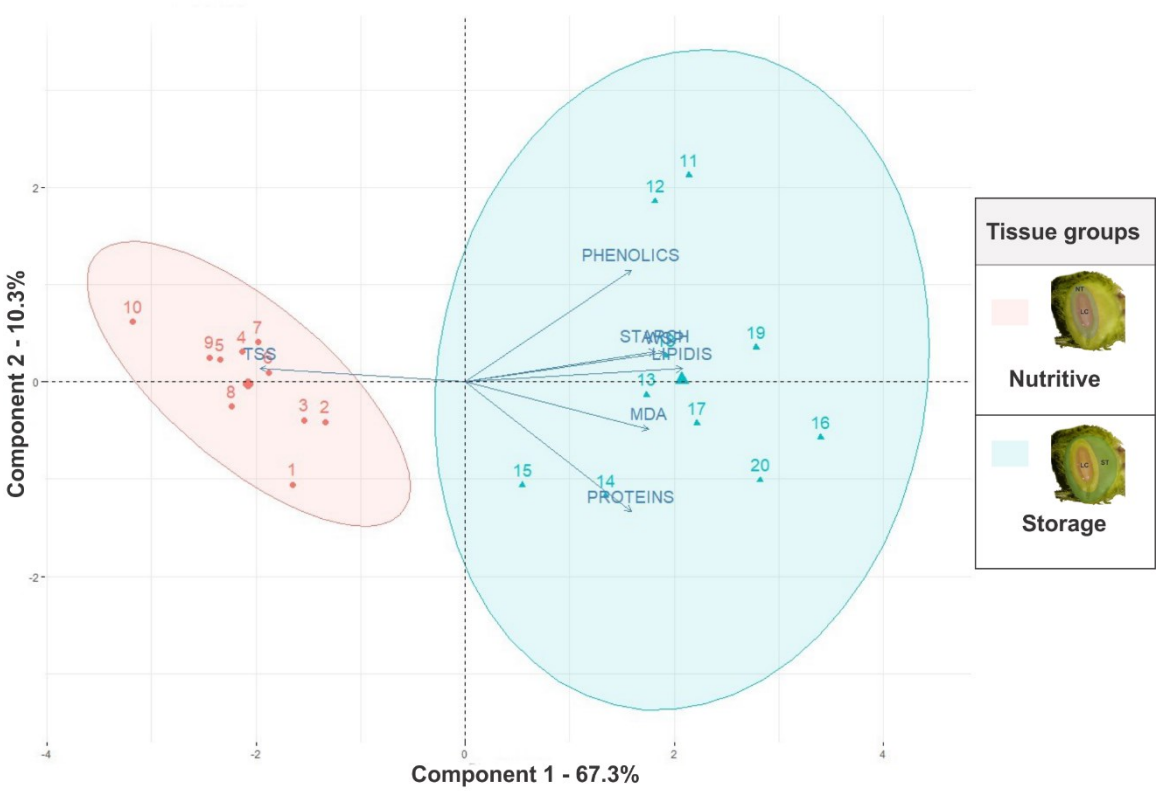

60
40
20
0
Proteins (mg g-1 DW)
a
b
a
80
60
40
20
0
Lipids (mg g-1 DW)
a
b
b
NT
LC
LC
ST
Nutritive
Storage
Tissues


Water-soluble polysaccharides (mg g-1 DW)
30
20
10
0
a
b
a
Total soluble sugars (mg g-1 DW)
200
150
100
50
0
a
a
b
Starch (mg g-1 DW)
80
60
40
20
0
a
a
c
Red gall
Green gall

849

850

851



852

853

854

855