Compartmentalization of Metabolites and Enzymatic Mediation in Nutritive Cells of Cecidomyiidae Galls on *Piper Arboreum* Aubl. (Piperaceae)

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Abstract

Galling insects commonly change the chemical profile of their host plant tissues during gall induction and establishment. As a consequence, galls accumulate a wide range of metabolites in specialized cells, which may be organized in a nutritive tissue and in outer storage cells. The nutrients compartmentalized in nutritive cells may be directly assessed or metabolized via enzymatic mediation, while the gall outer cortex may accumulate secondary metabolites. These secondary metabolites may configure a specialized chemical barrier against the attack of natural enemies. Either the nutritive inner cells or the outer cortical cells, with their specific metabolic apparatus, should differentiate under the chemical constraints of each host plant-galling herbivore interaction. This premise is herein addressed by the investigation of the histochemical profile of the non-galled leaves and galls induced by Diptera: Cecidomyiidae on *Piper arboreum*. The spatial compartmentalization of the nutritive and defensive metabolites indicates the new functions assumed during the redifferentiation of the host plant cells. The enzymatic mediation of the primary metabolites by sucrose synthase and invertases favors the nutritive requirements of the galling Cecidomyiidae or the structural maintenance of the gall. The accumulation of secondary metabolites is restricted to the tissue layers not involved in nutrition, and may act in the chemical protection against predators or parasitoids. Current results systematically document metabolites compartmentalization, evidence the impairment of toxic compounds storage in cells surrounding the larval chamber, as well as, detect the redirection of nutritive substances to the site of the Cecidomyiidae feeding. The activity of sucrose synthase is restrict to the nutritive tissue in the galls on *Piper arboreum*, and reinforces previous detection of this enzyme mediation in carbohydrate metabolism in Cecidomyiidae galls.

Keywords: chemical profile, cecidomyiidae, galling insect diet, gall metabolism

1. Introduction

Galling insects alter the morphogenetical patterns of their host plant organs by inducing cell redifferentiation (sensu Lev-Yadun, 2003), division and growth (Oliveira & Isaias, 2010a; Isaias, Oliveira, & Carneiro, 2011; Isaias, Oliveira, Carneiro, & Kraus, 2014; Magalhães, Oliveira, Suzuki, & Isaias, 2014). The new morphogenetical patterns generate specialized cells and tissues at the gall site, whose chemical and functional features are distinct from those of the host organs (Oliveira, Carneiro, Magalhães, & Isaias, 2011; Castro, Oliveira, Moreira, Lemos-Filho, & Isaias, 2012). The gall metabolic requirements generate a sink of primary and secondary metabolites, which may compartmentalize in protective and/or nutritive tissues (Carneiro, Castro, & Isaias, 2014). The carbohydrates drained to the gall developmental site are responsible for plant cell machinery support (Oliveira, Christiano, Soares, & Isaias, 2006; Castro et al. 2012), and can also act as potential signaling molecules for cell division and growth (Koch, 2004; Wind, Smeenkens, & Hanson, 2010; Isaias, Oliveira, Moreira, Soares, & Carneiro, 2015). The signaling function in gall sites has also been attributed to reactive oxygen species (ROS) (Oliveira & Isaias 2010a; Oliveira, Moreira, & Isaias, 2014; Isaias et al., 2014). ROS
accumulation demands phenolics scavenging (Isaias et al., 2015), which, in turn, influence IAA regulation (Bedetti, Modolo, & Isaias, 2014).

Proteins, lipids, reducing sugars or starch accumulate in the inner layers of the gall, i.e., the nutritive tissue, and can provide resources for the nutrition of the galling insect (Price, Waring, & Fernandes, 1986, 1987; Bronner, 1992). Nevertheless, in some systems, the availability of these metabolites to the galling insect or to the maintenance of cell metabolism depends on enzymatic activities (Bronner, 1992; Oliveira Magalhães, Carneiro, Alvim, & Isaias, 2010; Oliveira & Isaias, 2010b; Carneiro et al., 2014). The patterns of enzymatic activities depend on the feeding behavior of the galling insects (Bronner, 1992), and on the chemical profile of the host plants (Oliveira et al. 2010, 2014; Oliveira & Isaias, 2010b).

The enzymatic mediation of carbohydrates metabolism has been documented just for four galling herbivore-host plant systems in the Neotropics (Oliveira et al., 2010, 2011; Oliveira & Isaias, 2010b; Carneiro et al., 2014). These four systems involve gall inducing Cecidomyiidae and Psyllioidea with their divergent peculiarities, and demonstrated the non-exclusiveness of the carbohydrates accumulation to Cecidomyiidae galls. Among the investigated carbohydrates, sucrose is synthesized in the cytosol from photosynthetically fixed carbon, starch reserves or lipid metabolism (Wind et al., 2010), and is transported via phloem to other plant parts. The main enzymes responsible for sucrose metabolism are sucrose synthase (SuSy) and invertases, which catalyse the conversion of sucrose into glucose and fructose (Koch, 2004). In general, SuSy activity is associated to sink tissues and starch accumulation, while invertases mediate cell respiration, tissue growth and development (Koch, 2004; Wind et al., 2010). In galls, the metabolism of these enzymes was also related to the formation of histochemical gradients and maintenance of the gall tissues in Aspidosperma australi (Oliveira & Isaias, 2010b). The sites of the galling herbivore’s nutrition seems to be crucial for the redifferentiation of the nutritive cells, as documented for Notothrioza cattleiani galls on Psidium cattleianum (Carneiro, Pacheco, & Isaias, 2015).

Outside the nutritive cells, Cecidomyiidae galls usually have sclerenchymatic cells, forming a mechanical protective layer, and an outer parenchymatic cortex. These outer cell layers usually accumulate secondary metabolites, such as alkaloids, flavonoids, phenolics and tannins (Nyman & Julkunen-Titto, 2000; Oliveira et al., 2006; Formiga, Soares, & Isaias, 2011; Isaias et al., 2014), and can protect the galling herbivores against the attack of parasitoids and predators, and the gall structure against cecidophagous (Price, Waring, & Fernandes, 1987).

Cell redifferentiation and metabolism in gall outer and inner tissue layers are herein revisited in a Cecidomyiidae - Piper arboreum system. We assume that primary and secondary metabolites accumulate in distinct tissue compartments, as expected, but some metabolic steps of this gall should be dependent on the host plant carbohydrates metabolism rather than on the Cecidomyiidae feeding mode. If this is true, some similarities between the compartmentalization and metabolism of Cecidomyiidae and Psyllioidea galls should be found.

The intralaminar lenticular galls on P. arboreum have nutritive cells limiting the larval chamber, where carbohydrates should accumulate, as previously observed in other two Neotropical Cecidomyiidae galls (Oliveira et al., 2010, 2011). Similarly, the accumulation of carbohydrates in nutritive like cells has been documented in a Psyllioidea gall (Oliveira et al., 2011; Carneiro et al., 2014). Both gall inducers should not come into contact with non-palatable secondary metabolites, and therefore, enzymes mediation and a spatial compartmentalization of nutritive and defensive metabolites in gall developmental site are expected. Current analyses focus on the following questions: (I) does the accumulation of primary and secondary metabolites follow the expected compartmentalization? (II) Is there any disruption for metabolites accumulation in response to the galling stimuli of the Cecidomyiidae on P. arboreum? (III) Are there any similarities between the enzymatic mediation of Cecidomyiidae and Psyllioidea galls? And (IV) should ROS signaling involve sugar mediation during gall development on the Cecidomyiidae intralaminar leaf galls on P. arboreum?

2. Methods

Samples of non galled leaves (NGL) and mature leaf galls (MG) induced by an unidentified species of Diptera: Cecidomyiidae on Piper arboreum were accompanied and collected from September 2013 to March 2014 at the ecological station of Universidade Federal de Minas Gerais in Belo Horizonte, Minas Gerais state, Brazil.

2.1 Histochemical Assays

Fresh samples (n ≥ 5) were free-hand sectioned and submitted to histochemical tests with the following reagents: saturated solution of sudan Red in 70°GL ethanol during 5 min to detect lipids (Brundett, Kendrick, & Peterson, 1991); Fehling’s reagent (Solution “A” - 7.9% copper sulfate, and solution “B” - 34.6% sodium potassium tartrate and 1% sodium hydroxide) heated to pre-boiling temperature for detecting reducing sugars
(Sass, 1951); Lugol’s reagent (1% potassium iodine-iodide solution) during 5 min for starch detection (Johansen, 1940); 0.1% bromophenol blue in a saturated solution of magnesium chloride in ethanol during 15 min, and later washed in acetic acid and water, for the detection of proteins (Baker, 1958); 1% ferric chloride during 5 min, for phenolic compounds detection (Johansen, 1940); Dragendorff’s reagent (Solution "A" - 12.5% bismuth nitrate in 25% acetic acid, and solution "B" 40% potassium iodide) during 5 min for the detection of alkaloids (Johansen, 1940); Wiesner’s reagent (2% phloroglucinol in acidified solution) during 5 min for lignins detection (Johansen, 1940); fixation in 0.5% caffeine sodium benzoate in 90% butanol, followed by incubation in 1% p-dimethylaminocinnamaldehyde (DMACA) during 30 min for the detection of flavonoids (Feucht, Schmid, & Christ, 1986); 1% α-naftol and 1% dimethyl-p-phenylenediamine in phosphate buffer (pH 7.2) (NADI) during 30 min for the detection of terpenoids (David & Carde, 1964), and Lieberman-Buchard’s reagent (concentrated solution of sulfuric acid and acetic acid, 1:1, v/v) during 1 min for the detection of triterpenes (Wagner, Bladt, & Zgainski, 1984). The sections were washed in water and photographed under an optical microscope (Zeiss Primo Star®) with a digital camera (Canon Power Shot A 630®). Blank sections were used for the comparison of results.

2.2 Enzymatic Activity

For the detection of the activity of acid phosphatase, the sections were incubated in 0.012% lead nitrate and 0.1M potassium sodium glicerophosphate in 0.5M acetate buffer (pH 4.5) for 24 hours, at room temperature. The sections were washed in distilled water, and incubated in 1% ammonium sulfate for 5 min. As a control, the samples were not submitted to potassium sodium glicerophosphate (Gomori, 1956). For the detection of phosphorylase activity, the sections were incubated for two hours in 1% glucose-1-phosphate in 0.1M acetate buffer (pH 6.0), at room temperature, and subsequently subjected to Lugol's reagent for 5 min. For control, the samples were not incubated in glucose-1-phosphate (Jensen, 1962). For observation of invertase activity, the sections were incubated for 3 hours at room temperature in a solution containing 0.024% tetrazolium blue (NBT), 0.014% phenazin methosulfate, 30U of glucose oxidase and 30 mM of sucrose, 0.38mM sodium phosphate buffer (pH 7.5). The control was subjected to the reaction media without sucrose (Zrenner, Salanoubat, Willmitzer, & Sonnewald, 1995; Doehlert & Felker 1987). For detection of sucrose synthase activity, the sections were fixed in 2% paraformaldehyde with 2% polyvinylpyrroldone and 0.005M of dithiothreitol (pH 7.0) for 1 hour at 4°C. Later, they were incubated in a solution containing 5µL of 150 mM NADH, 5µl (1U) of phosphoglucomutase, 5µl of 3mM glucose 1,6-bisphosphate, 5µl (1U) of glucose-6-phosphate dehydrogenase, 5µl (1U) of UDPG pyrophosphorylase, 280µL of 0.07% aqueous solution of blue tetrazolium (NTB), 350 µl of buffer and 50µL substrate during 30 minutes. The buffer contained 10 mM MgCl2, 2 mM EDTA, 100 mM HEPES, 0.2% BSA and 2mM EGTA (pH 7.4). The substrate consisted of 15 mM UDP, 0.75 mM sucrose, 15 mM pyrophosphate. Two controls were used. In the first control, the glucose 1,6-bisphosphate and pyrophosphate were not added, and for the second control, the sucrose was suppressed (Wittich & Vreugdenhil 1998).

2.3 Histochemical Test for Reactive Oxygen Species (ROS)

ROS were detected by immersion of the sections in 0.5% 3,3’-diaminobenzidine (DAB) during 15 - 60min, in the dark (Rossetti & Bonatti 2001). The sections were washed in water and photographed under an optical microscope (Zeiss Primo Star®) with a digital camera (Canon Power Shot A 630®). Blank sections were used for the comparison of results.

3. Results

3.1 General Features

Leaf galls on *Piper arboreum* are intralaminal and lenticular. They project to both leaf surfaces, and has a uniseriate epidermis, an outer parenchymatic cortex, an inner cortex composed of sclerenchymatic cells, and a nutritive zone involving the larval chamber, which houses the galling Cecidomyiidae (Figure 1 A-C).
Figure 1. Leaf galls on *Piper arboreum* Aubl (Piperaceae)

A: Leaf with galls viewed by the adaxial surface. B: Hemisection of a mature gall evidencing the adaxial outer cortex, abaxial outer cortex, inner cortex, larval chamber, and nutritive tissue. C: Cecidomyiidae larva. ab, abaxial outer cortex; ad, adaxial outer cortex ic, inner cortex; lc, larval chamber; nt, nutritive tissue. Scale bars = 1 cm (A), 0.5 mm (B), 2 mm (C).

Table 1. Histochemical detection of metabolites and enzymes in non-galled leaves of *Piper arboreum* Aubl (Piperaceae)

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>ADEP</th>
<th>ADHP</th>
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<th>ABHP</th>
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Note. ABEP = abaxial epidermis; ABHP = abaxial hypodermis; ADEP = adaxial epidermis; ADHP = adaxial hypodermis; PP = palisade parenchyma; SP = spongy parenchyma; VB = vascular bundles. (+) positive reaction, (-) negative reaction.
3.2 Histochemical Profile of Non-Galled Leaves (NGL).

Phenolic compounds, flavonoids, alkaloids, and triterpenes were detected in the cells of hypodermis in black, dark blue, brown and red, respectively (Figure 2A-D, Table 1). Terpenoids were detected in blue in the cells of the hypodermis, and also in the idioblasts located between the palisade and spongy parenchymas (Figure 2E, Table 1). Lignins were evidenced in red in the cell walls of the xylem and of the pericyclic fibers (Figure 2F, Table 1). Lipids were detected as red droplets in the cells of the abaxial hypodermis, while reducing sugars, forming red precipitates, were observed in the adaxial and abaxial hypodermis (Figure 2G-H, Table 1). Starch grains and proteins were detected as dark blue grains or precipitates in the palisade and spongy parenchyma (Figure 2I-J, Table 1). The activity of invertases was evidenced as a dark blue precipitate in the hypoderm and parenchyma cells of the veins (Figure 2K, Table 1). The activity of sucrose synthase (SuSy) was detected as a
purple precipitate in the vascular bundles (Figure 2L, Table 1). The activity of phosphorylase and acid phosphatase was not observed. The ROS were detected in the epidermis and chlorophyllous parenchyma of the NGL (Figure 2M, Table 1).

3.3 Metabolites Compartmentalization in Cecidomyiidae Induced Galls on Piper Arboreum

The outer cortical cells of the mature galls (MG) developed from the hypodermis of the NGL, and accumulated phenolic compounds, flavonoids, alkaloids and terpenoids (Figure 3A-C, Table 2). Terpenoids occurred in idioblasts located between the palisade and spongy parenchyma of NGL, but they were not observed in the gall inner cortex (Figure 3D, Table 2). Triterpenes were not evidenced in MG (Table 2). Lignins were detected in the cell walls of the xylem and of the pericyclic fibers both in NGL and MG. In MG, lignins were also observed in the cell walls of the sclerenchyma surrounding the nutritive tissue (Figure 3E, Table 2). Lipids were observed in the outer region, and also in the nutritive tissue of the MG (Figure 3F, Table 2). Reducing sugars were revealed in the cells of the adaxial and abaxial outer cortices (Figure 3G, Table 2). The detection of starch was more intense in the cells of the inner cortex and next to the larval chamber, increasing laterally towards the non-galled region. Starch was also detected in the lignified-walled cells (Figure 3H, Table 2). Proteins were detected in the nutritive tissue (Figure 3I, Table 2).

3.4 Enzymatic Activity

The activity of invertases was detected in the cells adjacent to the larval chamber, and formed a centrifugal gradient towards the non-galled region (Figure 3J, Table 2). The activity of SuSy was detected homogeneously throughout the nutritive tissue and vascular bundles (Figure 3K, Table 2). The activity of phosphorylase and acid phosphatase was not observed either in NGL or MG.

3.5 ROS Detection. In MG, the ROS accumulated in a centrifugal gradient, decreasing towards the outer cortical tissue layers (Figure 3L, Table 2).

Table 2. Histochemical detection of metabolites and enzymes in Cecidomyiidae galls on Piper arboreum Aubl (Piperaceae)

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<thead>
<tr>
<th>Metabolites</th>
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Note. AB = abaxial outer cortex; AD = adaxial outer cortex; ABEP = abaxial epidermis; ADEP = adaxial epidermis; IC = inner cortex; NT = nutritive tissue; VB = vascular bundles. (+) positive reaction, (-) negative reaction.
4. Discussion

4.1 Compartimentalization of Metabolites

Two distinct compartments with accumulation of primary and secondary metabolites were observed in the leaf galls on *P. arboreum*. Primary metabolites have been especially detected in the inner tissues, while secondary metabolites accumulated only in the outer cortical parenchyma, which corroborates the expected spatial functional division of gall tissues. The outer cells were converted from photosynthetic and respiratory compartments towards defensive tissues, while the inner cells assumed a specialized nutritional role in
redifferentiated gall tissues.

The defensive compartment, i.e. the outer cortex of _P. arboreum_ leaf gall, accumulated alkaloids, terpenes, and phenolics, which have been considered waste products of plant metabolism (Roberts & Wink, 1998). Nevertheless, they have been contemporarily evaluated as sources of nitrogen or energetic lipids, and ROS scavenging molecules (Isaias et al., 2015), all of them necessary for the maintenance both of the host plant and gall metabolism. In spite of their involvement in antioxidant mechanisms (Blokhina, Virolainen, & Fagerstedt, 2003; Detoni, Vasconcelos, Rust, Isaias, & Soares, 2011), alkaloids, terpenes, and phenolics may secondarily deter or discourage the attack of predators, due to their toxicity (Rhodes, 1994; Róstas, Maag, Ikegami, & Inbar, 2013). In galls, because of the high oxidative stress, the role of phenolics and flavonoid derivatives has been discussed as ROS dissipation, an efficient strategy to recover the redox-potential homeostasis (Isaias et al., 2015). Also, the phenolics are involved in IAA metabolism and consequently cell hypertrophy at gall site (Bedetti et al., 2014).

Even though the terpenes accumulated all over leaf mesophyll, they are detected exclusively in the outer compartment of the galls on _P. arboreum_, reinforcing the chemical protective function of the gall outer tissue layers. The impairment of the terpenic idioblasts differentiation in the nutritive tissue of the galls on _P. arboreum_ should have favored the gall inducer, which did not come into contact with the toxic potential of the terpenes, and their anti-herbivore properties (Gershenzon, 1994). The strategy of disrupting the differentiation of terpenic idioblasts have been previously observed in the galling herbivores- _Lantana camara_ systems (Moura, Isaias, & Soares, 2005; Moura, Isaias, & Soares, 2008).

### 4.2 Double Metabolites Accumulation in the Inner Compartment

As a host plant potentiality, lipid droplets were detected in the cells of the cortical parenchyma, originated from the NGL mesophyll, their intrinsic location. The lipids accumulated in the inner tissue layers may function as an energetic resource both for the galling Cecidomyiidae’s nutrition and gall development. Even though attributed to Cynipidae (Bronner, 1992) and Lepidoptera galls (Vecchi, Menezes, Oliveira, Ferreira, & Isaias, 2013), lipidic droplets have been previously detected in some Cecidomyiidae galls of _Aspidosperma spruceanum_ (Oliveira et al., 2010), _Copaifera langsfordii_ (Oliveira et al., 2011), and _Marcetia taxifolia_ (Ferreira & Isaias, 2014). The accumulation of lipids in such galls has been related to the potential of the host plants for such accumulation (Oliveira et al., 2011; Ferreira & Isaias, 2014), as is true for _P. arboreum_.

The inner compartment of the galls on _P. arboreum_, i.e., the nutritive tissue, also accumulates proteins, similarly to the Cecidomyiidae galls on _Aspidosperma spruceanum_ (Oliveira et al., 2010). Proteins are excellent nutritive resources for the gall-inducing herbivores, and may have accumulated as a cellular response to the increased oxidative and respiratory stresses established during the galls development (Schönrogge, Harper, Lichtenstein, 2000). The increased level of proteins is followed by high levels of hexoses (Sturm & Tang, 1999), which are products of the activity of sucrose synthase and invertases (Roitsch & Gonzalez, 2004). The detection of invertases indicates the fast conversion of sucrose, and the activation of a mechanism of plant defense by increasing the synthesis of secondary metabolites (Wind et al., 2010; Sturm & Tang 1999). The double accumulation of nutritive compounds, such as lipids and proteins, in the nutritive tissue of a Cecidomyiidae gall is not the expected pattern, which should be carbohydrates storage (Bronner, 1992; Oliveira et al., 2010; Oliveira et al., 2011). This double stimuli is therefore a novelty for Cecidomyiidae galls in the Neotropics, and indicates a surplus for the galling herbivore nutrition.

### 4.3 Enzymatic Mediation of Carbohydrates Accumulation

The activity of sucrose synthase (SuSy) and invertases detected in the nutritive tissue of the galls on _P. arboreum_ corroborated the metabolic similarity between Pyroloidea and Cecidomyiidae galls. Both enzymes have been previously detected in galls induced by _Pseudophascoterion aspidospermii_ (Malenovský, Burckhardt, Queiroz, Isaias, & Oliveira, 2015) on _Aspidosperma australre_ (Oliveira et al., 2010) and by a Cecidomyiidae on _A. spruceanum_ (Oliveira & Isaias, 2010b). This enzymatic detection indicates a host plant metabolic requirement or potential rather than a dependence on the galling herbivore mode of feeding.

Moreover, current results demonstrate a common site for the activity of SuSy in the Cecidomyiidae galls on _P. arboreum_, and on _A. spruceanum_ and _Copaifera langsfordii_, which diverges in the _P. aspidospermii_ galls on _A. australre_, where the activity of SuSy was restricted to the vascular bundles. Based on such comparison, we can conclude that the feeding sites of the galling herbivores does not determine the host plant cells metabolism, but may determine the sites of enzymes activity. The activity of SuSy is commonly responsible for the reversible cleavage of sucrose into fructose and UDP-glucose (Amor, Haigler, Johnson, Wainscott, & Delmer, 1995; Koch, 2004), but may be especially related to the synthesis of starch observed in the nutritive tissue of the galls on _P.
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