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The early developmental stages of gall-inducing insects define final gall structural and histochemical profiles - The case of *Bystracoccus mataybae* galls on *Matayba guianensis*

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Abstract

The gall morphotypes depend on continuous chemical and feeding stimuli of the gall inducer, which promotes specific structural and metabolic changes in plant tissues. The galling insect manipulates host plant tissues and may stimulate the production of primary and secondary metabolites. The type of chemical arsenal and the storage site varies according to the developmental potentials of the host plant and the insect's feeding habit. Here, we investigate if the structural and histochemical profiles of the gall system of *Matayba guianensis* (Sapindaceae) - *Bystracoccus mataybae* (Eriococcidae) are dependent on the instars of the galling insect. Standard anatomical analyses were carried out, as well as histochemical evaluation of reactive oxygen species and primary and secondary metabolites. Structural changes induced by the first instar nymph were detected on the stem, while deep changes induced by the second instar nymph and adult female were detected in leaflet galls, indicating that the host plant tissue can impose more limits on gall development and complexity than the stages of galling insect development. The compartmentalization of primary and secondary compounds is established during the early stages of development of the leaflet gall by the second instar nymph. Despite deep structural differences between stem and leaflet galls, there were no significant changes in the establishment of the histochemical profiles.

Key words: Morphotype, Histochemical profile, Metabolic compounds, Instars of the insect.

Introduction

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

Some guilds of galling insects can develop a typical nutritive tissue around the larval chamber, with primary metabolite accumulation used in the galling insect's diet (Bronner 1992; Ferreira et al. 2017), and nutritive tissue formation and maintenance depending especially on the developmental stage of the galling insect (e.g. Rezende et al. 2018). The Cecidomyiid galls usually accumulate carbohydrates, while galls induced by Hymenoptera and Lepidoptera accumulate lipids (Raman and Dhileepan 1999; Raman et al. 2007; Vieira and Kraus 2007; Bedetti et al. 2013; Ferreira and Isaias 2013; Vecchi et al. 2013; Ferreira et al. 2017; Rezende et al. 2018). In contrast, galls induced by phloem-sucking insects do not develop a true nutritive tissue since the feeding activity may be restricted to the phloem cells (Bronner 1992). Nonetheless, some galls of phloem-sucking insects can accumulate primary

metabolites in their tissues, as is the case for galls of *Pseudophacopteron aspidospermi* (Hemiptera) on *Aspidosperma australe* and induced by *Euphalerus ostreoides* (Hemiptera) on *Lonchocarpus muelhbergianus* (Fabaceae) (Oliveira et al. 2006; Oliveira and Isaias 2010a). The galls can also accumulate secondary metabolites such as phenolic compounds, alkaloids, and terpenes (Hartley 1998; Bragança et al. 2017), which supposedly protect the galls against natural enemies (Bennett and Wallsgrove 1994; Formiga et al. 2009) and promote the scavenging of oxidative stress molecules (Isaias et al. 2015).

The histochemical approach has been used to clarify tissue organization in galls by the localization of primary and secondary metabolites, revealing differences and patterns of metabolite compartmentalization and functions (Bragança et al. 2017; Kuster et al. 2019). Therefore, changes in the metabolic profile have been tested during gall development in different systems (e.g. Oliveira et al. 2006; Carneiro and Isaias 2015) based, mostly, on gall size (i.e. young, mature stages). Until now, no study has reported if the galling insects' developmental stages may trigger structural and histochemical steps in gall developmental processes. Here, in our model of study, the gall-inducing *Bystracoccus mataybae* (Hemiptera) has a peculiar strategy to survive when the host plant, *Matayba guianensis* (Sapindaceae), loses its leaves. The first instar nymph moves to the stem before leaf fall and induces a stem gall. When the leaf sprouting begins, the first instar nymph develops into the second instar nymph, which moves to the leaves and induces the leaf galls (Pfeffer et al. 2018). The main goal of this study is to analyze if the different sites of induction and the stages of galling insect development can drive the structural and histochemical profiles of the gall.

Material and methods

Host plant-galling insect system

The galling insect *Bystracoccus mataybae* (Eriococcidae, Hemiptera) (Hodgson et al. 2013) induces galls on the stem and leaflets of *Matayba guianensis* Aubl. (Sapindaceae). The galling insect is parthenogenetic and has three instars throughout its development, all of them occurring on the same host in a synchronized cycle. When the leaf falls (dry season), the insect in the first instar moves from the leaflets to the branches, while the second instar induces galls in the new sprouting leaflets, then turning into an adult (= third instar) and becoming pregnant (Pfeffer et al. 2018).

Study area and sampling

The study was carried out at Estação Ecológica do Panga (19° 10'S, 48°24'W) and Fazenda Experimental do Glória (18°57'S, 48°12'W), Uberlândia city, Minas Gerais State, Brazil. Ten individuals of *M. guianensis* with galls induced by *B. mataybae* were selected in each population. Non-galled leaflets, as well as stems and galls were collected from August 2017 to March 2018 for structural and histochemical characterization. The analyses were performed in triplicate. Young and mature non-galled leaflets were used, as well as mature non-galled stems and galls at different stages of galling insect development. The stem galls with galling insects were used to represent the first instar nymph (named crawler) (Figure 1A). The young leaflet galls had a second instar nymph (Figure 1B), while mature leaflet galls had adult insects (= third instar) - with eggs and/or crawlers - (Figure 1C). All gall stages were defined as reported by Hodgson et al. (2013) and Pfeffer et al. (2018).

First-instar nymphs were collected on the stem galls from July to August, young leaflets and galls induced by the second instar nymph were collected from August to September, while adult insects were collected in October. From November to February, the

adult has eggs and/or crawlers, which can stay inside the insect or around it. Histochemical tests were performed when the adult was not pregnant and when it presented eggs and/or crawlers. Analyses were also carried out on senescent galls (living tissue) (Figure 1D) in order to observe possible changes at the site of metabolite accumulation and the presence or absence of primary and secondary substances.

Histological analysis

Structural features were examined in samples fixed in 0.1 mol/L Karnovsky's solution in phosphate buffer, pH 7.2 (2.5% glutaraldehyde and 4.5% formaldehyde in 0.1 M phosphate buffer) (Karnovsky 1965). The samples were dehydrated in an ethanol series and embedded in 2-hydroxyethylmethacrylate (Historesin[®], Leica Instruments, Germany) or used without embedding. Transverse 5 µm-thick sections were obtained with a rotary microtome (YD-315 model, China) from samples embedded in historesin, stained with 0.05% toluidine blue, pH 4.6 (O'Brien et al. 1965) and mounted with Entellan[®]. Freehand transverse sections of the stem were made, clarified using sodium hypochlorite, stained with astra blue and safranin (in the proportion of 9:1 v/v), and mounted on Kaiser glycerinated jelly (Kraus and Arduin 1997).

Histochemical analysis

For the detection of primary and secondary metabolites, only fresh samples were used, and handmade sections were prepared using razor blades, followed by immersion in specific reagents. For protein detection, the samples were immersed in 0.1% bromophenol blue in a saturated solution of magnesium chloride in 70% alcohol (Mazia et al. 1953). For lipids and starch, samples were submitted to a saturated solution of Sudan III in 70% alcohol

(Sass 1951) and to a Lugol solution (1% iodine-potassium iodide solution) (Johansen 1940), respectively. The localization of reducing sugars was determined by using Fehling's reagent, which consists of the combination of solution "A" (6.93% II copper sulfate w:v) and "B" (34.6% sodium potassium tartrate and 12% sodium hydroxide w:v), with subsequent heating of the slide containing the samples to pre-boiling (Sass 1951). The sections were treated with Ehrlich's solution to determine the presence of auxins (Bedetti et al. 2014) and with ruthenium red solution to determine the presence of pectins (Jensen 1962).

The histochemical localization of some secondary metabolites, such as phenolics, alkaloids, and terpenoids, was investigated. Total phenolics were detected with 2% ferrous sulfate in 10% formalin (v/v 1:1) (Gahan 1984) and Dragendorff's reagent was used for alkaloid detection, followed by washing in 5% sodium nitrite (Svendsen and Verpoorte 1983). For the detection of flavonoids, the samples were pre-fixed in 0.5% caffeine sodium benzoate in 90% butanol and then incubated in 1% *p*-dimethylaminocinnamaldehyde (DMACA) (Feucht et al. 1986). Terpenoids were detected with 1% α -naphthol and 1% dimethyl-*p*-phenylenediamine in phosphate buffer solution, pH 7.2 (NADI test) (David and Carde 1964). Structural lignin was detected using 2% phloroglucinol in acidified solution (Johansen 1940).

For the evaluation of reactive oxygen species (hydrogen peroxide), the sections were immersed in DAB reagent (3,3'-diaminobenzidine) in the dark (Rossetti and Bonatti 2001). For each metabolite group tested, a negative-control test and blank sections were conducted in parallel, and the glass slides were mounted in distilled water or the reagent itself. All the structural and histochemical sections were photographed with a Leica® DM500 photomicroscope coupled to a Leica® ICC50HD camera.

Results

Structural traits of non-galled stems, leaflets and galls

The non-galled stem has a uniseriate epidermis, with a rectangular cell wall shape, covered with a thick cuticle (Figure 2A) or already with periderm. The cells of periderm of non-galled stems have a rectangular cell wall shape (Figure 2A, detail). The phellem consists of 20-30 compactly arranged layers, the outer ones dead and the 5-7 layers close to the phellogen living (Figure 2A, detail). The phellogen and the phelloderm are composed of a rectangular cell layer and consist of living cells. The cortical tissues are predominantly parenchymatic with brachysclereids in small groups (Figure 2A). The vascular system usually has secondary growth (Figure 2A). Fibers surround the phloem (Figure 2A) and form a complete circle. The first instar nymphs (crawlers) induce galls on the stem surface (Figures 1A and 2B), which result in an increase of phellogen activity and consequently cell hypertrophy and periderm hyperplasia, especially the phelloderm (Figure 2B). After galling insect induction, there were no changes in the inner tissues of the periderm (phelloderm), as well as the cortical or vascular ones.

The young leaflets already have a uniseriate epidermis, 8-10 layers of chlorophyllous parenchyma with secretory cavities throughout the parenchyma and vascular bundles undergoing differentiation (Figure 2C). The mature leaflets are hypostomatic, with a uniseriate epidermis covered with a thick cuticle, and a dorsiventral mesophyll (Figure 2D). A uniseriate layer of hypodermis with a rounded cell wall shape occurs under the adaxial surface (Figure 2D). The palisade parenchyma is compact, has 3-4 layers and presents a columnar cell wall shape (Figure 2D). The spongy parenchyma contains large intercellular spaces, about 6-7 layers, and a rounded cell wall shape (Figure 2D). In the middle of the chlorophyll parenchyma there are collateral vascular bundles (Figure 2D).

Galls induced by the second instar of the galling insect on young leaflets are globoid, intralaminar, green, with an adaxial surface opening (Figure 1B). The young galls have a uniseriate epidermis with ordinary cells of isodiametric shape, a thin cuticle and non-glandular trichomes. The cortex is homogeneous and consists of parenchyma cells (> 50 layers) of various sizes and shapes (Figure 2E), with the occurrence of tissue hyperplasia and cell hypertrophy. New vascular bundles differentiate and are then displaced by the hyperplasia of the parenchyma, and are ultimately located near the nymphal chamber (Figure 2E).

The adults of *B. mataybae* (third instar) are reddish and build a second nymphal chamber (Figure 2F) below the adaxial surface where they start to lay their eggs. The galls (Figure 1C), kept covered by the uniseriate epidermis and the cortex, can be divided into outer and inner compact cortex types. The outer cortex is composed of homogenous parenchyma (> 50 layers) (Figure 2E), while the inner one occurs around the nymphal chamber and is mostly formed by sclerenchyma cells (10-15 layers) (Figure 2F). Collateral vascular bundles are located near the nymphal chamber, facing the adaxial surface of the gall (Figure 2E). The senescent phase (Figure 1D) occurs when the crawlers leave the adult female, followed by her death.

Histochemical characterization of non-galled organs and galls

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

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

In the non-galled stem, auxins, proteins, total phenolics, and flavonoids are detected in the parenchyma cells (Table 1). Terpenoids and lignins are detected only in cortical parenchyma cells, while alkaloids are detected also in the phloem, and central parenchyma cells (Table 1). Reactive oxygen species (ROS) prevail in cortical and phloem cells (Table 1).

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

Non-galled leaflet and galls with galling insect in the second instar nymph

Non-galled leaflets showed a positive reaction for reducing sugars and auxins in mesophyll cells, and for proteins only in cells of epidermis and vascular bundles (Table 1). Alkaloids and flavonoids were detected in the palisade parenchyma, but terpenoids occurred in the adaxial epidermis only (Table 1). The epidermis and parenchyma cells of vascular bundles showed a positive reaction for pectins (Table 1). Lignin and phenolic compounds were detected only in mature leaflets in the cell walls of pericyclic fibers and mesophyll cells, respectively (Table 1). ROS were detected in the chlorophyllous parenchyma, as well as in the vascular bundles of non-galled leaflets (Table 1).

The leaflet galls induced by the second instar (Figure 4, Table 1) showed a positive reaction for reducing sugars (Figure 4A) and auxins (Figure 4B) in the outermost cell layers

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

Leaflet galls with galling in the third instar (adults)

The chemical compounds detected histochemically in the galls induced by the first and second instar nymphs (Figure 5, Table 1) were also detected in leaf galls with adult insects (Figure 5A-L). Starch (Figure 5A) was an exception, occurring as a few grains in the cortex cells near the nymphal chamber only in adult galls. Phenolics did not occur in galls induced by the second instar nymph, but were detected in adult galls (Figure 5G). There were no differences in the location of the metabolites based on whether the galls contained pregnant or non-pregnant females.

Senescent galls – when the crawlers come out

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

Discussion

Despite deep changes in the host stem and leaflet morphology induced by the phenotype manipulator, both galls on *M. guianensis* showed similar histochemical profiles. The histochemical profile of the leaflet galls does not change according to the developmental stage of the galling insect. This histochemical profile is determined by the second instar nymph during the early stages of development of leaflet galls. An exception is noted for starch, which appears only in the mature galls, where the galling insects are adults (i.e. third instar). Although the mouth apparatus of the galling insect seems to be determinant for gall shape and tissue specialization (Bronner 1992; Ferreira et al. 2017), the host plant morphogenesis is also relevant to final gall structural and histochemical profiles of the gall. Nevertheless, here the early determination of the gall histochemical profile was determined from the onset of galling insect development.

The stem galls induced by the first instar nymph of *B. mataybae* showed low complexity compared to leaflet galls induced by the second instar nymphs. These stem galls develop from the host tissues that grow around the first instar nymph of the galling insect,

forming a single nymphal chamber. Therefore, the galls provide better conditions of moisture and temperature to the first instar nymph than those found external to the galls, acting as a micro-habitat, keeping the galling insect safely (Lill and Marquis 2003). The simple structure of the stem galls compared to leaflet galls clearly shows how the morphogenetic constraints of the host can play a key role in the determination of gall morphology, regardless of the developmental stage of the galling insect (Formiga et al. 2014). Stem organs seem to be less plastic than leaves and, thus, could impose stronger constraints (Pugnaire and Valladares 2007) to gall development. Thus, *B. mataybae* manipulates the host plant tissues, stems and leaves, in a different way. In this context, the extended phenotype concept (Dawkins 1982; Stone and Cook 1998) can be revised here, considering that the same insect species, a parthenogenetic one, induces different morphotypes in the same host plant species.

Regarding the histochemical differences between stem and leaflet galls, it is important to emphasize that stem galls do not accumulate starch. The absence of this metabolite may be related to the phloem-sucking behavioral habits of *B. mataybae*, which does not feed during the first instar phase (Gulan 2005) and thus does not appear to stimulate a strong sink of photoassimilates. However, galls induced by other phloem-sucking (Hemiptera) and scraping-sucking (Cecidomyiidae) insects can store starch when the gall inducer stops feeding and consequently reduces gall tissue metabolism (Bronner 1992). The primary metabolite compartmentalization is normally indistinct in leaflet galls among the developmental stages of *B. mataybae*. One example of metabolite gradient in gall tissues is the reducing sugars which has a centripetal gradient in the outer cortex among all the instar stages of the insect. This production and accumulation of carbohydrates occur in *Calliandra brevipes* galls (Fabaceae), indicating the relevant role of carbohydrates as metabolic

resources in some galls (Detoni et al. 2010). Starch is a carbohydrate resource for galls, observed as grains in small numbers near the nymphal chamber in the adult female phase, indicating low cell metabolism (e.g. Oliveira et al. 2017). Starch can be enzymatically cleaved by enzymes into sucrose, fructose and glucose during gall development and be used as a metabolic cell resource (Oliveira and Isaias 2010b).

The few lipid droplets found in the leaflet gall cortex, induced by different insect instars of the galling insect *B. mataybae*, probably do not seem to be related to insect feeding needs, but rather to the maintenance of the cellular machinery. Lipids represent a high energy reserve related to the maintenance of gall structure, as shown for the *Lonchocarpus mulherbergianus* – *Euphalerus ostreoides* (Oliveira et al. 2006) and *Lantana camara* – *Aceria lantanae* (Moura et al. 2008) systems. Protein synthesis and storage is common in nutritive tissues of other gall-inducing groups such as Cecidomyiids (Oliveira et al. 2010, 2011b; Ferreira and Isaias 2014) and Cynipids (Bronner 1992). Proteins accumulated in vascular bundles and perivascular parenchyma cells, as found in the *B. mataybae* leaflet galls, have also been reported for *Nothotrioza cattleiani* (Trioziidae) - *Psidium cattleianum* (Myrtaceae) galls (Carneiro and Isaias 2015). Carneiro and Isaias (2015) suggested that these phloem-sucking insects can induce the redifferentiation of nutritive tissue around the vascular system, and this may be related to the well-developed ROS scavenging apparatus. In addition, the increase of proteins in gall tissues seems to be a good indicator of high oxidative stress (Schonrogge et al. 2000).

The outermost layers of the gall cortex also accumulate ROS, which can be related to the high metabolic activity of the photosynthesis process, as shown for the Cecidomyiidae - *Piper arboreum* system (Bragança et al. 2017). The ROS, especially hydrogen peroxide, are the main stressors described for gall tissues. However, they can also act on molecular

signaling and contribute to gall tissue morphogenesis (Oliveira et al. 2011a; Isaias et al. 2015). ROS detection on the chlorophyllous parenchyma of non-galled leaflets and during all instar stages of *B. mataybae* galls indicates that ROS are intrinsic substances for cell metabolism, but the high intensity of ROS labeling in the gall cells reveals that the biological activity of the galling insect is also a stressing agent for the plant tissues. Galls containing adult insects accumulate ROS in vascular bundles, which may be associated with an increase of the insect's sucking activity.

In view of the oxidative stress generated in the gall, some mechanisms of stress dissipation are necessary for tissue homeostasis (Isaias et al. 2015), such as phenolic compound production (Bedetti et al. 2014; Suzuki et al. 2015; Oliveira et al. 2017). The occurrence of phenolics in the chlorophyllous parenchyma and around the nymphal chamber can be a response to ROS production as a result of the photosynthetic process and to damage from insect movement, respectively, as already reported by Kuster et al. (2019). Phenolic derivatives such as lignin can be an additional mechanism of stress dissipation in the cell walls of gall tissues (Akhtar et al. 2010; Isaias et al. 2015, Oliveira et al. 2017). Lignin synthesis depends on ROS generation, especially the hydroxyl radicals, which are consumed and dissipated while lignin is deposited on the cell walls (Grace and Logan 2000; Blokhina et al. 2003). Thus, the lignin on the perivascular and sclerenchyma cell walls from the first to the last instar of the insect could be the secondary pathway for ROS dissipation in *B. mataybae* galls.

Recent studies have shown that some phenolic derivatives can act on the signaling and stimuli of gall growth when synergistically associated with the auxins (Bedetti et al. 2014). This process acts on the inhibition of AIA-oxidases, thus increasing the action of auxins and leading to cell hypertrophy during gall formation, as demonstrated in *Piptadenia*

gonocantha galls (Bedetti et al. 2014). For the *B. mataybae* leaflet galls, the histochemistry results support this function, since auxin and phenolics were present synchronously inside the cells, mainly in the cortical parenchyma and near the nymphal chamber. The presence of alkaloids and terpenoids during leaflet gall instars may have some defensive function against the attack of parasitoids and predators, reducing competition for food by inhibiting the attack of other phytophagous insects (Janzen 1977; Cornell 1983).

Main considerations

The phenotype manipulator *B. mataybae* changes the morphogenetic patterns of stem and leaves of *M. guianensis* in a different way. Despite deep structural differences between stem and leaflet galls, there were no significant changes in the establishment of the histochemical profiles. In addition, the instar stages in the leaflet gall were not the triggers of structural or histochemical changes, indicating that the host organ potentialities probably restrict the primary and secondary traits of the galls. Thus, the *Bystracoccus mataybae*-*Matayba guianensis* system demonstrated that the early developmental stages of the galling insect can define final gall structural and histochemical profiles.

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Figure captions

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

Figure 2. Anatomical features of non-galled and galled tissues of stem and leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A- Non-galled stem with details of

the periderm in inset photo. Stem in secondary growth; B- Gall with first instar in the stem; C- Young non-galled leaflets with many secretory cavities; D- Mature non-galled leaflets; E- Gall with second instar in the leaflets. Vascular bundles in differentiation; F- Gall with third instar in the leaflets, showing the first and second nymphal chambers. *Abbreviations:* AF- Adult female with eggs; CX- cortex; Ep- Epidermis; FB- Fibers; HP- Hypodermis; NC- Nymphal chamber; 1st NC- First nymphal chamber; 2nd NC- Second nymphal chamber; PC- Parenchymatous cells; CC- Cortical cells; SC- Sclereids; PE- Periderm; P- Phellem; PG- Phellogen; PD- Phelloderm; PP- Palisade parenchyma; SC- Secretory cavities; SMA- Sclerenchyma; SP- Spongy parenchyma; VB- Vascular bundle; XY- Xylem.

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

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

the gall tissues, especially in the cortex (circle); D- Lipids on the epidermis; E- A few droplets of lipids in the cortex (arrow); F-H- Secondary metabolites in the outer cortical parenchyma; F- Terpenoids; G- Alkaloids; H- Flavonoids; I-J- Structural polymers (arrow); I- Lignin in cells of the sclerenchyma (arrows); J- Pectin in cell walls of the cortex (arrow); K- ROS in all cells of the cortex, darker labeling in the middle of the gall, region of the vascular bundles (arrow). *Abbreviations:* 1st NC- First nymphal chamber; 2nd NC- Second nymphal chamber; VB- Vascular bundle; TC- Trichomes.

Figure 5. Positive histochemical results for a gall with a third instar nymph in the leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A-E- Primary metabolites; A- Starch in the cortex near the nymph chamber; B- Lipids in cortical cells; C- Auxins in the outermost cortex; D- Reducing sugars in the cortical cells; E- Proteins in the vascular bundles; F-I- Secondary metabolites in the cortical cells; F- Terpenoids in the cortical cells near the nymph chamber and in the sclerenchyma; G- Phenolics; H- Alkaloids; I- Flavonoids; J- ROS with dark labeling in the vascular bundles and sclerenchyma that surround the chamber of the insect; K-L- Structural polymers; K- Pectin in the cortical cell walls (arrow); L- Lignin in the sclerenchyma and vascular bundles. *Abbreviations:* CX- Cortex; NC- Nymphal chamber; 1st NC- First nymphal chamber; 2nd NC- Second nymphal chamber; VB- Vascular bundle; SMA- Sclerenchyma.

Figure 6. Positive histochemical results for the senescent gall phase in the leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A-E- Primary metabolites; A- Starch in the cortical cells (arrow); B- Lipids in the cortical cells (arrow); C- Reducing sugars in the outermost cortex; D- Auxins; E- Proteins in the cells of the vascular bundles; F-G-

Secondary metabolites in the cortical cells; F- Phenolics; G- Alkaloids; H- Flavonoids; I- Lignin in the middle of the cortex; J- ROS – cortical cells labeled for ROS and cells of the sclerenchyma without labeling. *Abbreviations:* CX- Cortex; SMA- Sclerenchyma.

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

Metabolite groups	Non-galled organs			Galls			
	Stem	Leaflets		Stem	Leaflets		
		Young	Mature		1 st instar	2 nd instar	3 rd instar
Primary metabolites							
Starch	-	-	-	-	-	+	+
Proteins	+	+	+	+	+	+	+
Lipids	+	-	-	+	+	+	+
Reducing sugars	+	+	+	+	+	+	+
Pectins	-	+	+	+	+	+	+
Auxin	+	+	+	+	+	+	+
Secondary metabolites							
Alkaloids	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	-
Phenolics	+	-	+	+	-	+	+
<i>Lignins</i>	+	-	+	+	+	+	+
<i>Flavonoids</i>	+	+	+	+	+	+	+
Others							
ROS	+	+	+	+	+	+	+

+ = positive; - = negative

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

Figure 2. Anatomical features of non-galled and galled tissues of stem and leaflets of *Matayba guianensis* induced by *Bystracoccus mataybae*. A- Non-galled stem with details of the periderm in inset photo. Stem in secondary growth; B- Gall with first instar in the stem; C- Young non-galled leaflets with many secretory cavities; D- Mature non-galled leaflets; E- Gall with second instar in the leaflets. Vascular bundles in differentiation; F- Gall with third instar in the leaflets, showing the first and second nymphal chambers. **Abbreviations:** AF- Adult female with eggs; CX- cortex; Ep- Epidermis; FB- Fibers; HP- Hypodermis; NC- Nymphal chamber; 1st NC- First nymphal chamber; 2nd NC- Second nymphal chamber; PC- Parenchymatous cells; CC- Cortical cells; SC- Sclereids; PE- Periderm; P- Phellem; PG- Phellogen; PD- Phelloderm; PP- Palisade parenchyma; SC- Secretory cavities; SMA- Sclerenchyma; SP- Spongy parenchyma; VB- Vascular bundle; XY- Xylem.

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