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BIOLOGICAL SCIENCES

Pseudophacopteron longicaudatum (Hemiptera) induces intralaminar leaf galls on *Aspidosperma tomentosum* (Apocynaceae): a qualitative and quantitative structural overview

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Abstract: The structural complexity of galls depends on species-specific interaction driven by the galling *taxa*. However, the host plant and environment stressors can impose limits on gall developmental patterns and impact the establishment of gall morphology. Herein, we employed qualitative and quantitative approaches in order to elucidate how cell divisions, elongation patterns, and tissue organization are determinant for the development of intralaminar gall morphology induced by *Pseudophacopteron longicaudatum* Malenovský, Burckhardt, Queiroz, Isaias & Oliveira (Hemiptera: Psylloidea: Phacopteronidae) on leaves of *Aspidosperma tomentosum* Mart. (Apocynaceae). In addition, we aimed to determine which anatomical process can discriminate the stages of gall development, plus, examine the histochemical and cytological profiles of the galls. The differentiated structures, mainly abaxial epidermis and spongy parenchyma, are associated with gall closure, with hyperplastic events concentrated in the young phase of the galls. Thus, epidermis and spongy parenchyma hypertrophy and are responsible for the determination of the nymphal chamber formation and gall shape. The mature galls do not differentiate into a typical nutritive cells and do not develop a histochemical gradient in their tissues. The cytological features of galls such as plastoglobules and multivesicular bodies are related to ROS scavenging mechanisms due the high oxidative stress.

Key words: Galls, histochemical, cell elongation, perMANOVA.

INTRODUCTION

During the evolution of plant-insect interaction, an extremely specialist guild of insects arose and became able of manipulating host plant tissues to develop a new organ, the gall (Mani 1964, Giron et al. 2016, Oliveira et al. 2016). This new organ convers to the galling insects a marked adaptive advantage in relation to their free-living ancestors (Stone & Schönrogge 2003), providing an abundance of nutrients (Diamond et al. 2008), and protecting them against environmental stresses and natural enemies (Fernandes & Price 1992). The gall develops through continuous galling stimuli triggered by its oviposition and feeding behavior (Bronner 1992, Giron et al. 2013, Hori 1992). Thus, the biotic oxidative stress induced by the galling insect triggers a series of cellular responses which leads to changes in host plant morphogenesis and, consequently, gall development (Carneiro et al. 2014a, Magalhães et al. 2014). Herein, we describe some structural and histochemical steps of galls induced by *Pseudophacopteron*

longicaudatum (Hemiptera) on leaves of *Aspidosperma tomentosum* (Apocynaceae) in all pre-established stages of development.

In the neotropical region, different studies have discussed the morphological features of galls induced by different galling guilds, indicating that the structural complexity of the gall depends on species-specific interaction driven by the galling *taxa* (Ferreira & Isaias 2014, Isaias et al. 2011). However, galling insects from distinct *taxa* and with distinct feeding behaviors induce the development of similar gall shapes, while galling insects with similar feeding behavior can develop different gall shapes (Carneiro et al. 2014a). The limits imposed by the host plant tissue and environmental stressors can lead to different patterns of cell division and elongation during gall development, thus determining the morphology of galls (Magalhães et al. 2014, Oliveira & Isaias 2010a). We employed here qualitative and quantitative approaches in order to elucidate how cell divisions, elongation patterns and tissue organization are determinant for the development of intralaminar gall morphology. In addition, we aimed to determine the anatomical features that can discriminate the stages of gall development.

The histochemical profiles of galls can be associated with structural changes and tissue compartmentalization, especially the formation of an outer cortex (storage tissue), a mechanical layer, and an inner cortex (nutritive tissue) (Bragança et al. 2016, Ferreira et al. 2016, Isaias et al. 2015, 2018, Guedes et al. 2018). Sugars, lipids and proteins have been detected in some phloem-sucking gall systems, even when the galls contain no typical nutritive tissue (Ferreira et al. 2016). However, hemipteran gall systems show distinct histochemical profiles. *Euphalerus ostreoides* Crawf. - *Lonchocarpus muehlbergianus* Hassl. (Isaias et al. 2011, Oliveira et al. 2006) and *Psidium myrtoides* O. Berg –*Nothotrioza myrtoidis* Burckhardt. (Carneiro et al. 2014b) systems have a bivalve shape with low metabolism for starch, retrieving energy from reduced sugars. *Pseudophacopteron aspidospermi* Mart. - *Aspidosperma australe* Müll. Arg. (Oliveira & Isaias 2010b) and Cecidomyiidae – *Aspidosperma spruceanum* Mart. & Zucc. (Oliveira et al. 2010) accumulate starch and seem to consume it throughout gall development. Our current objective was to determine whether galls induced by *P. longicaudatum* on leaves of *A. tomentosum* fit the pre-established patterns found in galls induced by other phloem-sucking insects. In addition, we wanted to determine constraits imposed by the host plant on the developmental structure and histochemical profile of the gall and to discuss the histochemical and cytological profiles of galls induced on leaves of *Aspidosperma* genus by *P. longicaudatumon* and other gallers.

MATERIALS AND METHODS

Sampling and study area

Non-galled leaves and intralaminar leaf galls (Isaias et al. 2013) induced by *P. longicaudatum* on *A. tomentosum* were sampled in an area of Cerrado *sensu strictu* vegetation, at "Clube Caça e Pesca Itororó de Uberlândia" (CCPIU), west of Uberlândia municipality, MG (18°60'S - 48°W), Brazil. The galls (n = 70) were sampled in three different stages of development based on size and color: young galls (first white green callus induced on young leaves), mature galls (larger and dark green protuberance, sampled on mature leaves), and senescent galls (opened galls). Non-galled leaves (n = 70) were randomly sampled in three distinct stages of development based on nodal position, size, color and hardness: leaf primordium (shortly after leaf sprouting), young leaf (light-green with a membranous aspect), and mature leaf (fully expanded with a coriaceous aspect).

Histological measurements

Samples were fixed in glutaraldehyde (2.5% in 0.1M phosphate buffer , pH 7.2 (Roland & Vian 1991), dehydrated in an ethanol series (50 to 95 %), embedded in Historesin® (Leica ®), sectioned with a rotary microtome (YD315, ANCAP, Brazil) with 6-10 µm, stained with 1% toluidine blue, pH 4.7 (Kraus & Arduin 1997), and mounted in Vitral® varnish (Paiva et al. 2006). Cytometric and histometric measures were performed in transverse sections of non-galled leaves (n = 5) and galls (n = 5). Four measurements were made in fifteen cells of the adaxial epidermis, abaxial epidermis, spongy parenchyma, and palisade parenchyma per sample in all previously determined different stages using ImageJ® software. The following parameters were recorded: cell width, length, cellular area and circumference. We measured the diameter of the vascular bundles when possible. We extracted a total of 17 variables from the measurements.

Statistical analyses

To check if the galling insect induces changes in the axes of cell anisotropy, the histometric variables were submitted to exploratory analysis. Multivariate normality, linearity and homoscedasticity were tested, and a principal coordinate analysis (PCoA) (Zuur et al. 2007) was performed. All relevant co-variables without a collinear effect were selected using the Variance Inflation Factor (VIF) < 3 (Craney & Surles 2002), and a subsequent permutational multivariate analysis of variance was carried out using the Vegan package in R studio (Team 2015), based on the Euclidian similarity matrix. P values were obtained with 999 permutations of residuals under a reduced model. To check differences between leaves and gall tissues, the RandomForest algorithm (Liaw & Wiener 2002) and the Classification Tree (Hothorn et al. 2006) were applied in order to rank the most changeable tissue to create each cluster.

Electron transmission microscopy

Samples were fixed in Karnovsky solution 4%, 0.1 M, pH 7.2, for 24 hours (Morris 1965). The samples were then post-fixed in 1% osmium tetroxide in a 0.1 M phosphate buffer solution, pH 7.2, dehydrated through an ethanol series (O'Brien & McCully 1981), and gradually embedded in Spurr® resin. The samples were sectioned with a diamond knife on a Reichert-Jung Ultracut ultramicrotome (Leica, Wetzlar, Germany), fixed in nets, contrasted with uranyl acetate and lead citrate (Reynolds 1963), and analyzed with a transmission electron microscope (Jeol, JEM-2100) equipped with EDS (energy dispersive x-ray detector) at Laboratório de Microscopia de Alta Resolução (LabMic), Universidade Federal de Goiás, Brazil.

Histochemical analyses

Free hand sections of fresh galls and non-galled tissues in pre-established developmental stages (n=10) were obtained with razor blades, and submitted to standard histochemical tests for starch, reducing sugars, protein, lipids and reactive oxygen species (ROS) within a two-hour window, according to the methods of the authors listed in Table I. We mounted the slides in water and used non-stained tissues as control. The samples were analyzed with a Leica® ICC50HD digital camera coupled to a Leica® DM500 (USA) light microscope. The most congruent distributions, when comparing all images, were selected as representative models.

RESULTS

Gall development

Pseudophacopteron longicaudatum induced intralaminar galls on leaves of *A. tomentosum*

Table I. Histochemical assays applied to *Aspidosperma tomentosum* galls induced by *Pseudophacopteron longicaudatum* and to non-galled leaves.

Test/Reagent substance	Procedure	Reference
Lugol – Starch	Immersion of samples into 1% potassium iodide for 5 minutes	Johansen (1940)
Fehling Reagent – Reducing sugar	Immersion of the samples in equal parts of "A" (6.93% cupric sulfate II) and "B" (34.6% sodium and potassium tartrate and 12% sodium hydroxide) heated to pre-boiling	Sass (1951)
Blue bromophenol mercury – Proteins	Immersion of samples in 0.1% bromophenol blue in a saturated solution of magnesium chloride in 70% alcohol for 15 minutes	Mazia et al. (1953)
Sudan III Reagent – Lipid.	Immersion of samples in a saturated solution of Sudan III in 70% alcohol for 5 minutes	Sass (1951)
DAB – Reactive oxygen species (ROS)	Immersion of samples into 3,3 'diaminobenzidine for 20 minutes in the dark under refrigeration. Quick wash and mount in distilled water	Rossetti & Bonnatti (2001)

(Fig. 1a, b and c). Both galling insect oviposition and gall induction occurred on the abaxial surface of young leaves (Fig. 1d and f). The mature galls are green to dark-green coloration, with a rounded projection for both leaf surfaces, hairy on the abaxial surface (Fig. 1e and f, respectively). These galls had just one galling insect per nymphal chamber, and no parasitoids were found. The senescent stage of the gall started with the galling insect leaving the gall structure, although the cells maintained their size, shape and previous function.

First-instar nymphs of galling insects induced tissue hyperplasia in all tissues. Compared to non-galled leaf tissues (Fig. 2a and b), the gall cells lose shape and hypertrophy especially in the spongy parenchyma of the host (Fig. 2b and c). The abaxial epidermal cells decreased in size by anticlinal division, increasing the numbers of ordinary cells and trichomes (Fig. 2d, arrow), followed by width expansion after the complete nymphal chamber formation in the mature stage (Fig. 2e). In contrast, although the area of palisade parenchyma cells was increased after gall induction and formation, they had a variable number of layers (one to three) (Fig. 2f arrow). The vascular bundles and inner parenchymal cells of the galls kept dividing, and increased 54% and 10% in size, respectively, in maturation (Fig. 2g). Moreover, the spongy parenchyma changed the cell elongation patterns and expanded to develop the gall cortex. In the mature gall cortex, hypertrophied cells had a cylindrical shape, which took part in gall closure and in the consequent formation of the nymphal chamber (Fig. 2c). The adaxial epidermis did not divide as the abaxial one.

Histometric analysis

Cells and tissues significantly changed in area and elongation during gall development (Fig. 3). The non-galled adaxial epidermis (NAdE) increased in size during leaf development (Fig. 3a). During gall development, the adaxial

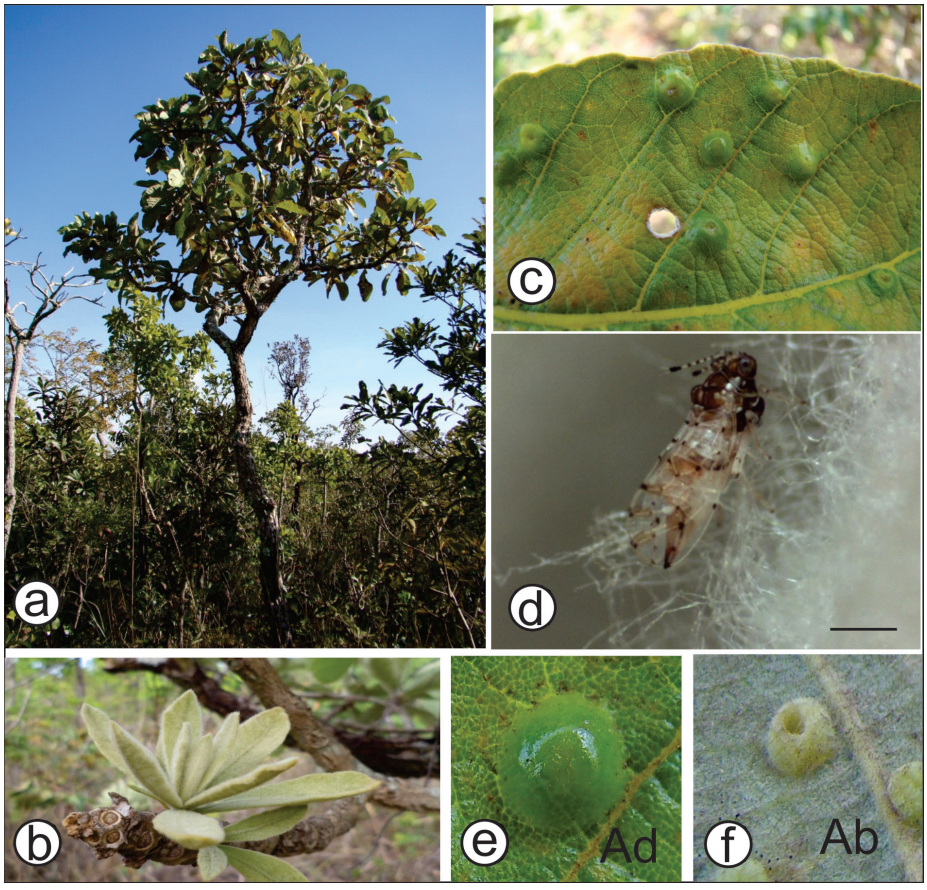

Figure 1. ***Aspidosperma tomentosum*** **(Apocynaceae) leaves and galls induced by** ***Pseudophacopteron longicaudatum*****.** **(a)** ***Aspidosperma tomentosum*** **in a natural Cerrado area. (b) Young leaves. (c) Intralaminar galls on a mature leaf with green color. (d)** ***Pseudophacopteron longicaudatum*** **on young leaves. (e) Adaxial surface of the gall (Ad). (f) Abaxial surface of the gall (Ab).**

epidermal cells slightly hypertrophied and divided with fluctuating size values after gall induction, although, overall, it kept the same size (Fig. 3b). When the stages of leaf development were compared, the cell area of the palisade parenchyma (NPP) continued to increase, from 97 to 160 μm^2 (Fig. 3c), dividing into a periclinal axis forming two layers. In contrast, although the gall palisade parenchyma (GPP) cells were larger than non-galled leaf cells, they decreased in area from 267 μm^2 when young to 200 μm^2 when mature, with a subsequent increase of 236 μm^2 when senescent (Fig. 3d). Young gall cells expanded from the non-galled leaf spongy parenchyma (NSP) and were significantly larger than the cells of the young non-galled leaves (353 and 53 μm^2, respectively) (Fig. 3e, f). Gall cortical parenchyma (GP) cells expanded from 223 to 682 μm^2 during development (Fig. 3f). The area of the non-galled abaxial epidermal (NAE) cells triplicated from 36 μm^2 at the young gall stage to 114 μm^2 at the mature stage (Fig. 3g). Compared to the non-galled tissues, the gall abaxial epidermis (GAE) cells were larger, increased in size through maturation, and decreased after senescence (Fig. 3h).

Discriminatory analysis

Exploratory analysis suggested collinearity in 9 of the 17 variables obtained from histometric measurements, with a Variance Inflation Factor > 3. Eight variables: cell length and width of the abaxial epidermis, adaxial epidermis, spongy parenchyma and palisade parenchyma were further tested for linearity and homoscedasticity, and used for principal

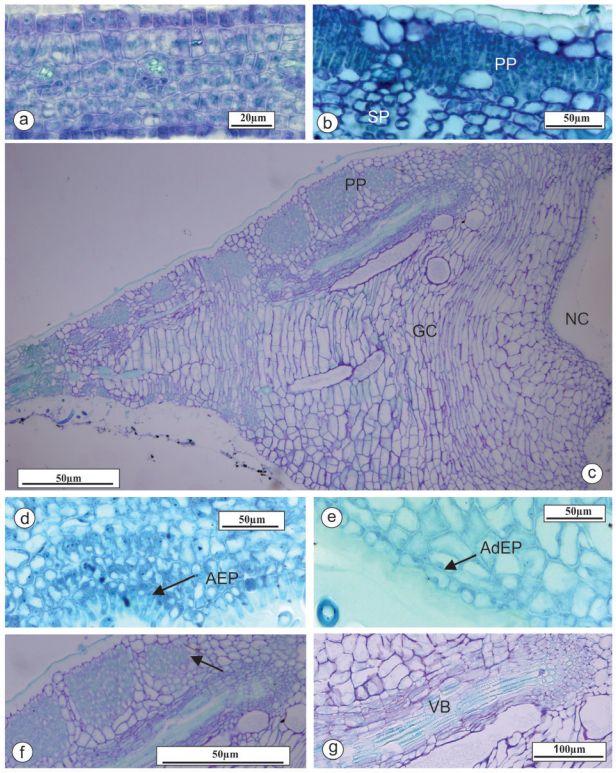

Figure 2. Gall structure of *Pseudophacopteron longicaudatum* induced on *Aspidosperma tomentosum* leaves. (a) Young leaf tissues. (b) Mature leaf tissue. (c) Mature gall. (d) Abaxial epidermis with trichomes in young galls. (e) Expanded cells of the abaxial epidermis following gall closure in a mature gall. (f) Mature gall with palisade parenchyma (arrows). (g) Hypertrophied vascular bundles. PP – Palisade parenchyma. SP – Spongy parenchyma. GC – Gall cortex. NC – Nymphal chamber. AEP – Abaxial epidermis. AdEP – Adaxial epidermis. VB – Vascular bundles.

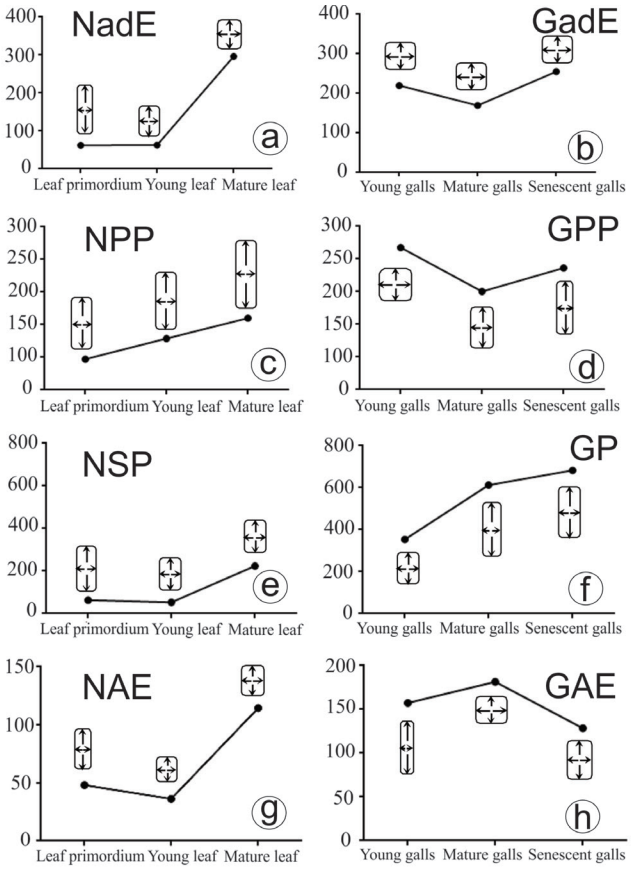

Figure 3. Cellular growth and anisotropy of *Pseudophacopteron longicaudatum*- induced galls in *Aspidosperma tomentosum* tissues in all stages of development. (a) Non-galled adaxial epidermis (NAdE). (b) Gall adaxial epidermis (GadE). (c) Non-galled palisade parenchyma (NPP). (d) Gall palisade parenchyma (GPP). (e) Non-galled spongy parenchyma (NSP). (f) Gall parenchyma (GP). (g) Non-galled abaxial epidermis (NAE). (h) Gall abaxial epidermis (GAE).

coordinates analysis (PCoA) and Permanova. Clustering using principal coordinates analysis (PCoA) showed a marked shift between gall and leaf development (Fig. 4). Thus, in Permanova with One-Way MANOVA the Euclidean test was significant ($F_{(5.234)}$ = 60.8 p<0.001), with gall and non-galled leaf tissue forming separate clusters, showing a drastic change in cell anisotropy. The developmental feature changes in each tissue differed between gall and leaf, and the Random Forest and Classification tree was used to create a diagram (Fig. 5). Young galls, despite the changes in all tissues, did not yet show a strong hypertrophy of the spongy parenchyma, or a reduction of palisade cells, forming a group apart from mature and senescent gall. Later, the formation and complete expansion of gall cortex in mature galls rendered a new cluster, with senescent galls and mature galls, which differed between them only by the remaining palisade parenchyma after the start of tissue deterioration on senescence.

Cytological analysis

Non-galled tissues showed typical palisade and spongy parenchyma with developed chloroplasts (Fig. 6a). Palisade parenchyma cells were rich in chloroplasts (Fig. 6b) and spongy parenchyma cells had large vacuoles and few chloroplasts organized in the peripheral region (Fig. 6c). Multivesicular bodies, as well as small vesicles were detected, especially in the spongy parenchyma (Fig. 6d).

The young gall cortex had sinuous and thin cell walls due to hypertrophy of the spongy parenchyma (Fig. 7a). Furthermore, the number of chloroplasts apparently decreased, and many plastoglobules and starch grains were present in mature galls (Fig. 7d). Mitochondria and multivesicular bodies were common (Fig. 7b), and multivesicular bodies showed different

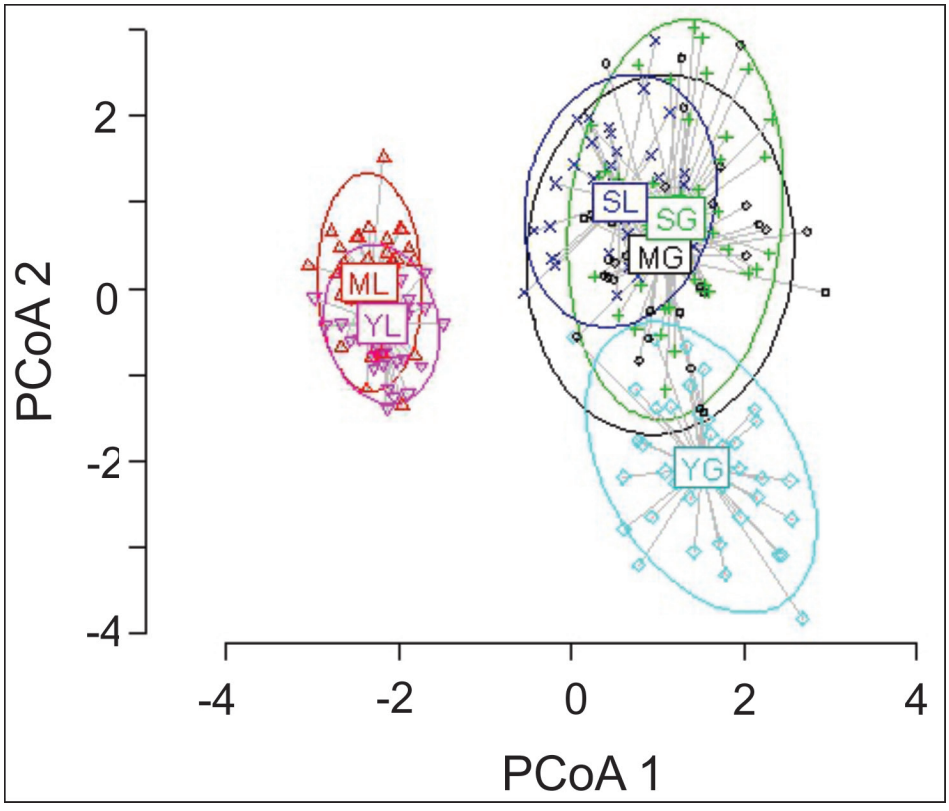

Figure 4. Ordination samples with principal coordinates analysis (PCoA) obtained by beta dispersion. Centroids of tissue combinations showing separation in leaf and gall structure assemblages. (MG) Mature gall. (ML) Mature leaf. (SG) Senescent gall. (SL) Senescent leaf. (YG) Young gall. (YL) Young Leaf.

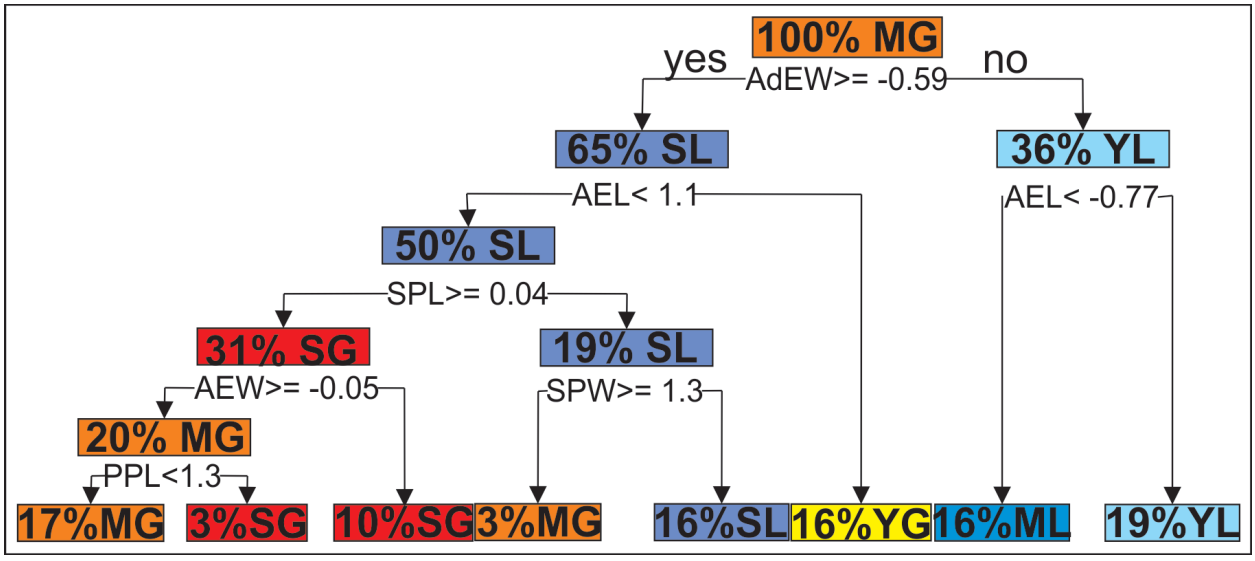

Figure 5. Development of a *Pseudophacopteron longicaudatum* gall in *Aspidosperma tomentosum* leaves determined by histometric measurements in Permanova. (MG) Mature gall. (ML) Mature leaf. (SG) Senescent gall. (SL) Senescent leaf. (YG) Young gall. (YL) Young leaf. (PPL) Palisade parenchyma length. (AEW) Abaxial epidermis width. (AdEW) Adaxial epidermis width. (SPL) Lacunose parenchyma length. (SPW) Spongy parenchyma width. (AEL) Abaxial epidermis length.

sizes and shapes (Fig. 7b, c). Both the adaxial and abaxial cortex lost intercellular spaces, and the cell membrane system appeared to be deteriorated (Fig. 7d).

Histochemical results

The patterns of distribution of the primary compounds of leaves and gall tissues were slightly different (Table II). The 3,3 'diaminobenzidine reacted with hydrogen peroxide in all cell walls and in the protoplast of epidermal cells in non-galled (Fig. 8a) and galled tissues (Fig. 9a). In galls, this positive reaction occurred especially in the epidermal cells around the nymphal chamber, with cells being more densely organized in the adaxial cortex (Fig. 9a). Starch was detected in cells adjacent to the midrib vein of non-galled tissues (Fig. 8b), and around the vascular bundles of young and mature galls. Starch grains were mostly present in the gall outer cortex and spread close to the nymphal chamber during the developmental processes (Fig. 9b). Reducing sugars were detected in the palisade parenchyma of non-galled tissues and in young and mature galls and were especially associated with chlorophyll tissues on the adaxial side (Fig. 9c). Proteins were distributed in cells of the mesophyll and vascular bundles of non-galled tissue (Fig. 8d). In galls, proteins were detected in the vascular bundles and chlorophyll tissues during the first stages of development, and only in the vascular bundles at senescence (Fig. 9d). Lipids were detected in the palisade parenchyma, lactiferous channels and cuticle of non-galled tissue (Fig. 8c). In galls, they were abundant in the gall cortex after the mature stage near the feeding site of the galling insect and also within the chlorophyll tissues (Fig. 9e), and during senescence, they were restricted to chlorophyll tissue (Fig. 9f).

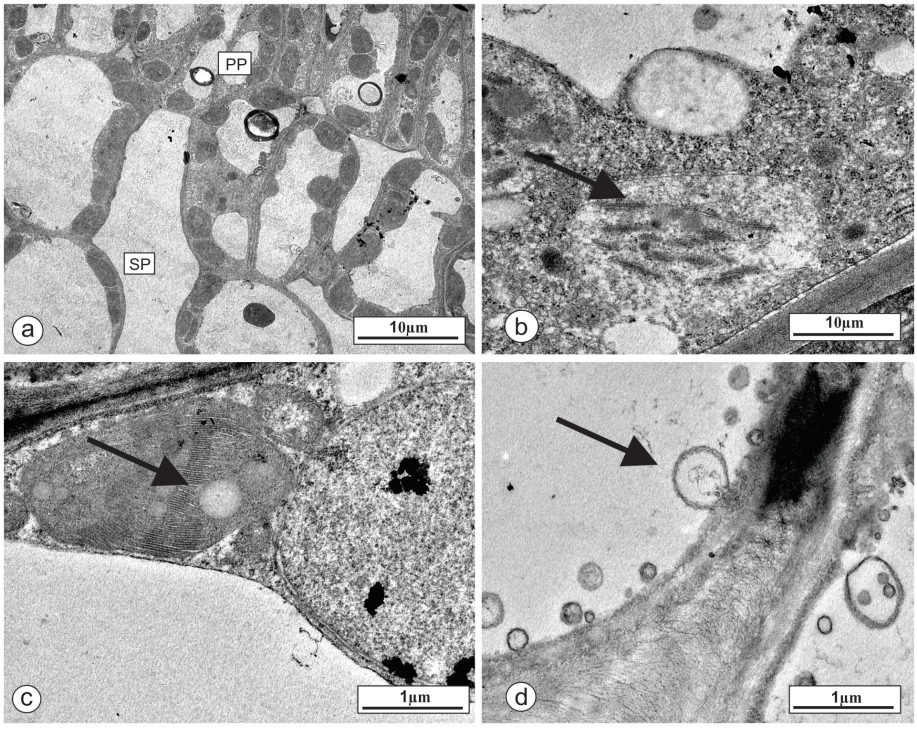

Figure 6. Electromyography transmissions from *Aspidosperma tomentosum* leaves (a) Palisade (PP) and spongy (SP) Parenchyma. (b) Developing membrane system chloroplast in young tissue (arrow). lysosome (L). (c) Chloroplast with some plastoglobules in a mature leaf (arrow). (d) Multivesicular corpuscle in a mature leaf (arrow). PP – Palisade parenchyma. SP – Spongy parenchyma.

DISCUSSION

Pseudophacopteron longicaudatum manipulates the host plant tissues, changing the patterns of cell division and elongation to develop the intralaminar leaf gall on *A. tomentosum*, as is also the case for other phloem-sucking insects on leaves of *Aspidosperma australe* (Oliveira & Isaias 2010b). The present histometric and cytometric analysis of the galls showed continuous cell growth and the RandomForest plus classification revealed patterns of cell hypertrophy and elongation associated with each developmental gall phase. Despite the differential distribution of lipids, carbohydrates and proteins in galls, there was no formation of histochemical gradients as well as found in galls induced by galling insects on leaves of *A. australe* (Oliveira & Isaias 2010b). Furthermore, gall tissues showed many cytological symptoms of oxidative stress, especially near the feeding sites. Proteins and lipids were detected in cells around the vascular bundles, a common occurrence in the intrinsic metabolism of the host plant (Demarco et al. 2006). However, during senescence, the galls showed positive reactions for proteins in phloem cells, indicating a strong sink generated by galling (Oliveira et al. 2017). The external cortex with hypertrophic cells has a low metabolic demand, and therefore accumulates starch grains. In addition, the gall tissues does not re-differentiate into a true nutritive tissue, or nutritive-like tissue as shown in other galls (Ferreira et al. 2016, Guedes et al. 2018). On the other hand, the histochemical pattern maintained most of the host plant cells primary distribution.

Anatomical features can discriminate gall development stages and cell functionalities

Galls induced by *P. longicaudatum* on leaves of *A. tomentosum* develop by intense cell division and elongation of all tissue systems compared to the non-galled leaves. These processes have been extensively described during gall

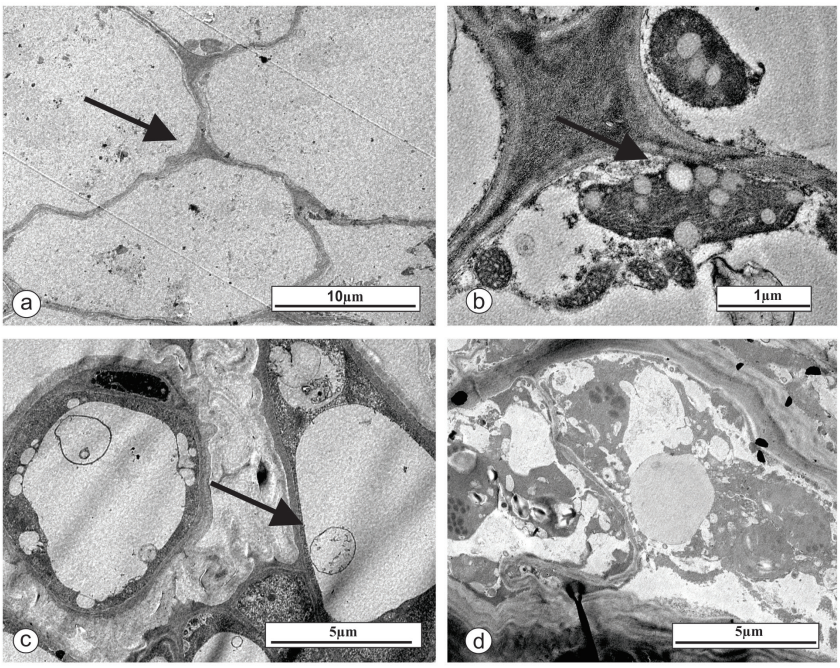

Figure 7. Electromyography transmissions from a *Pseudophacopteron longicaudatum*- induced gall in *Aspidosperma tomentosum.* (a) Spongy parenchyma differentiated into young gall parenchyma, with a thin and sinuous cell wall (arrow). (b) Chloroplasts with many plastoglobules in a mature gall (arrow) and associated mitochondria . (c) Multivesicular corpuscle in a mature gall (arrow). (d) Mature gall cell with fragmented protoplasm.

Table II. Summary of the histochemical results concerning *Pseudophacopteron longicaudatum* galls and *Aspidosperma tomentosum* leaves.

Detected substances	Organ	
	Leaf	Gall
Reactive oxygen species (ROS)	Cell wall; epidermis.	Cell wall; nymphal chamber epidermis in all stages. (Fig. 8a)
Starch	Midrib bundle internal cortex	Accumulated close to new vascular bundles in young galls (Fig 8b).
Reducing sugars	Palisade parenchyma	Chlorophyll tissue (Fig. 8c).
Proteins	Vascular bundle; palisade and spongy parenchyma	Within the chlorophyll tissue and vascular bundle. During senescence there is only protein in the vascular bundle. (Fig. 8d).
Lipid	palisade parenchyma; latex duct; cuticle	Cuticle. Spread in gall cortex (Fig. 8e) restricted to chlorophyll tissue at senescence (Fig. 8f)

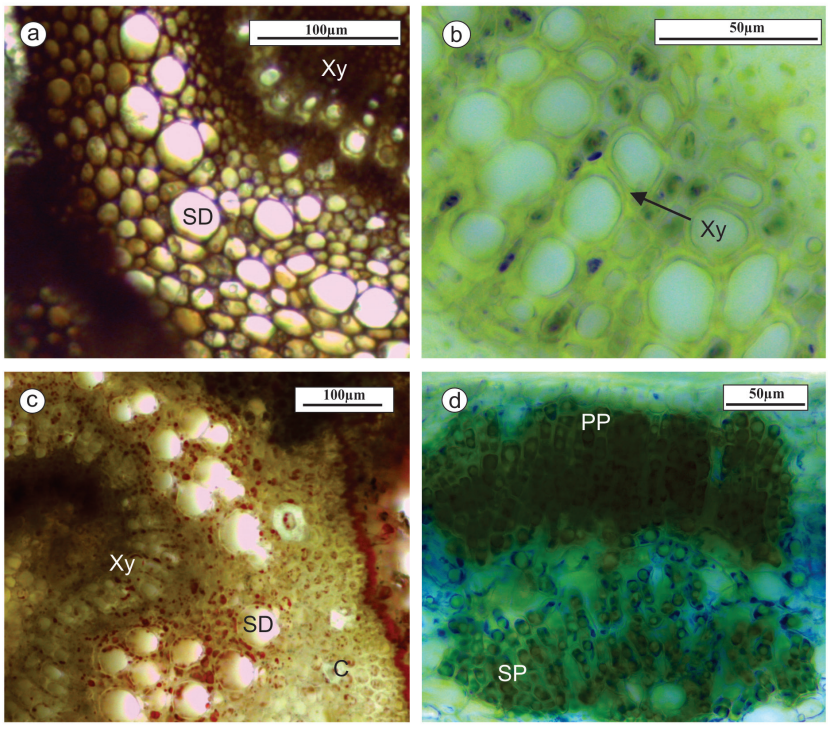

Figure 8. Photomicrographs of histochemical assays of *Aspidosperma tomentosum* leaves. (a) Reactive oxygen species (ROS). (b) Starch close to xylem (arrow). (c) Lipid in the cuticle and spread in the midrib. (d) Proteins distributed in leaf limbo. PP – Palisade parenchyma. Xy – Xylem. SD – Secretory Duct. C – Midrib cortex.

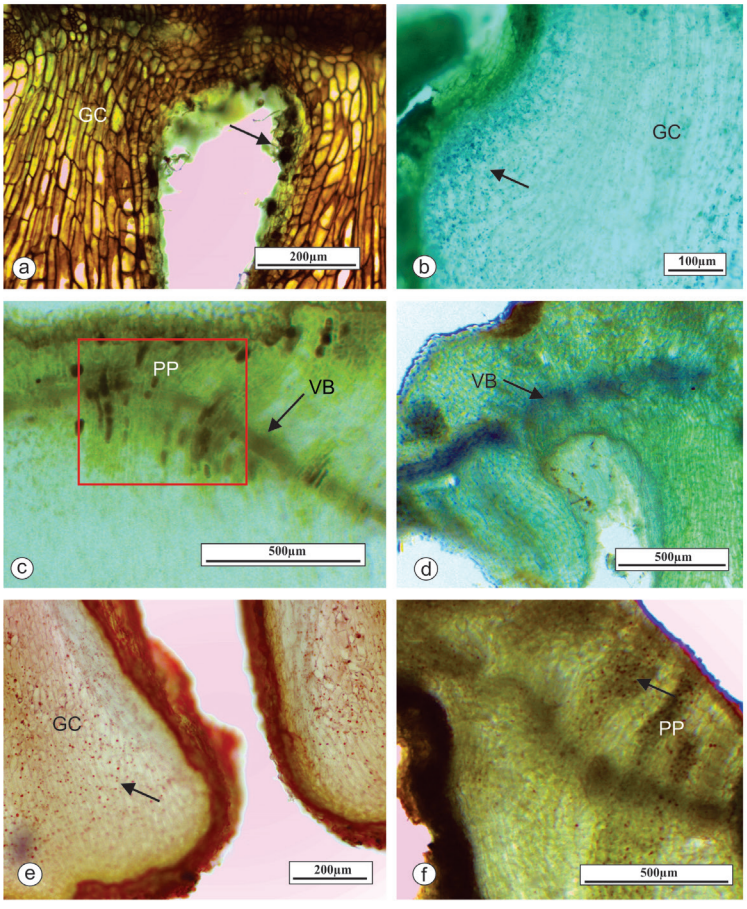

Figure 9. Photomicrographs of histochemical assays of *Aspidosperma tomentosum* galls induced by *Pseudophacopteron longicaudatum.* (a) Reactive oxygen species (ROS) in mature gall epidermis (arrow) and cell walls. (b) Starch close to the nymphal chamber (arrow). (c) Reducing sugars in a mature gall within chlorophyll tissue and close to vascular bundles (arrow). (d) Proteins restricted to vascular bundles in a senescent gall. (e) Lipid in the cuticle and spread in the gall cortex. (f) Lipids restricted to chlorophyll tissue in a senescent gall. PP – Palisade parenchyma. GC – Gall cortex. VB – Vascular bundles.

development induced by insects (Mani 1964, Moura et al. 2008, 2009, Oliveira & Isaias 2010a, Carneiro et al. 2014a), and can determine the gall morphology. In addition to notable hypertrophy in galls induced by *P. longicaudatum* on *A. tomentosum*, there are different patterns of cell elongation up to the formation of mature galls. As in other galls, the hyperplastic events are concentrated in the young phase (Carneiro et al. 2014a, Guedes et al. 2018), with full expansion occurring before senescence.

In *P. longicaudatum – A. tomentosum* system, during gall induction, there was a ubiquitous hypertrophy in all tissues, and leaf tissues are transformed into gall cortex. Although every cell grew, the abaxial epidermis and palisade parenchyma were the most altered tissues in the young galls. The classical palisade parenchyma shape showed increased cell width and became rounded; this tissue layer was lost and mixed with the spongy parenchyma in the new gall cortex. The growing gall cortex at the young gall, formed mainly by the spongy parenchyma cells, caused the gall to protrude towards the abaxial side in order to close the nymphal chamber. Thus, in order to maintain the continuous epidermis trough the gall, the abaxial epidermal cells strongly increased in numbers, first causing an increase in cell length. With the gall protruding from the leaf lamina during maturation, the abaxial epidermal cells increased in width stretching the epidermis. The hypertrophy continued and intensified during the gall maturation stage, the spongy parenchyma, which divides and forms the gall cortex, started anticlinal elongations, the gall cortex cells increased in length and became cylindric. These two events together form the nymphal chamber. Once the gall reached its maximal size in the mature stage, most of its anatomical features are maintained until the senescent stage, although some palisade cells increased in length throughout the senescence processes due to membrane loosening. A similar process may occur with senescent leaves, which clustered with the mature gall. These results indicate a rush of the galler to close the gall in order to offer an isolated and protected environment for nymph development.

The gall development can reduce the intercellular spaces in gall tissues creating a barrier to gas diffusion (Oliveira et al. 2017, Pincebourde & Casas 2016) but, in contrast, may function as an adaptive feature in order to avoid water loss, as proposed for the *Olea europaea* L. system (Kraus 2009). Water is essential to maintain turgor pressure in the vacuoles and consequently lead to cell growth (Peaucelle et al. 2012), being important during gall formation. Also, to minimize the consequences of low gas diffusion, some galls maintain photosynthetic activity, which plays an important role in hypoxia and hypercarbia avoidance (Oliveira et al. 2017, Pincebourde & Casas 2016). Moreover, keeping the photosynthetic apparatus unchanged can also be advantageous for sucking insects since galls may use the photoassimilates produced for their diet (Oliveira et al. 2017). The midrib vein in non-galled leaves shows bicollateral bundles (Rio et al. 2005) that are maintained in the gall, but with enhanced cell elongation. In comparison, an increase of vascular bundles, especially phloem cells, has been detected in galls induced by *Pemphigus betae* (Hemiptera) on *Populus angustifolia* and was associated with galling feeding (Richardson et al. 2017). Thus, the large demand for photoassimilates would justify the increase in conducting cells and ensure gall survival.

Cytological and histochemical symptoms of oxidative stress in galls

Hydrogen peroxide was detected in all gall tissues especially around the nymphal chamber. This accumulation has been described as a trigger to

gall establishment and participated in the changes of the cytological patterns (Oliveira et al. 2016). The increase in the formation of plastoglobules and multivesicular corpuscles observed here was associated with ROS scavenging (An et al. 2006, Austin et al. 2006), and is commonly found in different galls induced by Cecidomyiidae in *Aspidosperma spruceanum* (Oliveira et al. 2010), *Tibouchina pulchra* and *Marcetia taxiflora* (Ferreira et al. 2015, Vecchi et al. 2013), and in galls induced by Psyllidae on *Aspidosperma australe* (Oliveira & Isaias 2010b). These structures protect photosystems from ROS damage and consequently help to maintain the photosynthetic activity (Oliveira et al. 2011, 2017). Besides that, close to the feeding site, at the nymphal chamber, the chloroplasts shrink, and their membrane system is poorly developed. In addition, a fine balance among phenolic compounds, growth factors and ROS has been discussed as being essential for gall establishment (Bedetti et al. 2013, Oliveira et al. 2016) and for the maintenance of redox homeostasis (Isaias et al. 2015). These ROS molecules found especially in cell walls could interact with the pectin matrix and release the associated Ca^{2+}, causing relaxation and possibly tissue hypertrophy (Olson & Varner 1993, Bell et al. 2009, Braidwood et al. 2014, Oliveira et al. 2014, Teixeira et al. 2018). Moreover, although the response to ROS production are host dependent, the increase in ROS scavenging pathways could help the gall to avoid the hypersensitivity response and programmed cell death (Ferreira et al. 2018).

CONCLUSION

The present analysis showed clear qualitative and quantitative structural profiles in galled tissues and revealed how the galling insects manipulate the host plant tissues to build the gall. The differentiated structures are all mainly associated with the formation of nymphal chamber in mature galls. The hyperplasic events are concentrated in the young phase, reducing the intercellular spaces in the spongy parenchyma and compressing cells in the abaxial epidermis. With the increased number of cells in the gall cortex, all cells expanded in the mature stage, forming cylindric cells in the gall cortex, and wide epidermal cells. In addition, in the senescent stage, the gall cortex and epidermis maintained their size and the vascular bundles kept hypertrophying. revealing the presence of directional tissue manipulation.

Moreover, all structural changes and the galling insect's activity strongly affected the oxidative imbalance, triggering mechanisms that involve plastoglobules, multivesicular bodies or other ROS scavenging mechanisms in order to reach oxidative equilibrium. Considering that ROS are used by plants as active immune responses (Dodds & Rathjen 2010), it seems that galling organisms are able to overcome this defense. Therefore, we did not find any evidence indicating any host constraint against gall development. Although the partial maintenance of the host structures and histochemistry were related to constraints (Guedes et al. 2018), we here advocated that as all plant tissues were altered, and no necrosis or symptoms of hypersensitive reaction response was noted, a indicative that the insect has overcome any plant resistance.

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