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Responses of the Host Plant Tissues to Gall Induction in *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae)

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ABSTRACT

The ontogenetic characterization of the leaf galls induced in the internervural region and in the second and third order veins of A. spruceanum Müell Arg. (*Apocynaceae*) *aims to evaluate the distinct levels of cell reaction during the process of gall formation, and the relation between external gall morphology and the oviposition sites. The ground system had the most remarkable alterations, namely, the non differentiation of palisade parenchyma in both leaf sides, the hyperplasia of the spongy parenchyma and the neoformation of fibersclereids, a cell type not observed in non galled leaves. Changes of the feeding sites inside the larval chamber reveal distinct levels of cell competence to respond to the insects stimuli and explain the variations in the shape of the larval chamber.*

***Keywords*:** *Aspidosperma*, *Cell Competence*, *Gall Development*, *Leaf Anatomy*

1. Introduction

The size and shape of galls are determined by the mechanical injury, the salivary secretions, and the feeding activity of their related galling insects [1]. Consequently the morphology of a specific gall may be considered as an extended phenotype of its specific inducing insect [2,3], which must choose an adequate site of oviposition to guarantee the survival of its offspring. The eggs of the insects, as well as the secretions deposited with them at the site of oviposition, trigger gall induction. The exact location and number of eggs laid on the surface or inside the tissues of the host plant influence the final structure and size of the gall [3,4].

One of the most specialized groups of gall inducing insects is the Cecidomyiidae, whose galls complexity involves metabolical [5] and developmental patterns [6,7]. Processes of hyperplasia, hypertrophy, dedifferentiation or cell lysis [8] lead to the redifferentiation of specialized cells *sensu* Lev-Yadun [9] in the dermal, ground, and vascular systems. Such processes can occur in any plant cell that retains its nucleus at maturity [10] and are more intense the nearer the stimuli are. The number of cell divisions is intensely increased in the epidermis so as to accompany the development of the structure. The parenchyma adjacent to the larval chamber can be differentiated into a nutritive tissue that will nurture the insect [11]. New vascular bundles may differentiate and connect to the bundles of the host organ, forming a network for the translocation of substances. These bundles are directed to the larval chamber, and according to Mani [6] and Meyer and Maresquelle [7], commonly end up as sieve elements. The parenchyma adjacent to the vascular bundles is hyperplasic, with hypertrophied cells which may accumulate secondary metabolites, such as phenolic derivatives, which can be related with the occurrence of oxidative stress.

The *Aspidosperma spruceanum* Müell Arg. (Apocynaceae)-Cecidomyiidae system has been addressed in several focuses [5,12,13]. These galls can be induced in young or mature leaves, and develop in the second or third order veins, or in the internervural region where they are commonly more numerous [12].

Through the anatomical analysis of the developmental stages of the galls of *A. spruceanum*, the present study aimed to relate the final gall phenotype with the distinct

sites of oviposition, the internervural regions or on second and third order veins. At these sites, the levels of cell responses to the insects' stimuli may vary. Moreover, the location of phenolic derivatives and reactive oxygen species (ROS) in gall tissues was established through histochemical tests. This location may be related to the cecidogenetic field established by the midge's presence or to an investment in chemical defenses during the development of the galls.

2. Methodology

2.1. Collection and Fixation of Botanical Material

Non galled leaves and leaves with galls at different developmental stages and with different oviposition sites were collected from October 2001 to October 2002 from individuals (n = 6) of *A. spruceanum* located at the Pampulha Campus of the Universidade Federal de Minas Gerais in Belo Horizonte, MG, Brazil. The voucher material is deposited at BHCB herbarium under the registration number 46.274.

The samples were fixed in FAA (37% formaldehyde, acetic acid and ethanol 50˚ GL, 1:1:18, v/v) [14] for analysis at light microscope, and with 2% ferrous sulphate in 10% formalin [14] for detection of polyphenols.

2.2. Preparation of Histological Sections

For permanent slides, some samples, fixed in FAA, were dehydrated in an ethanol series and included in historesin (Reichert-Jung®). Some other samples were embedded in Paraplast® [15], after dehydration in n- buthyl series [14]. The transverse sections (5 μm) were obtained in a rotatory microtome (Jung-BIOCUT mod. 2035), and stained with 0.5% toluidine blue in 0.1% sodium carbonate at pH 11.0 [16]. The slides were mounted in water for immediate observation.

The material embedded in Paraplast® was sectioned (12 - 14 μm) in a rotative microtome (Jung-BIOCUT mod. 2035), and affixed to the slides with Bissing adhesive [15]. The staining was done with 0.5% safranin and astra blue (1:9 v/v) [15], and mounted with Entellan®. Transverse freehand sections of the non galled leaves, and of the galls in the 5 stages of development [12] induced in the internervural region and in the first and second order veins were done. These sections were clarified in 50% sodium hypochlorite, washed in distilled water, and stained in 0.5% safranin and astra blue (1:9 v/v) [15]. Sections were washed in distilled water, and mounted with jelly glycerin [15].

2.3. Histochemical Tests

The histochemical tests were done in freehand sections. The presence of lipids and starch was verified with Sudan Black B in 70% ethanol [17], and with Lugol's reagent (2% potassium iodide in 0.2% iodine) [18] for 15 minutes, respectively. The presence of polyphenols was verified with 2% ferrous sulphate in 10% formalin [14]. Proanthocyanidins and their oligomeric derivatives were tested by the fixation of the sections in 1% caffeine-sodium benzoate in 95% ethanol, for 5 minutes, and immersion in *p*-dimethylaminocynamaldehyde (DMACA) for 2 hours [19,20]. The slides were mounted in 50% gly-cerin. The detection of lignins was done with the reagent of Wiesner [21], in which the phloroglucinol in acidic conditions reacts with monomeric residues of the polymer (*i.e.* cinnamyl alcohol-derivatives) [22-24]. The sections were immersed for 5 minutes in phloroglucinol in 95% ethanol and mounted in 50% hydrochloric acid. Lignins were also detected with the reagent of Maule [22 -24], in which the sections were placed in 1% potassium permanganate for 5 minutes, washed in distilled water, transferred to hydrochloric acid for 1 minute, and mounted in 50% ammonia. This reagent causes the formation of colored derivatives by oxidation of the monomeric units of lignin.

2.4. Developmental Stages of the Galls

To determine the occurrence of the various stages of gall development in *A. spruceanum*, 25 leaves were randomly collected and the 137 galls found were classified according to the criteria described in Formiga *et al*. [12].

2.5. Detection of Sites of Reactive Oxygen Species (ROS)

The detection of the sites of ROS activity was performed using the DAB reagent (3-3'-diaminobenzidine). Free-hand cuts of fresh material were immersed in 0.5% DAB (Sigma) for 20 - 60 minutes in the dark [5,25]. The intensity of reaction was observed every 15 minutes.

3. Results

3.1. Non Galled Leaves

The leaves of *A. spruceanum* are isobilateral, hypostomatic, and hairy on the abaxial surface. The epidermis is uniseriate, covered by a thick cuticle. The palisade parenchyma is 3-layered at the adaxial, and 1-layered at the abaxial side of the lamina. The spongy parenchyma is 10 - 13 layered, with long armed cells. The vascular system consists of a first order vein, with initial cambial activity forming xylem and phloem in bicollateral arrangement. At the adaxial cortex, some small collateral vascular bundles are observed. In the second and third order veins, the bundles are collateral, involved by pericyclic fibers, parenchyma cells and the endodermis. Fibersclereids are common throughout the mesophyll.

3.2. General Aspects of Galls

The gall is closed, lenticular, verrucous, and green, no matter the site of oviposition (**Figures 1(a)-(e)**). The gall epidermis is similar to that of non galled leaf at all stages of gall development with apparently functional stomata. The main changes in relation to the non galled leaves are observed in the ground and vascular systems. The mesophyll of the gall loses the distinction between palisade and spongy parenchyma characteristic of the non galled lamina. The vascular bundles are disorganized due to the hyperplasia and hypertrophy of the associated parenchyma. The diverse orientation of the tracheary elements is evident.

3.3. Galls in the Internervural Region

Induction. The first signs of gall induction are noticed by the bulging of the leaf blade (**Figure 1(f)**), with sites of hyperplasia at the epidermis. The epidermal cells are small and isodiametric (**Figure 1(g)**). In the adaxial epidermis, the cells are anticlinallly hypertrophied, and the cuticle is thin **(Figure 1(h)**). The cortex is composed of size-varied parenchyma cells, in which hyperplasia and cell hypertrophy are common. The insertion of the ovipositor is noticed by a healing sheath (**Figures 1(j)-(k)**). In the central region, near the larval chamber, hypertrophied, round, sometimes binucleated cells and hyperplasic sites occur (**Figures 1(f), (j), (k)**). The vascular bundles of the second and third order veins are disorganized by hyperplasia of the associated parenchyma (**Figure 1(l)**).

3.4. Growth and Development

There is an increase in the number of cell layers in the peripheral portions of the galls. The prominence of the gall on the leaf surface increases (**Figure 2(a)**). The epidermis becomes locally hypertrophied, its cells elongate anticlinally and present signs of metacutinization (**Figure 2(b)**). In the peripheral portions of the gall cortex, the spongy parenchyma is hyperplasic, with long-armed hypertrophied cells (**Figures 2(c)-(d)**). Around the larval chamber, a sclerenchymatous ring limit the outer cortex and the nutritive tissue differentiates (**Figure 2(f)**). The larval chamber is elongated or circular (**Figure 2 (e)**). The circular shape occurs when the chamber is perpendicular to the proximal vascular bundle, and the vascularization is ensured by several bundles where the hyperplasia of the parenchyma leads to disorganization of the structure (**Figure 2(f)**). Fiberscleroids occur adjacent to the epidermis (**Figures 2(b)**, **(c)**) and in the mid portion of the gall parenchyma occur (**Figure 2 (f)**). The vascular bundles immersed in the parenchyma of the gall are more altered the closer the larval chamber is (**Figure 2(f)**).

3.5. Maturation

The dermal system is covered by a thick cuticle (**Figure 3(a)**). The ground system in the adaxial cortex is hyperplasic, with no palisade differentiation (**Figure 3(b)**). Sclereids and fiberscleroids occur throughout the cortex of the gall (**Figure 3(b)**). The spongy parenchyma is also hyperplasic, with hypertrophied cells. The larval chamber is round when sectioned perpendicular to the vein and is surrounded by a nutritive tissue (**Figures 3(c)**, **(d)**). The sclereids around the nutritive tissue have thick walls. The vascularization maintains the characteristics of the earlier stages, particularly near the larval chamber (**Figure 3(d)**).

3.6. Senescence

The prominence of the gall on the leaf lamina reaches its maximum by the time the insect abandons the gall. The surface of the gall is verrucous and the larval chamber is more elongated horizontally when sectioned parallel to the vascular bundles. The dermal system maintains the characteristics of the maturation stage. The ground system consists of parenchyma cells interspersed with sclereids and fiberscleroids adjacent to the epidermis and more numerous than in the earlier stages of development. The larval chamber is covered with small portions of nutritive tissue, surrounded by a thicker ring of sclereids (**Figure 3(c)**). The vascularization of the gall is maintained by one bundles which crosses the ring of sclereids (**Figure 3(d)**). The tracheary elements have spiral or pitted wall thickenings, and simple perforation plates (**Figure 3(e)**). At the end of this phase, the gall has a series of fundamental cristarque (**Figure 3(f)**), brachisclereids, tracheoidal sclereids, and fiberscleroids, interspersed to the spongy parenchyma. Reactions of cicatrization are observed in the cells of the nutritive tissue (**Figure 3(g)**).

All developmental stages of the galls occurred all over the year (**Table 2**).

3.7. Galls in the Midrib Region, Second and Third Order Veins

The galls developed on the veins have an asymmetrical increment of tissues (**Figure 4(a)**). The dermal system is formed by a uniseriate epidermis. This cell layer together with the exodermis has conspicuous wall thickening and metacutinization (**Figure 4(b)**). At the less developed side, the exodermis does not differentiate, and the epidermis has hypertrophied cells (**Figure 4(c)**). The cortex is parenchymatic with isodiametric and polygonal cells (**Figure 4(d)**), with long armed cells lateral to the larval chamber (**Figure 4(e)**). The larval chamber is placed within the vascular bundle and is surrounded by the nutritive tissue and the ring of sclereids (**Figures 4(e)-(f)**).

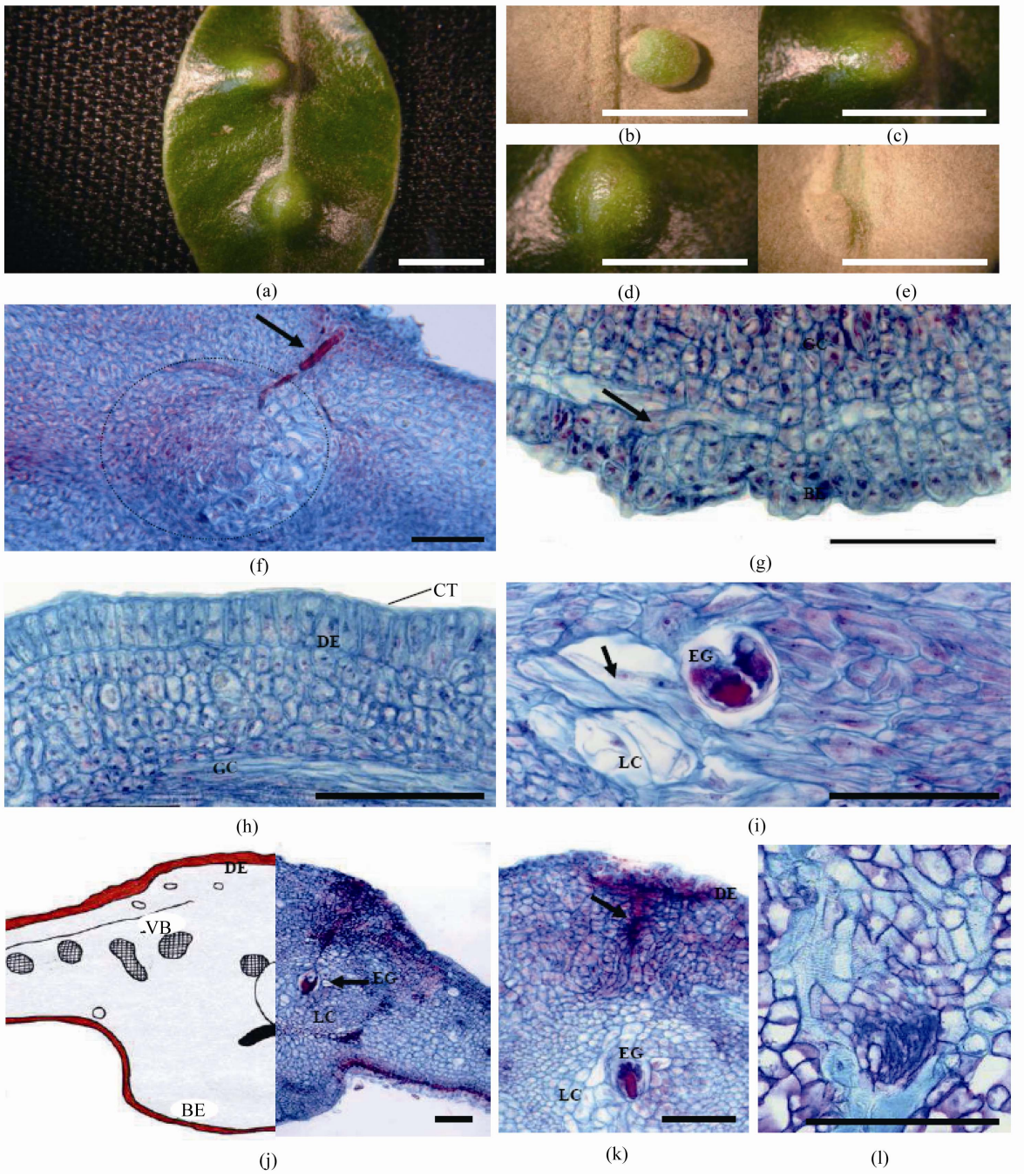

Figure 1. Morphological and anatomical aspects of the leaf gall on *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae). (a) View of the adaxial leaf surface with galls on the midrib and internervural region. (b), (c) Gall on the internervural region with projections to the abaxial and adaxial surface, respectively. (d), (e) Gall on the midrib with projections to the adaxial and abaxial surfaces, respectively. (f)-(l) Transverse sections of galls in the internervural region. (f)-(i) Induction phase—(f) General aspect in the moment of oviposition; the chamber is beginning to differentiate (dashed circle), we can see an oviposition scar (arrow). (b)-(g) Hyperplasia on the abaxial cortical portion with differentiating sclereids (arrow). (h) Hypertrophy of the adaxial epidermal cells, and hyperplasia of the adjacent cortical cells. (i) Hypertrophied cortical cells, binucleated cell (arrow) and hyperplasia around the egg of the Cecidomyiidae. Developmental phase. (j)—General aspect of the gall, with the insertion of the ovipositor and the egg (arrow) within gall cortex. (k)—Detail of of the site of oviposition scar (arrow) and egg location. (l) Disorganized vascular bundle. BE (abaxial epidermis), CT (cuticle), DE (adaxial epidermis), EG (egg), GC (gall cortex), HC (hypertrophied cells), HS (hyperplastic site), LC (larval chamber), NT (nutritive tissue), VB (vascular bundles), (Figures (a)-(e), bars = 5 cm, (f)-(l), bars = 100 µm.).

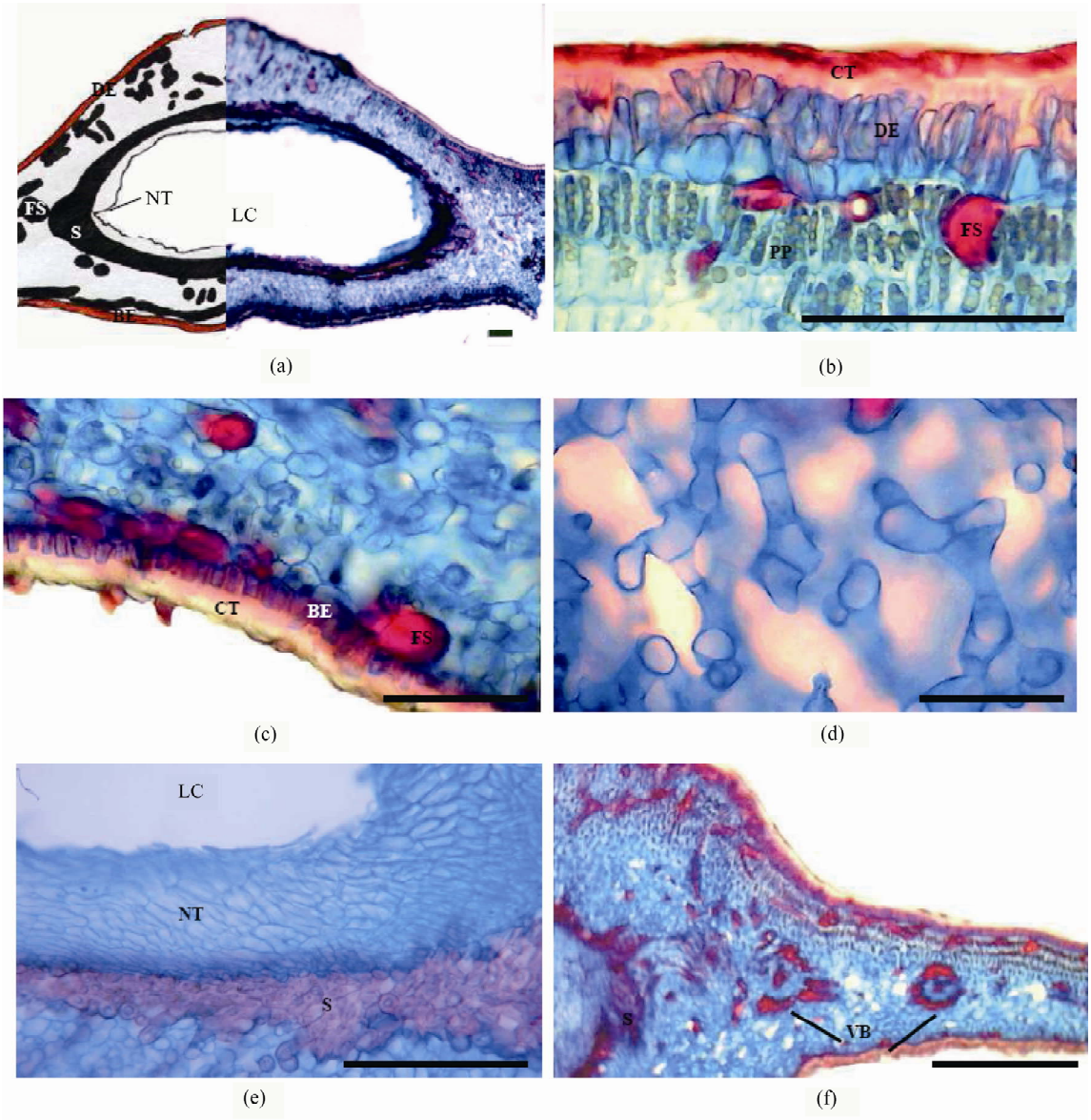

Figure 2. Anatomical aspects of the leaf galls on the internervural region on *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae). 3. (a) Developmental phase with large larval chamber. (b) Detail of gall on the adaxial cortical portion with metacutinization, and sites of cell division on the epidermis. The parenchyma is in palisade arrangement with sclereids, and a phenolic idioblast. (c) Detail of the gall on the abaxial cortical portion with metacutinization and sclereids. (d) Gall cortex with hyperplasic spongy parenchyma. (e) Detail of the nutritive tissue and sclerenchymatic zone around the larval chamber. (f)—Lateral portion of the gall with disorganized vascular bundles, some redirected to the larval chamber. BE (abaxial epidermis), CT (cuticle), DE adaxial epidermis), FS (fibersclereids), LC (larval chamber), NT (nutritive tissue), PP (palisade), S (sclereids), VB (vascular bundles). (In (a)-(g), bars = 100 µm. In h bar = 500 µm).

The collenchyma and the 1 - 3 layered palisade parenchyma are placed on both sides of the larval chamber. The vascular bundles of the second and third order veins diverge to the larval chamber.

3.8. Histochemistry of Galls

The various stages of development of the galls on the leaves of *Aspidosperma spruceanum* are histochemically similar in relation to the production and storage of the analyzed metabolites (**Table 1**).

The sites for lignins and ROS detection are similar, and there was no distinction between syringyl and Guaiacyl lignin either in non galled or galled tissues. The ROS are detected in the palisade parenchyma and around the larval chamber in the border line of the inner cortex. A centrifugal gradient is visualized, which is more intense

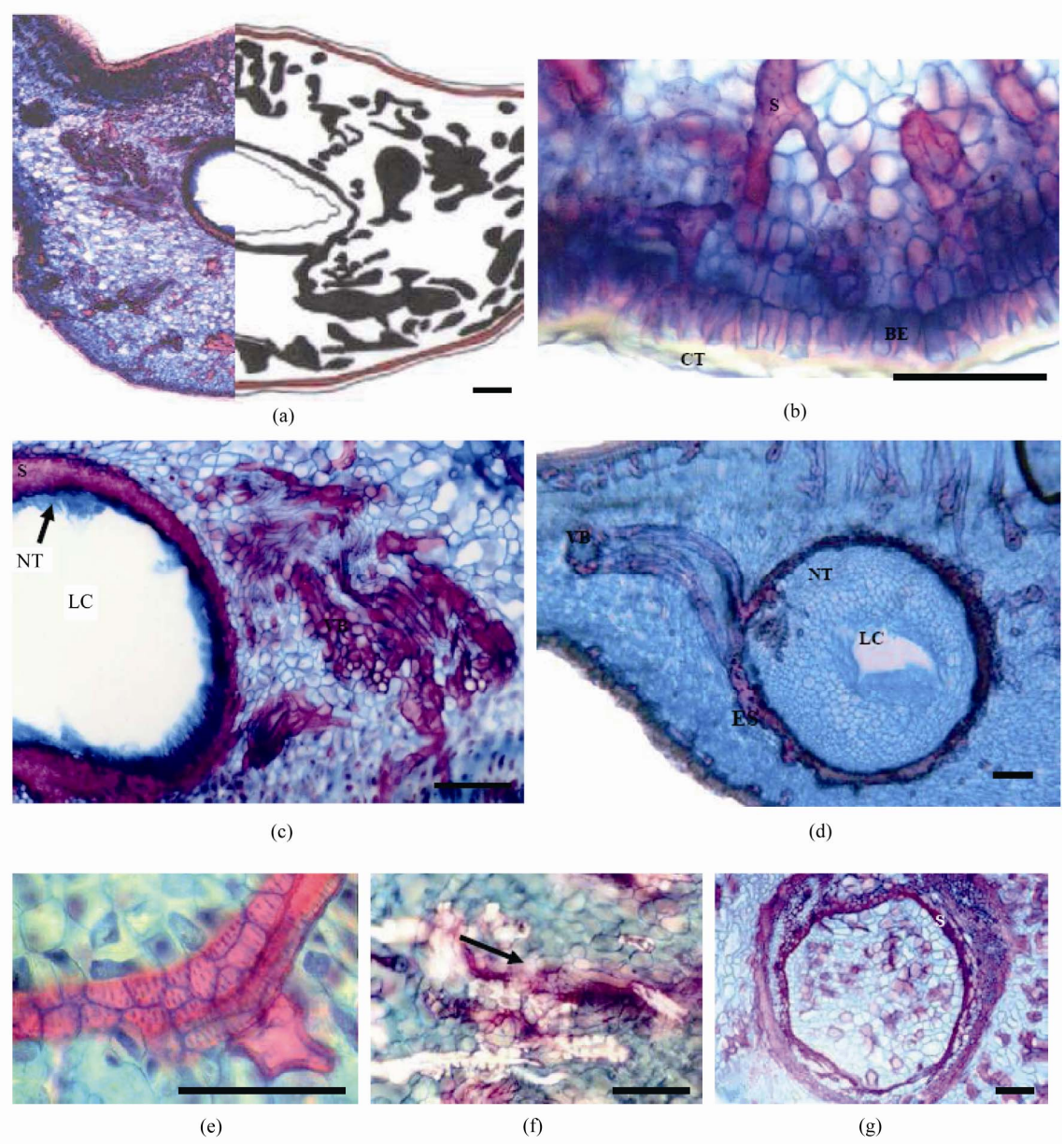

Figure 3. Anatomical aspects of the leaf galls on the internervural region on *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae). Stage 4. (a)—General aspect evidencing tissue zonation and round larval chamber. (b) Detail of the abaxial portion with numerous sclereids. (c) Detail of the larval chamber lined with nutritive tissue (arrow) and sclerenchymatic zone. (d)-(g) Maturation phase. (d)—Lateral portion of the gall with nutritive tissue surrounding the larval chamber and vascular bundle redirected towards it. (e) Tracheoidal sclereids with bordered pits. (f) Cristarque under polarized light (arrow). (g) Base of the larval chamber evidencing nutritive tissue, the sclerechymatic zone is interspersed by vascular tissues. Note early cicatriza- tion around the chamber. CT (cuticle), CL (larval chamber), EB (abaxial epidermis), ED (adaxial epidermis), ES (sclereids), FE (fibersclereids), FV (vascular bundle), NT (nutritive tissue), TC (scar tissue). (Bars = 100 µm).

in the maturation phase (**Figures 5(a)-(e)**).

4. Discussion

The Cecidomyiidae feeding activity caused alterations in dermal, ground and vascular systems of their host *A. spruceanum* leaves. The stimuli for gall development come from the insect, and the control of the growth and differentiation of the cells is directed to the morphogenesis of a new structure, as proposed by Dreger-Jauffret and Shorthouse [26]. This morphogenical redirection is directly dependent on the presence of the galling insect, and the changes cease after the senescence of the gall.

Table 1. Histochemical analysis of the gall tissue layers in *Aspidosperma spruceanum*.

	Lipids	Starch	Phenolic derivatives	Alcaloids	Flavanols
Cuticle	+	–	–	–	–
Epidermis	+	+	+++	–	–
Outer cortex	+	++	++	–	–
Inner cortex	–	+		–	–
Nutritive tissue	–	+	–	–	–

(+) positive reaction, (–) negative reaction. The number of signs indicates the intensity of the reaction.

Table 2. Percentage of occurrence of the stages of the development of the galls of *Aspidosperma spruceanum*.

Stages	Occurrence
Induction	8.8%
Development	24.8%
Maturation	24.1%
Senescence	42.3%

Gall developmental phases were determined according to the criteria described in Formiga *et al.* (2009). $N = 137$ galls in 25 leaves.

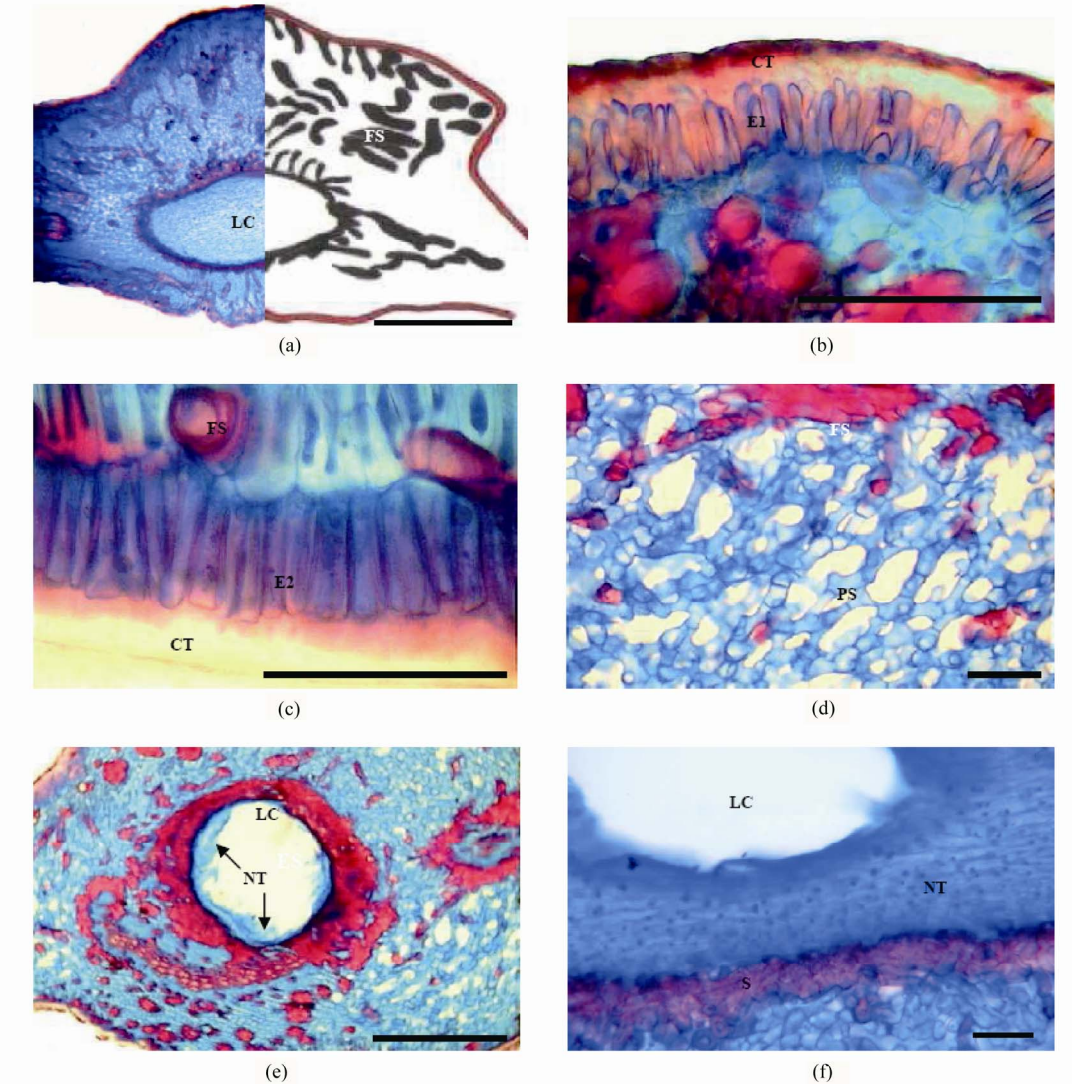

Figure 4. Anatomical aspects of leaf galls in the midrib region of *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae). (a) General aspect. (b) Adaxial portion with metacutinization and hypertrophy of epidermal cells, and adjacent cortex with numerous sclereids. (c) Abaxial portion with metacutinization and hypertrophy of epidermal cells. (d) Hyperplasic spongy parenchyma. (e) General aspect of the gall with round larval chamber, surrounded by the nutritive tissue (arrow), the sclerenchymatic zone and sclereids interspersed within parenchymatic cells. (f) Detail of the larval chamber surrounded by nutritive tissue, and sclerenchymatic zone. CT (cuticle), CL (larval chamber), CP (parenchymal cells), E1 (epidermal hypertrophy and hyperplasia), E2 (epidermal hypertrophy), FE (fibersclereid), FI (fibers), FL (phloem), IC (isodiametric cells), PS (spongy parenchyma), XI (xylem). (In a, bar = 500 µm. In (b)-(f), bar = 100 µm).

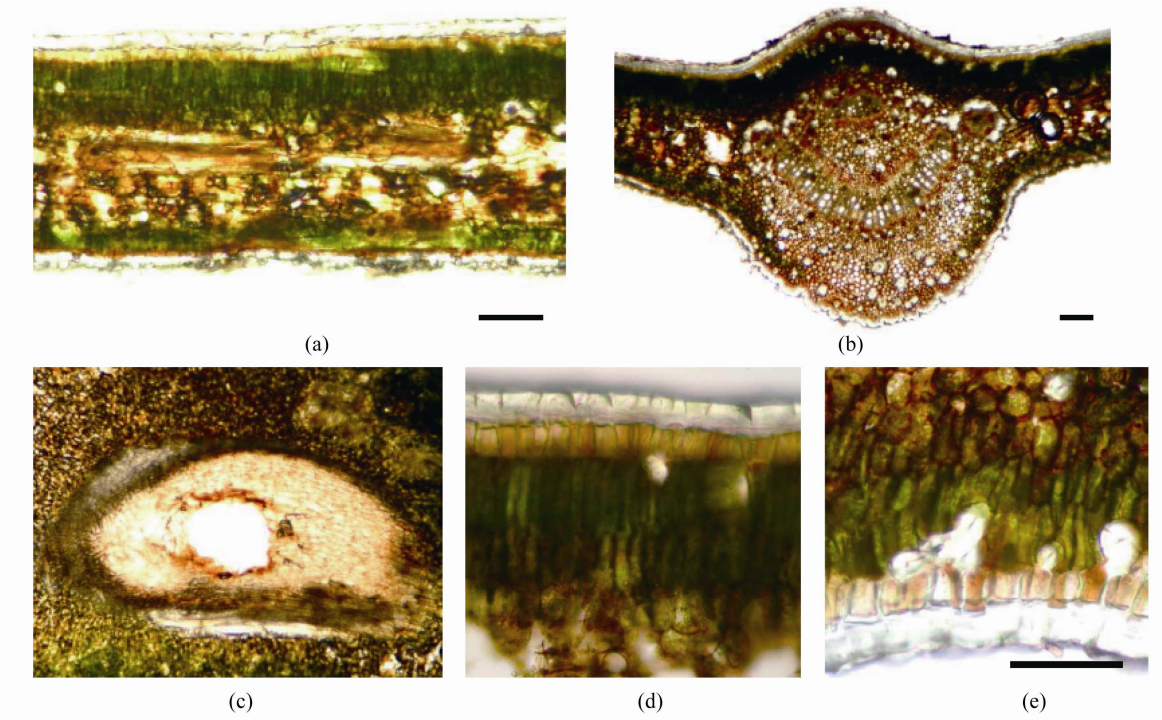

Figure 5. Histochemical tests for reactive oxygen species (ROS) on non galled leaves and galls of *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae). (a)-(b) Non galled leaf. (a) General apect of leaf lamina. ROS concentrated on spongy parenchyma. (b) Midrib region. ROS concentrated in palisade parenchyma. (c)-(e) Gall. (c) Inner cortex with larval chamber. ROS concentrated in the inner cells of the nutritive tissue and around the sclerenchymatic zone. (e) Adaxial cortical portion. ROS concentrated on epidermis and cortex. (e) Abaxial cortical portion with ROS concentrated on the epidermis and parenchyma. (Bars = 100 µm).

All the four basic developmental stages of the Cecidomyiidae galls *sensu* Rohfritsch [27] occur simultaneously in *A. sprucenum* (**Table 2**), indicating the multivoltinism of the insect, as proposed by Campos *et al*. [13]. The induction phase seems to start with oviposition, which occurs inside the leaf tissues, indicating that the female of this species has a strong and long ovipositor, called terebra [28], able to pierce the thick cuticle, the epidermis and the parenchyma. Moreover, the different degrees of cell hypertrophy immediately around the egg and the hyperplasia of the tissue facing the adaxial leaf surface indicate the concomitant stimuli of a fluid injected at the time of oviposition, and the mechanical injury caused by the ovipositor. This is an anatomical evidence of the insects' activity, difficult to visualize in nature due to its diminutive dimension.

The occurrence of galls in the induction phase in young and mature leaves denotes a wide range of oviposition sites for the galling herbivore. This behavior is relatively uncommon in gall inducing insects, which are referred to prefer to lay eggs on meristematic tissues [27]. In fact, the parenchymatic cells that react to the behavior of the Cecidomyiidae in *A. spruceanum* are considered to be partially differentiated or less specialized *sensu* Buvat [29]. The dedifferentiation of mature cells requires sophisticated changes in cellular morphogenetical programs, and in the case of the galls must proceed through the growth and developmental phase. At this phase, a conspicuous feature is the redifferentiaton of columnar sclereids, not observed in non galled leaves of the host species. Galling herbivores, in general, are not capable of inducing a new structure or tissue strange to the morphogenetical program of their host plant cells [6,30]. Nevertheless, this feature had been reported in some other dipteran [27,31-33], and may help in the mechanical support spongy tissue differentiated in the inner cortex of the gall.

Fibersclereids may develop from parenchyma cells, with the growth and elongation of the structure of the gall. This is only possible if the cells are still alive when the gall starts its development, as previously described by Arduin *et al*. [34] in Cecidomyiidae galls induced in *Struthanthus vulgaris*. In the galls of *A. spruceanum*, the number of fibersclereids increases from the induction through the growth and development, and maturation phases. Also, the fibersclereids increase in the vicinity of the larval chamber. The lignification plus the accumulation of phenolic compounds in gall parenchyma commonly occur in

Table 3. ROS analysis on the tissue layers of non galled leaves and of distinct gall developmental phases in *Aspidosperma spruceanum*.

	Non Galled Leaves	Gall Developmental Phases			
		Induction	Development	Maturation	Senescence
Cuticle	–	–	–	–	–
Epidermis	–	–	–	–	–
Palisade parenchyma	++++	abs	abs	abs	abs
Outer cortex	abs	++	++	++	++
Spongy parenchyma	++	abs	abs	abs	abs
Inner cortex	abs	+++	+++	++++	+++
Nutritive tissue	abs	++	++	++	++

(+) positive reaction, (–) negative reaction. abs = absent. The number of signs indicates the intensity of the reaction.

response to different types of injuries [35], and consists in a high mechanical resistance against the attack of predators and parasitoids. The sclerenchymatic layer, common in many midge induced galls and located between the vascular and the nutritive tissues [27], is evidenced in these galls since the phase of growth and development. Another feature of the sclerenchyma in the galls of *A. spruceanum* is the numerous connections with the surrounding layers, facilitating the transport of substances, in an acessory function to the vascular system.

In the maturation phase, the gall tissues are fully differentiated, and the final shape and dimensions are built. The most striking alterations are the non-differentiation of palisade parenchyma on both leaf surfaces, the hyperplasia of the spongy parenchyma and the increased formation and diversification of lignified cells. The inhibition of differentiation of palisade and spongy parenchyma is commonly cited in galls. Arduin *et al*. [34] and Isaias [36] demonstrated it in midge induced gall in *Struthanthus vulgaris* and *Machaerium* spp., and Vecchi [37], in galls induced by a microlepidoptera in *Tibouchina pulchra*. Kraus *et al*. [38] affirmed that these reactions are a convergent pattern in galls of several species of Brazilian flora. In fact, the variety of gall morphotypes induced in leaves seems to be mainly a result of changes in the ground system, whose cells respond more readily to the stimuli of the insect.

Even in the vascular system, the remarkable change is the hyperplasia of the vascular parenchyma, with some xylem bundles redirected to the larval chamber. The helical thickening of their cells is common in organs in primary growth, and should allow the elongation of the gall during the growth and development phase. The vascular system maintained the common pattern of the bundles of the non galled organs, with the maintenance of the formation of cristarque.

Alterations to a greater or lesser extent in the vascular system, and the neoformation of bundles were described by Meyer and Maresquelle [7] in various types of galls. According to Isaias [36], galls that develop on the first order veins have an increased translocation of assimilates towards to the area of the gall, and therefore should not have neoformation of bundles. Similarly, in the galls of *A. spruceanum* induced at the second and third order veins no new bundles are differentiated. Also, since the vascular system includes the highest levels of cell differentiation [39], the inducing Cecidomyiidae seems to have little ability to manipulate the vascular tissues.

The ability of the Cecidomyiidae to change the site of feeding inside de chamber can explain the different shapes of the larval chamber in the gall of *A. spruceanum*. It is elongated when parallel to the vein and round when perpendicular to the vascularization. This may be indicative of the rotational movements of the body of a Cecidomyiidae inside the gall, as observed by Arduin *et al*. [34] in the galls of *Struthanthus vulgaris*. Another possible cause of the variation in the shape of the larval chamber is the responsiveness of the tissues involved in the formation of gall, consisting of partially differentiated parenchyma cells *sensu* Buvat [29].

When the galls develop in the region of the veins, the differentiation of collenchyma in the abaxial portion is inhibited, mostly because the presence of a temporary tissue of support, common in growing organs [29], are not necessary in these galls. Considering that the gall is a temporary and fast growing structure, with limited size and shape, its support is given directly by the neoformation of numerous sclereids and fibersclereids.

Considering that the cells of the vascular system are highly specialized and anucleated, the feeding activity of the gall inducing insect should be restricted to the parenchyma cells. The cecidogenetic field is interrupted by the cells whose ontogenetical fate is reached, and therefore, the larval chamber necessarily have to stretch following the direction of the bundles, *i.e.*, the axial parenchyma cells, the pericycle, and the endodermis. This di-

rection explains the variations in the shape of the larval chamber.

In opposition to the structural changes, few metabolites accumulate in gall site. The reaction for lipids evidenced the metacutinization, and may be related to the protection against desiccation [40]. The absence of lipids as a reserve substance is consistent with the studies of Bronner [11,41] which proposes the formation of nutritive and reserve tissue rich in carbohydrates in galls of Cecidomyiidae. The accumulation of polyphenols in the peripheral layers are usually related to an effective chemical defense [1,42], however, this hypothesis does not apply to the galls of *A. spruceanum* since these galls have many natural enemies [5]. The lignification can restrict water loss inside the gall, defining a microenvironment that has enough moisture for the survival of the gall inducer.

The sites of positive reaction to ROS were similar to those of lignins (**Table 3**), a coincidence already detected by Hückelhoven [43]. Cell wall lignification and the accumulation of ROS were also intense around the larval chamber. This region has intense cell divisions and differentiation, and is in direct contact with the gall inducing insect, which can explain its high level of oxidative stress. It is relevant to mention that the coincidence of the sites for lignification and ROS detection reinforces the antioxidant role of lignin biosynthesis since this process consumes large amounts of hydroxyl radicals [44,45].

By the time the galls reach the phase of senescence, the feeding activity of the insect ends up, and the suberization of the cells lining the larval chamber is anatomically evidenced. This phase can occur even after the falling of the leaves, when the insects pupate in the soil and the imago emerges to start a new set of inductions [27]. This is true for galls induced either in the internervural or in the veins.

5. Conclusions

The development of the galls of *A. spruceanum* corroborates the pattern previously established for the galls of midges, with the oviposition in parenchyma layers, and significant changes in the three plant tissue systems. In this gall, the shape of the larval chamber followed the direction of the vascular parenchyma cells, the pericycle and the endodermis, which are very responsive tissues. The neoformation of fibersclereids deserves attention for this kind of cells is not observed in the host non galled leaves. Also they provide structural protection for the Cecidomyiidae, and may function as an accessory transport system.

The lignification of the cells at the same site of ROS accumulation is a further indication of protection to the gall inducer, which generates a safe microenvironment protected from pathogens such as fungi and bacteria, and effective against environmental factor such as dryness and diffusion of toxic free-radicals inside the gall. These galls are also efficient in nutrient supply, and their anatomical features evidence their adaptive value for the gall inducer.

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