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Influence of auxin and phenolic accumulation on the patterns of cell differentiation in distinct gall morphotypes on *Piptadenia gonoacantha* (Fabaceae)

Cibele Souza Bedetti[A], *Gracielle Pereira Bragança*[A] *and Rosy Mary dos Santos Isaias*[A,B]

[A]Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Caixa Postal 486, CEP31270-901, Belo Horizonte, Minas Gerais, Brazil.
[B]Corresponding author. Email: rosy@icb.ufmg.br

Abstract. The cascade of biochemical changes occurring at sites of gall development seems to involve a group of common metabolites in plants, namely, the phenolics. Phenolic accumulation has been commonly related to chemical defence, but their primary role seems to be the regulation of cell hypertrophy in galls. Such regulation implies phenolics–auxin (IAA) association at some cell re-differentiation sites, and determines final gall shapes. Herein, we investigated phenolic and auxin accumulation in four gall systems, grouped in two morphotypes, namely lenticular and globoid, induced on pinnulas of *Piptadenia gonoacantha* (Mart.) J.F.Macbr. Changes in the direction and type of cell expansion between non-galled pinnula and galls were also evaluated. Galling insects associated to lenticular and globoid gall morphotypes promoted changes in host plant cells, leading to the development of different cell sizes, different degrees of anisotropy, and different directions of cell expansion. The accumulation of IAA–phenolics compartmentalised on the basis of gall morphotype, i.e. in the cells of superior and lateral inferior cortices in the lenticular gall morphotypes, and throughout the outer cortex in the globoid gall morphotypes. The sites of accumulation of IAA and phenolics coincided with the most hypertrophied regions, influencing on the determination of the final gall shape.

Additional keywords: Cecidomyiidae galls, developmental anatomy, histochemistry.

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Introduction

Insect galls are products of a complex series of plant-tissue reactions, and have proved to be elegant models for the study of alterations in the morphogenesis of host organs (Arduin and Kraus 1995; Oliveira and Isaias 2010; Magalhães *et al*. 2014; Fleury *et al*. 2015; Suzuki *et al*. 2015), because they naturally concentrate cell and tissue alterations in repetitive and constant cycles occurring in nature. Gall morphogenesis relies on species-specific interactions, in which the galling herbivore alters the patterns of differentiation of their host organs (Mani 1964; Stone and Schonrögge 2003) through several processes, including the re-orientation of cellulose microfibrils and alterations in the direction of cell expansion during gall development (Magalhães *et al*. 2014; Suzuki *et al*. 2015). The expansion of cell walls is crucial for the establishment of the shape of plant organs, and involves the dynamics of isotropy and anisotropy. When cell walls expand in the same ratio in all directions, the isotropic pattern is established, and when there is a preferential direction of elongation, the anisotropic pattern occurs (Baskin 2005). Anisotropic cell expansion seems to predominate specifically in some gall tissue layers, which, together with the patterns of cell divisions, are controlled by the gall inducer (Carneiro *et al*. 2014*a*; Suzuki *et al*. 2015). This relationship has been recently demonstrated in fusiform galls induced by *Lopesia* sp. on *Lonchocarpus cultratus* (Fabaceae) (Suzuki *et al*. 2015). Cell expansion and division at gall sites disrupt the ordinary morphogenetic pattern of the host organ, and lead to new cell cycles in specific sites, which seems to be responsible for the diversity of final shapes observed in galls (cf. Oliveira and Isaias 2010; Isaias *et al*. 2011; Dias *et al*. 2013; Carneiro *et al*. 2014*a*; Magalhães *et al*. 2014; Fleury *et al*. 2015).

The manipulation of the host-plant organ morphogenesis by the galling insect extends to the control of the biochemical composition of gall tissues (Hartley 1998; Oliveira *et al*. 2006; Carneiro *et al*. 2014*b*; Bragança *et al*. 2017). Such manipulation is evidenced by the accumulation of storage substances that favour the nutritive requirements of galling insects, such as reducing sugars in galls of *Psidium myrtoidis* (Carneiro *et al*. 2014*b*), starch and lipid in galls of *Lonchocarpus muelhbergianus* (Oliveira *et al*. 2006) and *Piper arboreum* (Bragança *et al*. 2017). In gall tissues, there is also the overproduction of growth hormones such as auxin (indole-3-acetic acid, IAA;

Hori 1992; Bedetti *et al*. 2014; Suzuki *et al*. 2015), and accumulation of secondary metabolites, such as the phenolics. Phenolic synthesis in galls occurs mainly by the phenylpropanoid pathway. The concentration of phenylalanine ammonia lyase, which is the key enzyme in this pathway, is higher in gall tissues than in adjacent plant tissues (Hartley 1998, 1999). Phenolic accumulation is usually associated with defence mechanisms (Hartley 1998, 1999), but it has also been associated with the control of IAA concentration at the sites of gall development (Hori 1992; Bedetti *et al*. 2014; Suzuki *et al*. 2015).

Because galls develop as adventitious plant organs, the influence of IAA and other phytohormones is expected, and they certainly determine the metabolic turnover established in the cecidogenetic field, where the fate of host-plant cells is altered (Hori 1992; Bedetti *et al*. 2014; Suzuki *et al*. 2015). In fact, high concentrations of IAA have been detected in insect galls on *Solidago altissima* (Mapes and Davies 2001; Tooker and De Moraes 2011) and on *Triticum aestivum* L. cultivar Centennial (Tooker and De Moraes 2011). In pinnula, lenticular, concave-convex and fusiform rachis galls on *Piptadenia gonoacantha*, indole-3-acetaldehyde, which is the immediate precursor of IAA, was detected (Bedetti *et al*. 2014). One of the alternative metabolic pathways involved in gall development is the association of phenolics–IAA. Phenolics can inhibit IAA oxidases in gall tissue layers, which may result in high concentrations of auxin and cell hypertrophy (Hori 1992). Therefore, the primary role of phenolics at sites of gall development should be the regulation of growth, and chemical defences against natural enemies should be a secondary effect (Bedetti *et al*. 2014).

Here, we used a superhost of galling herbivores, *Piptadenia gonoacantha* (Mart.) J.F.Macbr. (Fabaceae), as a study model to comparatively evaluate the involvement of IAA and phenolics during gall development. *Piptadenia gonoacantha* hosts four galls grouped in two distinct morphotypes, namely, two globoid and two lenticular. The globoid morphotypes range from ellipsoids to spheroids, whereas the lenticular morphotypes usually form a halo on the leaf lamina, resembling either the biconvex or biconcave lenses (Isaias *et al*. 2013). We assume that the development of such shapes should require distinct sites of cell division and patterns of cell elongation. Compensatory mechanisms between these two processes may occur. The four gall systems studied here involve a unique plant genome under the influence of four distinct morphospecies of Diptera, Cecidomyiidae, and can elegantly demonstrate whether the accumulation of IAA and phenolics in specific gall-tissue compartments is related to the determination of the final gall shapes. We generate comparative structural data on each of two pairs of gall morphotypes to determine differences and similarities on the basis of developmental time and histology. In an effort to understand the generation of the two gall shapes, we analyse alterations in the degree of anisotropy, and the types of cell expansion in each gall-tissue layer, as well as in young and mature galls. We expect to co-detect IAA and phenolic accumulation at similar tissue sites, as well as convergent morphogenetic patterns in similar gall morphotypes, and along gall ages. Accordingly, divergences that explain the different structural solutions that determine the two distinct final shapes, may be possible.

Materials and methods

Host species and sampling

Piptadenia gonoacantha (Fig. 1*a*) is commonly known as 'pau-jacaré' (= alligator stick) and presents transverse and longitudinal ridges on the stem, which resemble the reptile leather (Souza and Lorenzi 2005) (Fig. 1*b*). Samples of non-galled pinnula (NGP), lenticular concave-convex galls (LC; Fig. 1*c*, *d*), lenticular plane-convex galls (LP; Fig. 1*e*, *f*), globoid with emergences galls (GE; Fig. 1*g*, *h*) and globoid hairy galls (GG; Fig. 1*i*, *j*), at young and mature stages, were collected at the Estação Ecológica da Universidade Federal de Minas Gerais, Pampulha Campus, Belo Horizonte, Minas Gerais, Brazil (UFMG–EE; 19°51′S, 43°59′W). The morphology, anatomy and ontogeny of GE were previously described by Arduin *et al*. (1994) and Arduin and Kraus (1995).

Histochemical methods

The samples were fixed in 2% ferrous sulfate in 10% formalin, so as to identify sites of phenolic accumulation (Johansen 1940). For IAA detection, free-hand sections of fresh samples of the four gall morphotypes were incubated in Ehrlich's reagent (Leopold and Plummer 1961) for 5 min at room temperature. Positive reactions were indicated by a pink colour as previously obtained on LC and rachis fusiform galls on *P. gonoacantha* (Bedetti *et al*. 2014). The results of the histochemical tests were compared with blank sections. Hereafter, the LC was used to compare with the other lenticular morphotype (LP), and with the globoid morphotypes (GG and GE). The results were photographed with a digital camera (Canon Power Shot A650, Tokyo, Japan) coupled to an optical microscope (Zeiss Primo Star, Jena, Germany).

Anatomical analysis

The samples were fixed in Karnovsky solution (Karnovsky 1965, modified to 0.1 mM phosphate buffer, pH 7.2) for structural analysis. The GE were immersed in 10% ethylenediamine for 2 weeks to soften the tissues (Carlquist 1982), and then dehydrated and embedded in polyethylene–glycol (PEG) at 60°C. The LC, LP and GG were dehydrated in *n*-butyl series and embedded in Paraplast (Kraus and Arduin 1997). The galls were cross-sectioned in a rotary microtome (Leica 2035 BIOCUT). The sections were stained in safranin–astra blue 9 : 1 (v/v, modified to 0.5%; Bukatsch 1972), and the slides were mounted with colourless Acrilex varnish (Paiva *et al*. 2006). Photographs were obtained in a digital camera (Canon Power Shot A650) coupled to an optical microscope (Zeiss Primo Star).

Cytometric and histometric analyses

For cytometric analyses and determination of the type of cell expansion in gall-tissue layers, the continuum NGP–galls were considered. The position of the vascular bundles and palisade–spongy parenchyma limit were also observed. The measurements of cell areas and the longest cell axis (periclinal and anticlinal) in the middle portions of the NGP ($n = 5$) and of the four gall morphotypes, at young and mature stages ($n = 5$, for each stage of gall morphotype), were performed with the Axion Vision 7.4 program (Carl Zeiss Microscopy GmbH, Jena, Germany). For each cell type, 75 measurements were taken (3 cells of each tissue

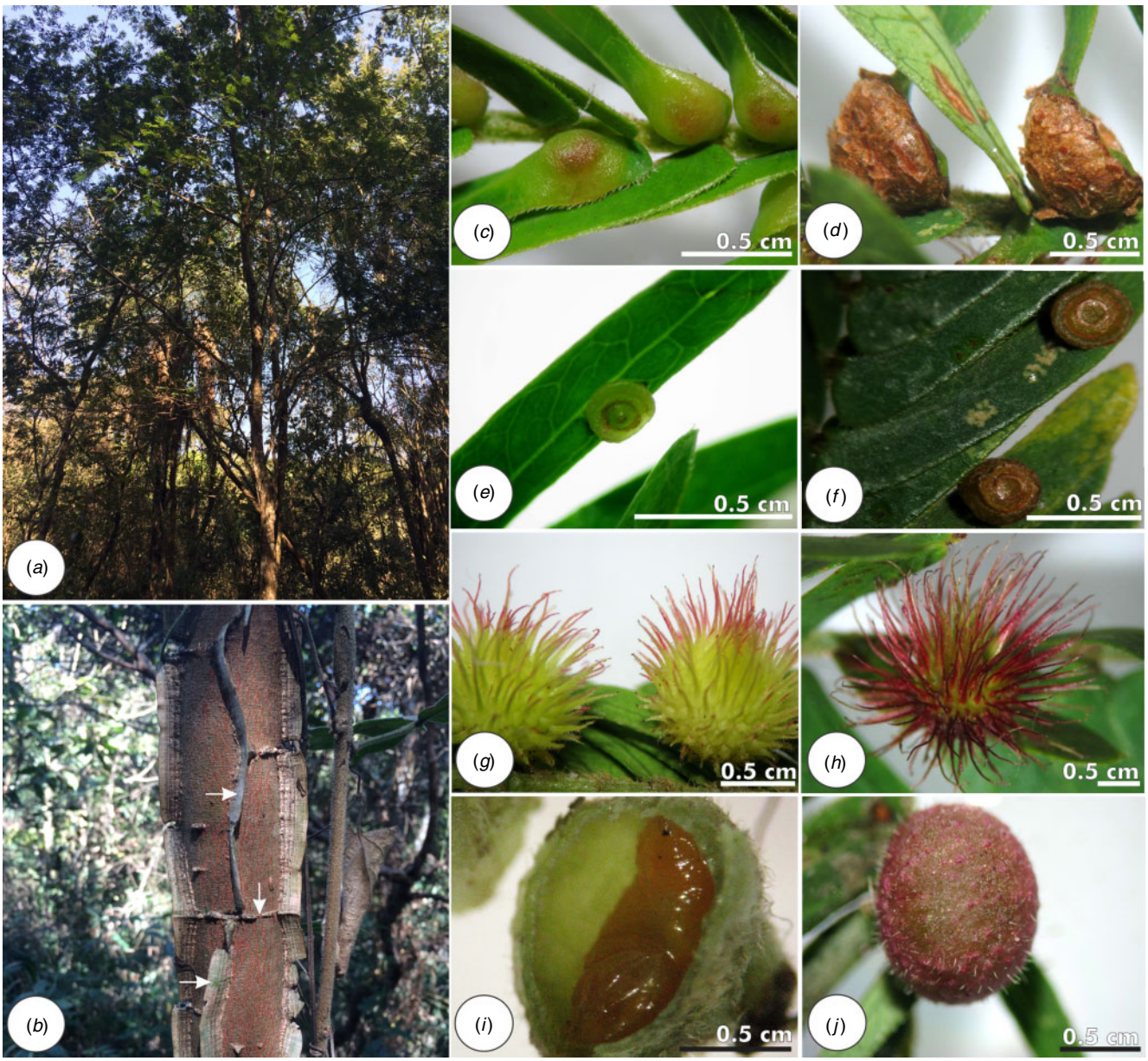

Fig. 1. General aspects of the *Piptadenia gonoacantha* (Mart.) J.F.Macbr. (Fabaceae) and galls induced on its pinnula. (*a*) *Piptadenia gonoacantha* at Estação Ecológica da Universidade Federal de Minas Gerais. (*b*) Detail of stem with transverse and longitudinal ridges (white arrows). (*c*) Lenticular concave-convex galls at young stage. (*d*) Lenticular concave-convex galls, with the dermal system suberised at mature stage. (*e*) Lenticular plane-convex gall at young stage. (*f*) Lenticular plane-convex galls, with the dermal system suberised at mature stage. (*g*) Globoid with emergence galls at young stage. (*h*) Globoid with emergence gall at mature stage. (*i*) Globoid gall at young stage, with the larva of gall-inducing insect. (*j*) Globoid galls at mature stage.

per image, 25 images per developmental stage), and the data were expressed as an average. For non-galled tissues, the axes and the area of the cells of the adaxial (AD) and abaxial (AB) epidermis, palisade parenchyma (PP) and spongy parenchyma (SP) were measured as was the total area of the vascular bundles (VB). For the galls, the cells of the dermal system (DS), parenchyma of the outer or superior cortex (OC/SC), parenchyma of the inner or inferior cortex (IC), and of the nutritive tissues (NT) were measured. The cortical sclerenchyma cells (SL) were measured, when present. The total area of the VB was measured.

Cell expansion, direction and degree of anisotropy

The type of cell expansion, represented by the cell major axes, was determined from measurements of the periclinal and anticlinal axes, using the orientation of the ellipse for reference. The degree of anisotropy was expressed by the ratio between the average of the maximum and minimum axes of the ellipse (Baskin 2005).

Statistical analysis

The morphological and anatomical data of the galls were compared by means of the principal-component analysis

(PCA) to determine whether their differences and similarities were supported on a quantitative basis. The PCA was based on the correlation matrix among morphometric variables (Table 1). For galls, the axes of cells that are common to all morphotypes (DS, OC/SC, IC and the area of VB) were analysed. The Paleontological Statistics Software Package (PAST; Hammer *et al*. 2001) was used for the PCA analyses.

Results

Anatomy of the non-galled pinnula (NGP)

The dermal system of the NGP is composed of a uniseriate epidermis. The ground system is composed of a dorsiventral mesophyll consisting of one-layered palisade parenchyma (PP), and three- to six-layered spongy parenchyma (SP; Fig. 2*a*). The vascular system has collateral bundles surrounded by fibres, whose number of layers varies according to vein dimensions, and which are located between the PP and the SP.

Lenticular concave-convex gall morphotype (LC)

The LC at the young stage (Fig. 2*b*) has uniseriate epidermis. The superior cortex has seven or eight layers of parenchyma cells, and three or four layers of sclerenchyma cells, both originated from the palisade parenchyma of the NGP. The nutritive tissue has five to seven cell layers originated from the division of the SP cells. Below the larval chamber, two or three layers of nutritive-tissue cells, and two or three layers of sclerenchyma cells are observed. The inferior cortex has 15–19 layers of elongated parenchyma cells, originated from the division of the SP cells. Between the two cortical regions, vascular bundles are located. In the mature stage (Fig. 2*c*), the epidermal cells remain uniseriate. The superior cortex has seven to nine layers of parenchyma cells, and six to nine layers of sclerenchyma cells. The larval chamber expands and the nutritive tissue has 9 or 10 cell layers. The inferior cortex has 30–45 layers of elongated parenchyma cells. The lenticular shape is formed by the process of tissue hyperplasia more evident in the inner cortical parenchyma, causing a curvature towards the abaxial portion of the pinnula. The vascular bundles maintain the collateral arrangement and are located in the outer limit of the nutritive tissue and the inferior cortex. At the end of this stage, the dermal system suberises.

Table 1. Principal components 1 (PC1) and 2 (PC2) obtained from the correlation matrix of cytometric characters of pinnula galls on *Piptadenia gonoacantha* (Mart.) JF.Macbr. (Fabaceae)

Measurements on the longest cell periclinal (↔) and anticlinal axis (↕). DS, dermal system; IC, parenchyma of the inner (or inferior) cortex; OC, parenchyma of the outer (or superior) cortex; VB, vascular bundles

Histometry	Axis	
	PC1	PC2
DS (↔)	–0.01558	0.9326
DS (↕)	0.7883	0.0384
DS (area)	0.7727	0.04913
OC (↔)	0.9167	0.1933
OC (↕)	0.8707	–0.1314
OC (area)	0.9247	0.02093
IC (↔)	0.386	0.3702
IC (↕)	–0.3031	0.7409
IC (area)	–0.06897	0.9448
VB (area)	0.7953	0.01382
Variance (%)	45.504	25.075

Lenticular plane-convex gall morphotype (LP)

The LP at the young stage (Fig. 2*d*) has uniserate epidermis. The larval chamber is surrounded by a one- or two-layered nutritive tissue and divides the cortex into superior and inferior portions formed by the hyperplasia and hypertrophy of the PP cells. Hyperplasia and cell hypertrophy are also observed in the parenchyma of the vascular bundles of the remaining non-galled portion of the pinnula. At the mature stage (Fig. 2*e*), the superior cortex has two to five layers of elongated parenchyma cells, and three to eight layers of sclerenchyma cells. The larval chamber expands, and is surrounded by three to nine layers of nutritive cells. The inferior cortex is formed by elongated parenchyma cells derived from the adaxial surface of the host pinnula. The vascular bundles remain with collateral arrangement.

Globoid with emergences gall morphotype (GE)

The GE is commonly induced on the abaxial surface of the pinnula and its indumentum has numerous emergences. Only the abaxial portion of the host pinnula originates the gall, with the abaxial epidermis originating the outer dermal system, and the SP originating the tissues of the outer cortex, inner cortex and the nutritive cells. The PP becomes homeogeneous. At the young stage (Fig. 2*f*), the outer cortex has 35–40 layers, and the inner cortex has 55–70 layers of parenchyma cells. The nutritive tissue is six- to nine-cell layered. Sclerenchyma cells differentiate mainly in the basal portion of the nutritive tissue surrounding the larval chamber. The vascular bundles are immersed in the outer cortex. At the mature stage (Fig. 2*g*), the outer cortex has 75–80 layers, and the inner cortex has 80–90 cell layers of parenchyma cells. There are four to six layers of sclerenchyma cells around the nutritive tissue, which is two- to four-cell layered.

Globoid hairy gall morphotype (GG)

The GG develops in the adaxial surface of the host pinnula. At the young stage, the epidermis is uniseriate, and the cortex has 14–18 layers of homogeneous parenchyma cells. The vascular bundles are located in the middle portion of the gall, limiting the outer and inner cortices. At the mature stage (Fig. 2*h*), the epidermis remains uniseriate with simple trichomes. The outer cortex, originated by the processes of hyperplasia and hypertrophy of the PP cells, has 4–10 layers of parenchyma cells. The inner cortex, originated from hyperplasia and hypertrophy of the SP cells, has three to five layers of sclerenchyma cells, and a one- to eight-cell layered nutritive tissue. The vascular bundles are located in the outer limit of the nutritive tissue. At the end of the mature stage, the nutritive tissue is one- to two-cell layered, because of its consumption by the galling insect.

Distribution of IAA and phenolics in the galls

In lenticular galls, the accumulation of IAA and phenolics was observed in the superior cortex and lateral inferior cortical cells, forming a strand from the abaxial epidermis towards the larval chamber (Fig. 3*a*, *d*). The accumulation of IAA and phenolics occurs mainly in the peripheral layers of the superior cortex,

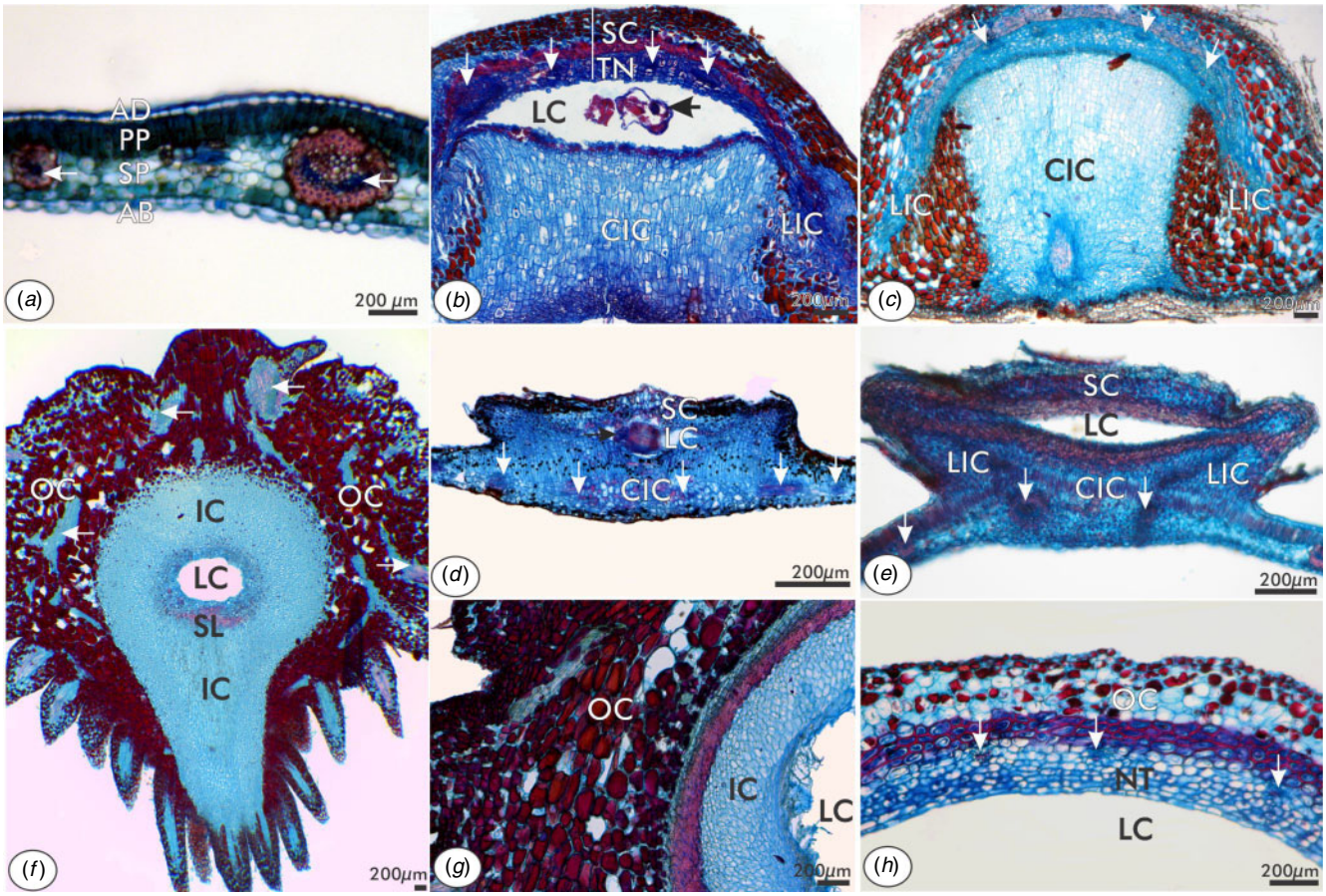

Fig. 2. Sections of non-galled pinnula (NGP) and galls on *Piptadenia gonoacantha* (Mart.) JF.Macbr. (Fabaceae). (*a–e*, *h*). Cross-sections. (*f*). Longitudinal section. (*a*) NGP showing dorsiventral mesophyll and collateral bundles (white arrows). (*b*) Lenticular concave-convex gall at young stage, with superior cortex composed of parenchyma, sclerenchyma and nutritive cells around of larval chamber, with the larva of gall-inducing insect (black arrow). The inferior cortex is composed of elongated parenchyma cells. Vascular bundles (white arrows) are located in the transition between the two cortical regions. (*c*) Lenticular concave-convex gall at mature stage in a section tangential to the larval chamber. (*d*) Lenticular plane-convex gall at young stage showing larval chamber with gall-inducing insect (black arrow), localised in the transition of the two cortical portions. Hypertrophy of vascular bundles (white arrow) in the remaining non-galled pinnula is observed. (*e*) Lenticular plane-convex gall at mature stage showing expansion of the larval chamber. (*f*) Globoid gall with emergences at young stage, with the outer cortex formed by parenchyma cells, and the inner cortex formed by layers of parenchyma cells, and nutritive tissue. (*g*) Globoid gall with emergences at mature stage. Transition zone between the inner cortex and the outer cortex is formed by sclerenchyma cells. (*h*) Globoid gall at mature stage, with uniseriate dermal system, outer cortex composed of parenchyma cells and inner cortex consisting of sclerenchyma and nutritive cells. The vascular bundles are located in the first layers of the nutritive tissue. AB, abaxial epidermis; AD, adaxial epidermis; CIC, central inferior cortical cells; DS, dermal system; LIC, lateral inferior cortical cells; NT, nutritive tissue; OC, outer cortex; PP, palisade parenchyma; SC, superior cortex; SL, sclerenchyma cells; and SP, spongy parenchyma.

and throughout the lateral cells of the inferior cortex (Fig. 3*a–d*). In globoid galls, the accumulation of phenolics and IAA occurs homogeneously throughout the outer cortex (Figs 3*e–h*, 4).

Principal-component analysis (PCA)

The characteristics of the outer cortical cells, height and area of epidermal cells, and the whole area of the vascular bundles strongly correlated with the PC1. The width of the epidermal cells and the height and area of the inner cortical cells correlated with the PC2. All cytometric variables, except the height of the outer cortical cells, were positively correlated with the PC2. The first two axes of PCA explained 70.6% of the total variance (Table 1).

The GG in young and mature stages also formed a single and isolated group, whereas the two developmental stages of the other gall morphotypes (GE, LC, LP) were separated by cell areas, and types of cell expansion in distinct tissue layers. The GE comprised the most separated group in relation to the NGP (Fig. 4).

The main features that separated the morphotypes intra- and inter-specifically, and among the stages, were the height, width and the area of the outer cell layers, the height and area of epidermal cells, and the whole area of the vascular bundles. The young GG and GE were separated by the PC1 and PC2, whereas mature GG and GE were separated mainly by the PC1. The young LP and LC were separated by the PC1, whereas mature LP and LC were separated by the PC2. The GG was

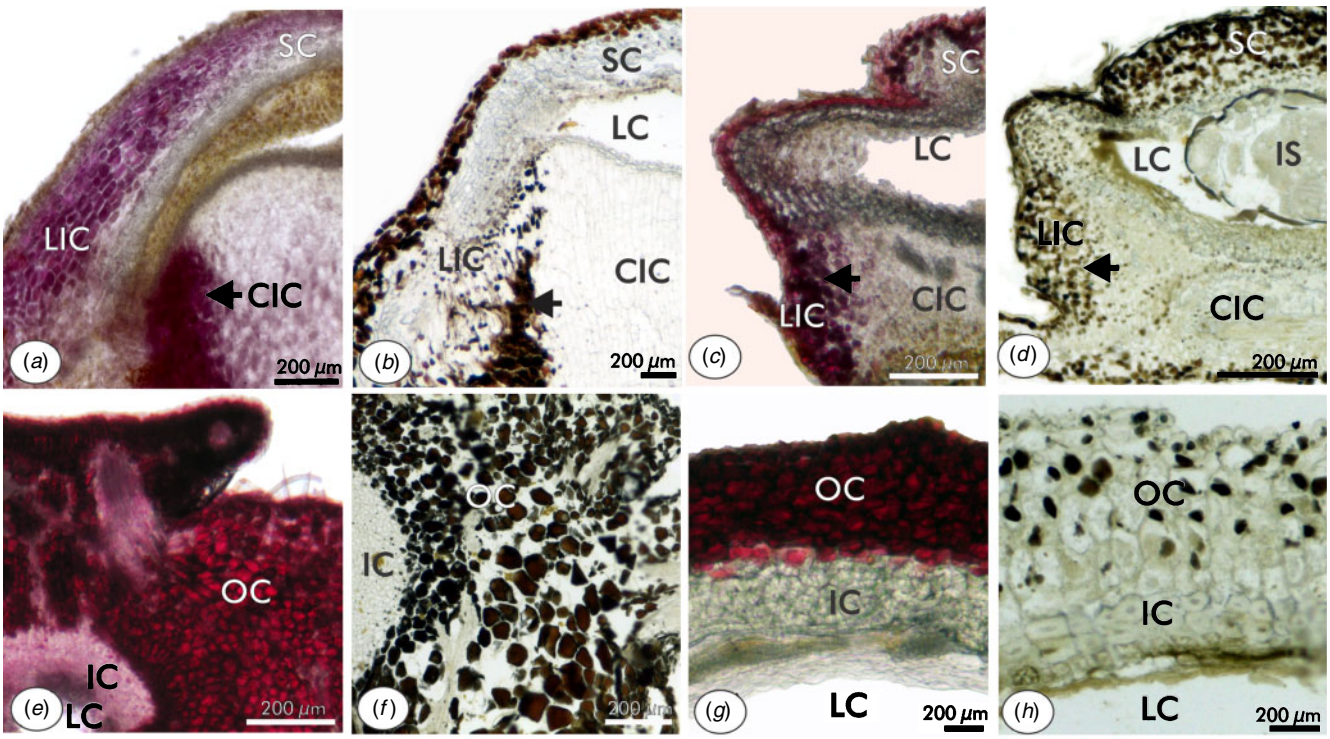

Fig. 3. Histochemical tests in sections of mature galls on *Piptadenia gonoacantha* (Mart.) JF.Macbr. (Fabaceae). (*a*, *c*, *e*, *g*) Detection of indole-3-acetic acid (IAA). (*b*, *d*, *f*, *h*). Detection of phenolics accumulation. (*a*, *b*) Lenticular concave-convex gall. (*c*, *d*) Lenticular plane-convex galls. Lenticular gall morphotypes show accumulation of IAA and phenolics in the superior cortical and lateral cells of the inferior cortex (black arrows). (*e*, *f*) Globoid gall with emergences. (*g*, *h*) Globoid galls. Globoid gall morphotypes show homogeneous accumulation of IAA and phenolics throughout the outer cortex, which involves the gall structure. CIC, central inferior cortical cells; LIC, lateral inferior cortical cells; IC, inner cortex; IS, gall-inducing insect; LC, larval chamber; OC, outer cortex; and SC, superior cortex.

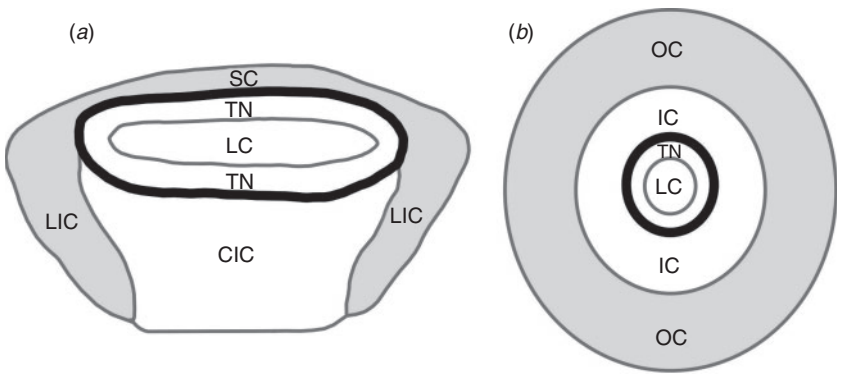

Fig. 4. Diagrams of transverse sections of galls showing sites of indole-3-acetic acid (IAA) detection (grey). (*a*) Lenticular galls and (*b*) globoid galls. CIC, central inferior cortical cells; IC, inner cortex; LC, larval chamber; LIC, lateral inferior cortical cells; OC, outer cortex; and SC, superior cortex, black (sclerenchymatic cells).

separated from the LC mainly by the PC2, and from the LP by the PC1 and PC2, at the young and mature stages. The young GE was separated from the young LC by the PC1, whereas the mature stages were separated by the PC1 and PC2. The GE was separated from the LP at both developmental stages by the PC1 (Fig. 5).

Degree of anisotropy, direction and type of cell expansion

In the NGP, the cells of the PP have a high degree of anisotropy, whereas the SP and the epidermal cells tend to have an isotropic expansion (degree of anisotropy next to 1; Table 2). The direction of cell expansion in the NGP is predominantly periclinal, except for the PP, where the cells expand anticlinally.

Most of the cells of the four gall morphotypes have an anisotropic expansion, with few cells tending toward isotropic expansion (Table 2). The cells of the LC expand periclinally, except for the IC cells at a mature stage. The cells of the SC are ~7.3-fold expanded in relation to the original size of the PP cells (Table 3). The cells of the LP expand periclinally both at young

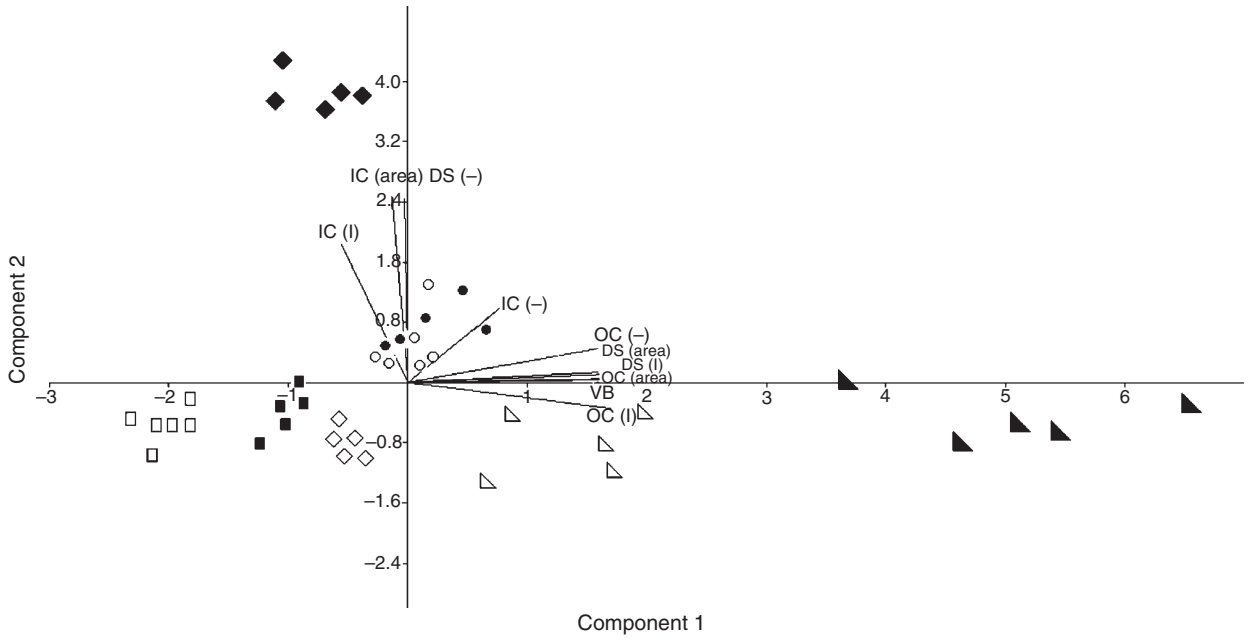

Fig. 5. Representation of the scores of the first two axes of the principal-component analysis (PCA) of the cytometrical and hystometrical traits of non-galled pinnula (NGP) and pinnula galls induced on *Piptadenia gonoacantha* (Mart.) JF.Macbr. (Fabaceae). Squares represent lenticular plane-convex galls, rhombus represent lenticular concave-convex galls, circles represent globoid galls, and triangles represent globoid galls with emergence. Black, mature stage of galls; white, young stage of galls; (I), height; (-), width.

Table 2. Degree of anisotropy represented by the ratio between the maximum and minimum average axes of the cells of non-galled pinnula (NGP) and pinnula galls on *Piptadenia gonoacantha* (Mart.) JF.Macbr. (Fabaceae)

AD, adaxial epidermis; DS, dermal system; GE, globoid with emergences; GG, globoid; IC, parenchyma of the inner (or interior) cortex; LC, lenticular concave-convex; LP, lenticular plane-convex; M, mature; NT, nutritive tissue; OC, parenchyma of the outer cortex; PP, palisade parenchyma; SC, parenchyma of the superior cortex; SL, sclerenchyma cells; SP, spongy parenchyma; Y, young; asterisk indicates cells of NGP

NGP gall/stage	Degree of anisotropy				
	AD*/DS	PP*/OC	SP*/IC	SL	NT
NGP	1.10	3.59	1.11	–	–
LC/Y	1.73	1.71	1.69	1.7	2.71
LC/M	4.56	1.95	2.24	2.24	1.58
LP/Y	1.83	2.18	3.73	-	1.51
LP/M	1.66	2.19	3.95	1.47	1.46
GE/Y	1.44	1.62	1.30	1.31	1.36
GE/M	1.16	1.81	1.36	1.56	1.27
GG/Y	1.30	1.32	1.44	–	2.17
GG/M	1.42	1.08	1.15	–	1.53

Table 3. Number of folds that the cells of galls at young and maturation stages increased when compared with the original cells of the non-galled pinnula of *Piptadenia gonoacantha* (Mart.) JF.Macbr. (Fabaceae)

DS, dermal system; GE, globoid with emergences; GG, globoid; IC, parenchyma of the inner (or interior) cortex; LC, lenticular concave-convex; LP, lenticular plane-convex; M, mature; NT, nutritive tissue; OC, parenchyma of the outer (or superior) cortex; VB, vascular bundles; Y, young

Gall/stage	DS	OC	IC	VB	NT
LC/Y	2.30	3.46	1.40	2.49	1.38
LC/M	1.71	7.30	5.45	4.85	4.10
LP/Y	1.55	1.92	1.87	1.46	0.59
LP/M	2.59	2.15	1.70	1.52	0.72
GE/Y	2.59	9.97	1.35	5.59	1.57
GE/M	3.25	30.13	1.60	14.51	3.38
GG/Y	2.60	3.62	2.92	0.56	2.82
GG/M	2.77	3.78	4.06	0.76	4.06

and mature stages. The cells of the DS are ~2.6-fold larger, and the cells of the SC are ~1.7-fold larger in relation to the original size of the PP cells (Table 3). All the cells of the GE and GL, in both developmental stages, expanded periclinally. The highest degree of cell expansion in GE occurs in the cells of the OC, that are ~30.1-fold larger in relation to the original cells of the SP (Table 3). In the GG, the cells of the outer and inner cortices are ~3.7 and ~4.0 -fold larger than the original cells of the PP and SP, respectively (Table 3). The transverse and longitudinal axes of the cells changed in the four gall morphotypes on *P. gonoacantha*, indicating that the hypertrophic processes contribute to the final shape of all the gall morphotypes.

Discussion

Developmental patterns and the complexity of gall morphotypes

The different galls are products of distinct host plant-cell reactions to the mechanical and chemical stimuli generated by different galling insects, which is shown by the distinctiveness of the generated morphotypes (Isaias *et al*. 2013, 2014). A similar result was observed for *P. gonoacantha.* The LP are very similar to the host leaves, both in anatomical and morphological features,

and accordingly can be considered the simplest galls analysed here. Such limited distinctiveness between the simple galls and their non-galled host leaves has previously been discussed for the galls on *Baccharis reticularia* DC. (Asteraceae) (Formiga *et al*. 2015). The GE were the largest of the gall morphotypes on *P. gonoacantha*, with the highest number of cortical cell layers, and the most differentiated cells on the dermal system, with trichomes arising from the emergences. The complexity of the GE was corroborated on a quantitative basis by discrimination of cytometric and quantitative characteristics as the most separated group on the PCA analysis. The more the galls differ from their host organs, the greater the complexity of their structure, as observed for the globoid gall with emergences on *P. gonoacantha*.

Differences and similarities on the basis of morphotypes and the developmental stage

The cytometric and histometric data separated the galls on *P. gonoacantha*, both on the basis of morphotypes and by the developmental stage. The four gall morphotypes were separated mainly by the features of the outer cell layers, i.e. the height, width and area, height and area of epidermal cells, and the whole area of the vascular bundles, which comprised the PC1. Cell features separated the gall morphotypes at different developmental stages, except for the globoid galls. The development of the lenticular shape on the pinnula galls on *P. gonoacanhta* seemed to be more related to the high hyperplasia of the inner cortical cells at the transition from young to mature stage. However, the development of the globoid shape was due to the greater and gradual hypertrophy of the outer cortical cells at young and mature stages. The mature LC was different from the other three gall morphotypes by the width of epidermal cells, and the height and area of the inner cortical cells. During the development of the pinnula galls on *P. gonoacantha*, the disruption of the standard cell-elongation axes was altered in two new pathways, towards both the lenticular and globoid structures. The periclinal direction of growth and cell elongation predominated in all the gall morphotypes on *P. gonoacantha*, as reported for other galls such as the bivalve-shaped gall morphotypes (Isaias *et al*. 2011). In addition, the anisotropic expansion predominated in the gall distinct shapes on *P. gonoacantha*, and was considered crucial for the establishment of new elongation patterns during the development of plant organs (Baskin 2005; Crowell *et al*. 2010).

The feeding sites of the larvae and, consequently, the shape of the larval chamber have been classically reported as the determinant pressures for the development of the various gall shapes (Rohfritsch 1992). No such relationship could be found for any of the four galls on *P. gonoacantha* studied here. The two pairs of similar gall morphotypes evaluated, namely lenticular and globoid, seemed to have distinct cellular mechanisms involved in the generation of the new forms. These new forms are apparently independent of the shape of the larval chamber. In the GG, the larval chamber is located in the centre of the structure, whereas in the GE, the size and location varies along developmental stages. In the lenticular galls, the larval chambers are periclinally elongated, and separate the superior and inferior cortices. The cells of the superior and inferior cortices elongate periclinally to the longest axis of the larval chamber, except in the inferior cortex of the LC at the mature stage, where the cells elongate anticlinally. On the basis of this analysis, the shape of larval chambers does not seem to directly influence the final gall morphotype. The independence among the developmental patterns, the shape of the larval chamber, and the mode of the galling insects feeding have previously been proposed for the horn-shaped galls on the leaflets of *Copaifera langsdorffii* Desf. (Fabaceae) (Oliveira and Isaias 2009), and is yet a challenge to be evaluated in other gall morphotypes. Nevertheless, our study model strongly showed that mechanisms other than the shape of the larval chamber must be involved.

As an alternative pathway for the determination of gall shapes, Isaias *et al*. (2015) proposed the influence of a functional gradient established by the accumulation of reactive oxygen species (ROS) at gall sites. The ROS should accumulate in response to the insect feeding and respiration, with the sites of cell responses being independent of the contact with the feeding mouth apparatus of the galling herbivore. The relationship of ROS with phenolics and IAA accumulation was also evidenced in the lenticular concave-convex galls (LC) and rachis fusiform galls on *P. gonoacantha* (Bedetti *et al*. 2014), and strongly reinforces the alterations in metabolic status of host organs towards gall morphogenesis. In this perspective, ROS, phenolics and IAA could act together in the morphogenesis of the other gall morphotypes on *P. gonoacantha*, influencing the final shapes.

Phenolics and IAA accumulation and the final shape of the galls

The accumulation of phenolics at sites of cell hypertrophy was evidenced in all the four galls on *P. gonoacantha*, and has been broadly reported for many galls. It is also one of the most remarkable features of gall development reported both for temperate and neotropical systems (Abrahamson *et al*. 1991; Isaias *et al*. 2011, 2014). Nevertheless, the relation of cell hypertrophy to accumulation of phenolics is rarely discussed, and has been herein highlighted as an important step in the metabolic arrangement of gall developmental sites. The pattern of phenolics accumulation previously reported for the globoid galls induced on leaves of *Psidium myrtoides* (Myrtaceae; Carneiro *et al*. 2014*a*) is consistent with the current results for the accumulation of phenolics and IAA at sites of cell hypertrophy in galls on *P. gonoacantha*. During the developmental stages of the galls on *P. myrtoides*, tissue hyperplasia and cell hypertrophy occur at different regions of the cortex, determining the globoid gall shape (Carneiro *et al*. 2014*a*). This temporal balance and the new functions of the cells also seem to occur in the globoid and lenticular galls on *P. gonoacantha*, whose cells have different features, both in relation to shape and developmental stage. Taking for granted that accumulation of phenolics mediate cell responses to galling stimuli (Isaias *et al*. 2015), its histolocalisation within plant cells is crucial for the morphogenesis of the gall.

The accumulation of IAA and phenolics was detected at distinct tissue sites and was dependent on the gall mophotypes on *P. gonoacantha*. The lenticular galls (LP and LC) had accumulation of IAA and phenolics mainly in the outer cortical cells and in the lateral cells of the inferior cortex, which are the regions with the most hypertrophied cells. The globoid galls

(GG and GE) had homogeneous distribution of IAA and phenolics all over the outer cortex. The similar sites of accumulation of IAA and phenolics in each pair of *P. gonoacantha* gall morphotypes show the role of IAA-phenolics in the determination of final gall shapes. The relationship between the changes in the patterns of growth and cell elongation during the development of galls has been demonstrated in galls induced on *Lonchocarpus muehlbergianus*, and is coherent with the final gall size and shape (Isaias *et al*. 2011). Also, a cecidogenetic field established in a centrifugal pattern from the centre of the structure develops under the influence of the galling herbivore (Mani 1964). In *P. gonoacantha*, the cecidogenetic field seems to be different in the lenticular and in the globoid galls, because cell divisions and gradients of cell hypertrophy are peculiar to each gall morphotype. In the lenticular galls, the larval chambers are periclinally elongated and divide the cortex into superior and inferior regions, with a higher rate of cell divisions in the inferior cortex. In the globoid galls, the larval chamber is round and located in the middle of the inner cortex. There is a gradient of cell divisions, higher in the inner cortex and decreasing towards the outer cortex. The hypertrophy of the cortical cells in the globoid galls on *P. gonoacantha* occurs in a centrifugal gradient from the inner towards the outer cortex, similarly to the globoid galls on *P. myrtoides* (Carneiro *et al*. 2014*b*). In the lenticular galls of *P. gonoacantha*, cell divisions are higher in the central cells of the inferior cortex. The hypertrophy occurred towards the outermost layers of the superior cortex and of lateral cells of the inferior cortex, in two centrifugal gradients.

This is the first histolocalised map of the accumulation of IAA in relation to gall size and shape, and sets lights on the generation of two specific gall morphotyes, namely the lenticular and the globoid. Each gall-inducing insect promotes specific changes in the same original host-organ cells, with similar morphogenetical potentialities generating distinct sizes, degrees of anisotropy, direction and types of cell expansion.

Conflicts of interest

The authors declare no conflicts of interest.

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