DEVELOPMENTAL MORPHOLOGY OF BUD GALLS INDUCED ON THE VEGETATIVE MERISTEMS OF *QUERCUS CASTANEA* BY *AMPHIBOLIPS MICHACAENSIS* (HYMENOPTERA: CYNIPIDAE)

PAULINA HERNÁNDEZ-SOTO^{1, 4, 6}, MIGUEL LARA-FLORES², LOURDES AGREDANO-MORENO³,
LUIS FELIPE JIMÉNEZ-GARCÍA³, PABLO CUEVAS-REYES⁵ AND KEN OYAMA^{1, 4}

¹Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México. Morelia, Michoacán, México.

²Escuela Nacional de Estudios Superiores Unidad León, Universidad Nacional Autónoma de México. León, Guanajuato, México.

³Departamento de Biología Celular, Facultad de Ciencias, Universidad Nacional Autónoma de México. México, D.F.

⁴Escuela Nacional de Estudios Superiores Unidad Morelia, Universidad Nacional Autónoma de México. Morelia, Michoacán, México.

⁵Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo. Morelia, Michoacán, México.

⁶Corresponding author: gerco777@yahoo.com

Abstract: A gall is the result of complex interactions between a gall inducing-insect and its host plant. Certain groups of insects have the ability to induce a new structure, a gall, on plant organs by altering the normal growth of the host involved plant organ. The gall usually provides shelter and nutrients, in addition to protection against adverse environmental conditions and natural enemies to the inducing insect and its offspring. The ecological uniqueness of a gall is that it allows the inducing-insect to complete their life cycles. In this study, we have described the structures of different stages of growth of a gall induced by *Amphibolips michoacaensis* on the buds of leaves on *Quercus castanea* (Fagaceae) to know the subcellular changes during development. The gall consist of various layers such as a nutritive tissue, a lignified sheath, a spongy layer and an outermost epidermis around a centrally located larval chamber. The nutritive cell of the larval chamber show nuclear and nucleolar hypertrophy in the early phases of growth. The granular profile of the nucleolus suggests an active synthesis of ribosomes indicating an accelerated protein synthesis in these cells. During early stages of growth, the cells of the spongy layers within galls are nucleate and nucleolate and include amyloplasts, and the cytoplasm is less abundant. During later growth stages, the spongy cells are enucleate and enucleolate. Chloroplasts occur in the epidermal cells during early stages of growth, indicating that galls are photosynthetically active in early stages of growth. During intermediate stages of growth, a gradual loss of cellular components occurs commencing in the epidermal cells and progressing towards the nutritive cells.

Key words: *Amphibolips michoacaensis*, Cynipidae, gall morphology, Hymenoptera, *Quercus castanea*

Resumen: La formación de una agalla es el resultado de una compleja interacción entre el insecto inductor de agallas y su planta hospedera. Ciertos grupos de insectos tienen la habilidad de inducir una nueva estructura, la agalla, sobre los órganos de las plantas alterando el crecimiento normal del órgano de la planta involucrado. Las agallas usualmente proveen refugio y nutrientes, además de protección contra condiciones ambientales adversas y enemigos naturales al insecto inductor y a su descendencia. La particularidad ecológica de la agalla es que permite al insecto inductor completar su ciclo de vida. En este estudio, nosotros hemos descrito la estructura de los diferentes estadios de crecimiento de la agalla inducida por *Amphibolips michoacaensis* sobre brotes de hojas de *Quercus castanea* Née (Fagaceae) para conocer los cambios subcelulares durante su desarrollo. La agalla consiste de varias capas tales como tejido nutritivo, una capa de células lignificadas, tejido esponjoso y una capa epidérmica externa que rodean a una cámara larval central. Las células del tejido nutritivo de la cámara larval presentan hipertrofia de núcleo y nucléolo en las fases tempranas de crecimiento. El perfil granular del nucléolo sugiere una síntesis activa de ribosomas indicando un incremento en la síntesis de proteínas en estas células. Durante fases tempranas de crecimiento, las células del tejido esponjoso de la agalla son nucleados y nucleolados e incluye amiloplastos y el citoplasma es menos abundante. Durante las fases tardías del crecimiento, las células del tejido esponjoso son enucleados y enucleolados. Los cloroplastos se presentan en las células epidérmicas en las fases tempranas del crecimiento, lo que indica que las agallas son fotosintéticamente activas en estadios tempranos de crecimiento. Durante las fases intermedias de crecimiento se observa una pérdida gradual de los componentes celulares que comienza en las células de la epidermis y avanzan hasta las células nutritivas.

Palabras clave: *Amphibolips michoacaensis*, Cynipidae, Hymenoptera, Morfología de agallas, *Quercus castanea*

Approximately 13,000 species of insects belonging to Thysanoptera, Hemiptera, Coleoptera, Lepidoptera and Hymenoptera are known today as gall inducing agents (Dreger-Jauffret and Shorthouse, 1992; Raman *et al.*, 2005). Species of the Cynipoidea (Hymenoptera) induce complex and diversified galls on various plants (Ronquist, 1995, 1999). The Cynipidae are unique because a majority of their species recognized under Aylacini, Diplolepidini, Eschatocerini, Pediaspidini and Cynipini, are specialist plant feeders and they induce galls and only those of the Syndergini are associated with gall-inducing Cynipidae (Ronquist, 1994). A majority of the Cynipidae are associated with Fagaceae and particularly with *Quercus* (Ronquist and Liljeblad, 2001; Csoka *et al.*, 2005).

Gall-inducing insects are specialist plant feeders and many of them remaining tied to specific plant. The gall is a product of the interspecific association of the plant with the insect, which develops a specific novel structure in response of the insect stimulus (Weis and Abrahamson, 1986; Raman, 2007, 2011). Gall-inducing insects actively manipulate the host plant to induce a structure that provides nutrition to their larvae and protection against adverse environmental conditions and natural enemies (Price *et al.*, 1987; Stone and Schönrogge, 2003). The galls generated by the Cynipidae are the most diverse and structurally complex and they are considered the most evolved galls produced by insects (Nieves-Aldrey, 1998; Raman *et al.*, 2005; Csoka *et al.*, 2005). The galls induced by the Cynipidae comprise multiple and highly differentiated cell layers. The nutritive tissue and lignified sheath enveloping the larva form the “inner gall” and the cortical tissue the “outer gall” (Bronner, 1977). The internal gall size is constant while the external gall usually varies according to species (Nieves-Aldrey, 1998).

Development of a gall can be seen to include three phases: initiation, growth and maturation (Maresquell and Mayer, 1965). The gall development starts with the wasp laying eggs in the meristematic tissue of the host plants (Bronner, 1973; Rey, 1992). The plant usually responds with necrosis of cells immediately beneath and around the egg, whereas the adjacent cell layers below and around begin to proliferate. During the growth phase, the cells surrounding the larva turn hypertrophied and hyperplased, both of which are the most visible responses in gall growth process (Meyer and Maresquell, 1983). The cells ligning the larval chamber are differentiated as nutritive cells, feeding the growing larvae (Bronner, 1992; Rey, 1992; Brooks and Shorthouse, 1997). The criticality of the developing cynipid larva for the growth and maturation of the gall has been demonstrated: the gall ceases to grow, should the larvae die prematurely (Rey, 1992; Brooks and Shorthouse, 1997; Nieves-Aldrey, 2001). Finally, the maturation phase is characterized by a decrease in cell division activity, most of the gall tissues turn lignified as the larva matures and pupates. The gall eventually desiccates.

Although the overall morphology of cynipid galls may vary, the patterns of the internal tissues layers of the gall are similar in the know galls induced by the Cynipidae, with an outer cortical parenchyma and an inner cavity that contains one to many larval chambers. Each larval chamber is usually encapsulated by a layer of sclerenchyma. Lining the sclerenchyma is the inner gall composed of nutritive parenchyma cells that accumulate lipids, sugars and amino acids (Bronner, 1992; Harper *et al.*, 2004). Starch concentration increases away from the larval chambers while enzymes, lipids and sugar concentration increase towards the larval chambers (Bronner, 1992). All Cynipidae generate this essential nutritive tissue that serves as a source of nourishment for the larva (Rey, 1992). Bronner (1992) reports specific characteristics for inner nutritive cells such as enlarged nucleus and nucleolus, fragmented vacuole, low content of starch and high contents of lipids and proteins.

About 80 % of all the Cynipidae galls form on oak (*Quercus*) and rose (*Rosa*) (Ronquist and Liljeblad, 2001; Harper *et al.*, 2004). The majority of the few studies about Cynipidae galls have been made in roses (LeBlanc and Lacroix, 2001; Leggo and Shorthouse, 2006), and one or two about the internal structure of galls induced by cynipid on *Quercus*; the knopper galls on acorns of *Q. robur* induced by *Andricus quercuscalicis* and “Oak Apple Gall” in buds of the English oak *Q. robur* by *Biorhiza pallida* (Harper *et al.*, 2004).

However, there are no reports of ultrastructural studies in *Quercus* galls induced by the Cynipidae in Mexico, with approximately 160 species; Mexico is one of the centers of species diversification of genus *Quercus* (Nixon, 1993). In this study, we described the morphological and anatomical characteristics in different stages of growth of the bud gall induced by *Amphibolips michoacaensis* on *Quercus castanea* an endemic species with a wide distribution in Mexico. We compared the cell structure of different stages of growth of galls using electron micrographs of the tissues to know the changes involved in the different layers during the development of the gall wasp.

Material and methods

Collection of specimens. Galls of *Amphibolips michoacaensis* Nieves-Aldrey & Maldonado (Cynipidae) were collected in April-May in 2011 and 2012 on trees of *Quercus castanea* in central Mexico (19°66'17.5" N; 101°16'70.2"W). Galls of different sizes that contained larvae were sampled and measured their diameter, smalls (7 mm diameter), mediums (14-24 mm diameter) and big (36-56 mm diameter) (n = 80). The collected galls were dissected and the tissue of larval chamber, spongy tissue and epidermis were taken for further analysis. *Amphibolips michoacaensis* is a new species described recently from galls from *Q. castanea* (Nieves-Aldrey *et al.*, 2012).

Electron microscopy. Tissues were prepared following the protocols described in Jiménez-García and Segura-Valdéz (2004). Briefly, tissues were fixed with 4 % paraformaldehyde plus 6 % glutaraldehyde for 24 h at room temperature 30 °C. They were then postfixed with 1 % osmium tetroxide for 3 h and dehydrated with a series of graded ethanol and propylene oxide. Embedding was performed with epoxy resin at 60 °C for 16 h. Thin sections 250-350 nm, were placed on copper grids, stain with 3 % toluidine blue and covered with Formvar. Sections were contrasted with 5 % uranyl acetate and 0.5 % lead citrate. Grids were observed with a transmission electron microscope (JEOL 1010, JEOL, Peabody, MA) working at 80 kV. Images were obtained with a charge-coupled device camera coupled to the microscope.

Results

Amphibolips michoacaensis induced galls in the buds of *Quercus castanea*. The spherical galls measured up to 60 mm of diameter; they are soft to human feel and spongy and green during early stages and turns brown on maturation (Figure 1A, B). The gall induced by *A. michoacaensis* includes a centrally located single larval chamber occupied by one larva (Figure 1C). Three layers of tissue occur in the galls induced by *A. michoacaensis*: the nutritive, the spongy and the epidermal cells.

The nutritive cells in growing galls of 6 mm of diameter showed a centrally located large vacuole, with a few amyloplasts in the cytoplasm (Figures 2A, 3A) and hypertrophy of nucleus and nucleolus (Figures 2A, 3B, C). The nucleolus appears granular (Figure 4B). In the cytoplasm, the endoplasmic reticulum and many ribosomes were present (Figures 4A, C). In the cells of spongy tissue, the number of amyloplasts were more than that were evident in the nutritive cells (Figures 2B, 3D) and the structure of nucleus and nucleolus present a certain amount of degradation in their structures compared with those of nutritive cells (Fig-

ures 3E, F). Changes in nucleus morphology were visible: a segregated nucleus, less compact and less dense is markedly reduced compared with nutritive cells while central nucleolus present blanching regions and less dense. The epidermal cells show thickened cell walls, many chloroplasts (Figures 2C, 3G, H) and numerous mitochondria (Figure 3I).

When gall size increases to 14 mm of diameter, some structural changes were observed in their cell layers. The nutritive cells showed hypertrophy of nucleus and nucleolus, the nucleus appears segregated less dense and less compact, the nucleolus present blanching regions, the morphology the nutritive cells are similar to the spongy cells in galls of 6 mm of diameter (Figure 5A). The number of amyloplasts increased in nutritive cells (Figure 5A), than in previous sizes (6 mm in diameter) (Figure 3A), and large vacuole fragmented occurred (Figures 2D, 5A). The cells of spongy tissue had a few amyloplasts, appear cytoplasmic remains, a thickened cell wall, few cells had nucleus and nucleolus and the vacuoles were fragmented (Figures 2E, 5B, C). In the epidermal cells thickened cell walls and few cytoplasmic remains can be observed (Figures 2F, 5D). The epidermal cells did not show chloroplasts compared with the smaller galls that had a large number of chloroplasts (Figure 3H); only thickened cell walls and few cytoplasmic remains can be observed (Figures 2F, 5D).

Some nutritive cells in galls of 24 mm of diameter showed a gradual degradation process of nucleus and nucleolus, while in other cells the nucleus and nucleolus remained intact in structure with a prominent nuclear envelope (Figures 6A-C). In some nutritive cells amyloplasts were evident (Figure 6B). The vacuoles of nutritive cells were fragmented and autophagic vacuoles were apparent (Figures 6C).

In the spongy tissue, cells with large vacuoles and cells with fragmented vacuoles were present (Figure 6D, E), the cell walls were thick and autophagic vacuoles were apparent (Figure 6E) and cytoplasmic debris were present in some cells with greater levels than the others (Figure 6E,

Figure 1. Galls induced by *Amphibolips michoacaensis* on *Quercus castanea*. A Galls in different stages of growth in the tree, the galls are smooth and green light in color. B Mature galls, the growth stop and the gall has a brown color and hard tissue; in these galls, the holes made by the insects from inside to leave the gall after a complete development can be observed (arrows). C Cross-section of mature gall, the single larval chamber (Lc) in the center of the gall surrounded by sclerenchyma lignified cells (light brown sheath) and the tunnel made by the insect can be observed.

Figure 2. Cross-section of gall induced by *Amphibolips michoacaensis*; single larval chamber (Lc), nutritive (N), spongy (S) and epidermal (E) cells (color photo at the left). A-C Light micrographs of the different layers in gall of 6 mm in diameter. A Nutritive cells present hypertrophied nucleus (N) and nucleolus (n) and big central vacuole (V). B Spongy cells present numerous amyloplasts (Am). C Epidermal cells show numerous chloroplasts (Ch). D-F Light micrographs of the different layers in gall of 14 mm in diameter. D Nutritive cells show hypertrophied nucleus (N) and nucleolus (n). E Spongy cells present fragmented vacuoles (V), few cells present nucleus (N) and nucleolus (n). F Epidermal cells had only cytoplasmic remains (Cr) and thickened cell walls (Cw).

F). The most epidermal cells include cytoplasmic debris (Figures 6G, H), whereas other cell were empty (Figure 6I). Both spongy and epidermal cell layers bore thickened cell walls.

The nutritive cells of galls of 36 mm of diameter rarely include a nucleus and a nucleolus (Figure 7A) and the number of autophagic vacuoles and the content of cytoplasmic remains increased (Figure 7B) compared with the nutritive cells in galls of 24 mm of diameter (Figure 6C), resembling more to the cells of spongy tissue of galls of 24 mm of diameter (Figure 6D). There are a few spongy cells with cytoplasmic remains and most of them kept the cell membrane (Figures 7C, D). The epidermal cells were vacant (Figure 7E). The morphology of cells in the spongy tissue and epidermal cells were nearly identical at this stage.

Nutritive cells of galls of 56 mm of diameter showed few cytoplasmic remnants (Figures 8A, B) similar to cells of spongy tissue in galls of 36 mm in diameter (Figure 7C). Most of the spongy cells were empty, cells had lignified walls and few cells still included cytoplasmic remains (Figures 8C, D). Epidermal cells were empty (Figures 8E, F). In this gall size, there was no real differences between the three different layers of cells that form the gall; nutritive, spongy and epidermal cells.

Discussion

The present study shows that the structure of gall induced by *Amphibolips michoacaensis* in different stages of growth is similar to galls induced by others Cynipidae such as hypertrophied nucleus and nucleolus and abundance of ribosomes in nutritive cells (Rothfritsch, 1974; Bronner, 1992; LeBlanc and Lacroix, 2001; Leggo and Shorthouse, 2006).

Bronner (1992) reported numerous plastids and mitochondrias in the nutritive cells of the Cynipidae galls, whereas in the galls induced by *Amphibolips michoacaensis* both mitochondria and chloroplasts occurred in epidermal cells of galls during early stages of growth. In galls induced by *Diplolepis rosaefolii* (Cynipidae) on leaves of *Rosa virginiana*, fragmented vacuoles occur in cells located at the periphery of nutritive tissue but they are either reduced or absent in cells closet to the larval chamber (LeBlanc and Lacroix, 2001). In galls induced by *A. michoacaensis*, the cells of nutritive and spongy tissues present large vacuoles during early stages of growth, whereas during later stages of growth they appear fragmented until their disappearance finally. In galls induces by the Cynipidae, *Andricus quercus-calycis* and *Biorhiza pallida* on *Quercus robur*, the immediate layers surrounding the larva had enlarged cells filled

Figure 3. Ultrastructural morphology of galls with 6 mm in diameter. A-C Cells in nutritive tissue. The nucleus (N) and nucleolus (n) are hypertrophied, the cytoplasm contains few amyloplasts (Am) and a big vacuole (V). D-F Cells in spongy tissue. The internal organization of the nucleus (n) is lost. An increment in the number of amyloplasts (Am) is observed in cytosol (Cyt). G-H Cells in epidermal tissue containing mitochondria (Mit) and numerous chloroplasts (Ch) (bar = 1 μ m).

Figure 4. A-C Ultrastructural morphology of nutritive cells in galls of 6 mm in diameter. The nucleus (N) and nucleolus (n) are dense; the nucleolus has a granular structure and the cytoplasm (Cyt) present rough endoplasmic reticulum (ER) and ribosomes (Ri) (bar = 1 μ m).

Figure 5. Ultrastructural morphology of galls of 14 mm in diameter. A Cells in nutritive tissue surrounding the larval chamber present engrossed cell walls (Cw), abundant amyloplasts (Am), hypertrophied nucleolus (n) and cytoplasm (Cyt). B-C Spongy tissue cells, single or some vacuoles (V) per cell and less amyloplasts (Am) than proximal cells to the chamber. D Cells in epidermal tissue show cytoplasmic remains (Cr) and engrossed cell wall (Cw) (bar = $1\mu\text{m}$).

Figure 6. Ultrastructural morphology of galls of 24 mm in diameter. A-C Cells of nutritive tissue present hypertrophied nucleus (N) and nucleolus (n) with loss of structure, present nuclear membrane (Nm) and in some cells appear autophagic vacuole (Va). D-F Cells of spongy tissue show big and fragmented vacuoles (V), autophagic vacuoles (Va) with poor cytoplasmic content (Cyt) and occasionally cells with nucleus (N) and nucleolus (n). G-I Epidermal cells show membranes disjoined to cell wall (Cw), remains cytoplasmic (Cr) and engrossed cell wall (bar = $1\mu\text{m}$).

Figure 7. Ultrastructural morphology of galls of 36 mm in diameter. A-B Nutritive cells present a great amount of autophagic vacuoles (Va) and few cells with nucleus (N) or nucleolus (n). C-D Spongy cells present cytoplasmic remains (Cr) and thickened cell wall (Cw). E The epidermal cells present cell membrane (Cm) and thickened cell wall (Cw) (bar = 1 μ m).

Figure 8. Ultrastructural morphology of gall of 56 mm in diameter galls. A-B Nutritive cells present few cytoplasmic remains (Cr). C-D Spongy tissue cells present cell wall (Cw) and cytoplasmic remains (Cr). E-F Epidermal cells (bar = 1 μ m).

with lipidic materials with enlarged nuclei and nucleoli (Harper *et al.*, 2004), agree with the found in nutritive cells in the galls induced by *A. muchoacaensis*. The presence of chloroplasts in epidermal cells suggest photosynthetic activity during early stages of gall growth.

The most impressive events of growth and metabolic process during gall development, such as proteins synthesis and generation of energy materials, occur during early stages of growth. The nucleolus, where the ribosomal subunits are assembled, appears granular (Hernández-Verdún, 2006). The nucleolar hypertrophy and the increment of ribosome biogenesis in plant cells stimulate induce proliferation as a result of greater biosynthetic demand (Montanaro *et al.*, 2008). In later stages of growth, the total absorption of this energy, the assimilation of proteins and the absorption of all cellular components occurred; these processes occurred from outer to inner cell layers. All these processes finished with a thickened cell wall maintaining the shape and hardness of the galls that provide protection to the wasp during the pupal stage (Stone *et al.*, 2002).

The larvae of gall-inducing Cynipidae can alter the physiology of host tissues (Leggo and Shorthouse, 2006), the structure and anatomy of layers of gall is related with the stage of development of insect inside of this structure. The changes in the nutritive, spongy and epidermal cells show the specific requirements of insect in growth and metamorphosis.

A complex interaction between insects and plants manifests in galls induced by insects, among which the most diversified group is the Cynipidae (Stone and Schönrogge, 2003). Galls provide food, shelter and protection to the larvae of the Cynipidae enabling them to complete their life cycles. In this study, we explain the differentiation and structure of cells that make the galls induced by *Amphibolips muchoacaensis* on *Quercus castanea* leaves.

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