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ARTICLE

Factors influencing the morphogenesis of galls induced by *Calophya mammifex* (Calophyidae) on *Schinus polygama* (Anacardiaceae) leaves

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Abstract: Environment, plant, and gall-inducing insect genotypes are key factors in determining the morphogenesis of galls. However, the exact roles of these factors have not been clarified. We used anatomical and histochemical methods to evaluate the determining factors in the final structure of galls induced by *Calophya mammifex* on leaves of *Schinus polygama* (Cav.) Cabrera under the Mediterranean climate conditions of southern Chile. Also, we compared mature galls with those induced by the congeneric *Calophya rubra* on the same host plant. *Calophya mammifex* develops a univoltine life cycle and a diapause period in the Mediterranean climate conditions of southern Chile. Morphogenetic and histochemical leaf patterns were altered by *C. mammifex* feeding activity. For the first time, two specialized tissue compartments, a nutritive-like tissue and a common storage tissue, are reported for Calophyidae-induced galls in the Mediterranean region of southern Chile. Galls induced by *C. mammifex* and *C. rubra* have sufficient anatomical and histochemical alterations to be diagnosed as complex structures, whose distinction in vascular system differentiation implies structural constraints imposed by host plant organs.

Key words: calophyids, gall, histochemistry, morphogenetic potentialities, Mediterranean climate, univoltinism.

Résumé : L'environnement, la plante et le génotype des insectes qui induisent la galle constituent des facteurs clés de la détermination de la morphogenèse des galles. Toutefois, les rôles précis que jouent ces facteurs n'ont pas été clarifiés. Les auteurs ont utilisé des méthodes anatomiques et histochimiques pour évaluer les facteurs déterminants de la structure finale des galles induites par *Calophya mammifex* sur les feuilles de *Schinus polygama* (Cav.) Cabrera, dans les conditions climatiques méditerranéennes du sud du Chili. Ils ont aussi comparé les galles matures de celles induites par le congénère *C. rubra* sur la même plante hôte. *Calophya mammifex* adopte un cycle de vie univoltin et une période de diapause dans les conditions climatiques méditerranéennes du sud du Chili. Les profils morphogénétiques et histochimiques des feuilles étaient modifiés par l'activité trophique de *C. mammifex*. On décrit pour la première fois deux compartiments tissulaires spécialisés, un tissu de type nutritif et un tissu d'entreposage commun, dans les galles induites par les Calophyidae dans la région méditerranéenne du sud du Chili. Les galles induites par *C. mammifex* et *C. rubra* présentent suffisamment de modifications anatomiques et histochimiques pour être diagnostiquées comme structures complexes, dont la distinction dans la différenciation du système vasculaire implique des contraintes structurales imposées par les organes de la plante hôte. [Traduit par la Rédaction]

Mots-clés : calophyides, galle, histochimie, potentialités, climat méditerranéen, univoltisme.

Introduction

Gall inducers are biotic factors capable of altering the morphogenetic fate of host plant cells, generating specific gall phenotypes for each host plant (Isaias and Oliveira 2011). *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) responds differently to four different gall-inducing insects: two Hemiptera (Calophyidae) and two Lepidoptera (Guedes et al. 2018). *Calophya mammifex* Burckhardt &

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Basset (Calophyidae) induces globoid leaf galls, the most common morphotype, whereas *Calophya rubra* Blanchard (Calophyidae) induces conical stem galls on *S. polygama* (Burckhardt and Basset 2000; Guedes et al. 2018). Insects with similar feeding habits, such as the calophyids, are expected to influence host plant tissues similarly (Rohfritsch 1992). Moreover, plant responses to gall inducer feeding habits should also be considered, for they are specific and specialized (Meyer 1987). Galls are host plant cell build-ups, and their development must be constrained by host plant organ and cell morphogenetic potentialities (Isaias and Oliveira 2011), to consequently determine whether galls may develop complex or simple structures (Formiga et al. 2015).

Histological, histochemical, and histometric alterations, as well as the differentiation of specialized storage cells and tissues, imply distinct levels of structural complexity among galls (Ferreira et al. 2017*a*). Galls with little organization and tissue differentiation, several layers of parenchymatic cells, and no metabolic storage tissues are considered simple galls (Rohfritsch and Anthony 1992; Isaias et al. 2014; Ferreira et al. 2017*a*). Gall complexity may be attributed to vascular bundle development and primary metabolite storage (Ferreira et al. 2017*a*), features that confer an intermediate level of structural complexity to Calophyidae-induced galls (*Calophya* cf. *duvauae* and *C. rubra*) (Dias et al. 2013*a*; Guedes et al. 2018).

Although dense trichomes, large nymphal chambers, and tissue hyperplasia have been described in *C. mammifex* mature globoid leaf galls on *S. polygama* (Guedes et al. 2016), their anatomical development remains unknown. Phenological, anatomical, and histochemical peculiarities of the *S. polygama* – *C. rubra* system associated with Mediterranean climate conditions of southern Chile have been recently studied (Guedes et al. 2018), and could be extended to the *S. polygama* – *C. mammifex* system, as evaluated in this study. Therefore, the current study model is nongalled *S. polygama* leaves, as control organs, and galls induced by *C. rubra* (Guedes et al. 2018), a congeneric species of *C. mammifex*, for comparative analysis of structural alterations.

Although synergistic interactions among environment, host plant, and inducer genotypes can determine specific gall phenotypes (Weis et al. 1988; Abrahamson and Weis 1997), their roles in determining gall morphology is not clearly understood (Moura et al. 2008). The *S. polygama* superhost and *C. mammifex*/*C. rubra*-induced galls constitute an appropriate model to study the role of host organ constraints on gall development and on the extended phenotypes of galling herbivores. The following questions are addressed: (*i*) What are the diagnostic features of *C. mammifex* leaf galls on *S. polygama*? (*ii*) Should galls induced by *C. mammifex* and *C. rubra* have similarities? And (*iii*) are there morphogenetic constraints in host leaves expressed on *C. mammifex* induced galls?

Materials and methods

Sampling and collection site

A *S. polygama* population located in the Mediterranean climate of southern Chile, Bio-bío Region, Ñuble Province, at kilometre 4 on the Itata highway (36°39′32″S, 72°16′43″W at 150 m a.s.l.) was studied. The Mediterranean climate of southern Chile has cold, wet winters (June–August) and hot, dry summers (December–February) (Giorgi and Lionello 2008); particularly, the climatic regime of central Chile is characterized by the absence of summer rainfall and associated thunderstorm activity (Armesto et al. 2007). Meteorological data were obtained from Weatherbase (http://www.weatherbase.com). During the study period, July 2015 and March 2016, the highest temperatures were recorded in January 2016 (36 °C) and the lowest in June, July, and August 2015 (–3, –1, and –3 °C, respectively). The month with the highest average precipitation was June (185.4 mm), and the month with the lowest average precipitation was February (2.5 mm).

For vegetative phenology, we randomly marked 10 *S. polygama* individuals and observed them monthly. Leaf sprouting, senescent leaves, and leaf fall were evaluated visually. We collected branches with and without galls from each tree monthly, which were stored in plastic bags and transferred to the Laboratory of Natural Product Chemistry at the Universidad de Concepción. On each branch, we assessed 20 leaves (n = 20) with galls by direct observation and registered gall position as follows: apex, mid-portion, basal regions, leaf margin, and leaf lamina.

We randomly marked five trees for anatomical and cytohistometric analysis and collected five (n = 5) nongalled mature leaves (NGL) and globoid leaf galls (GLG) from each tree at growth and development, maturation, and senescence stages (n = 5 per developmental stage). Leaf galls were sorted according to insect instar (Guedes et al. 2018), as follows: growth and development, first to fourth instar; maturation, fifth instar; and senescence, open gall and absent gall inducer. In addition, conical stem galls (CSG) in maturation phase (n = 5) were collected for comparison with GLG. Also, some GLG were dissected with a razor blade under the stereomicroscope and grouped according to insect instar. Nymphs were collected and preserved in 70% ethanol, then sent to Daniel Burckhardt at the Natural History Museum Basel (NHMB) for species identification. Several voucher specimens were deposited in NHMB under accession numbers NMB-PSYLL0004288 – NMB-PSYLL0004293.

Anatomical and cytohistometric analysis

Following each collection, the NGL, GLG, and CSG samples (n = 5) were fixed in 4% Karnovsky (2.5% glutaraldehyde and 4.5% formaldehyde in phosphate buffer (0.1 mol·L^{-1}, modified to pH 7.2) (O'Brien and McCully 1981), 2.5% glutaraldehyde (Karnovsky 1965), or FAA (37% formaldehyde, glacial acetic acid, and 50% ethanol, 1:1:18 *v*/*v*/*v*), and subsequently stored in 70% ethanol.

Fixed samples were dehydrated in *n*-butyl series (Johansen 1940), embedded in Paraplast (Kraus and Arduin 1997), sectioned (12–18 μm) with a rotary microtome (Leica 2035 Biocut), stained with 0.5% astra blue and safranin (9:1 *v*/*v*) (Bukatsch 1972), and mounted with clear varnish (Paiva et al. 2006). The histological slides were observed and photographed using a light microscope (Leica DM500) coupled with a digital camera (Leica ICC50 HD).

Histometric and cytometric data were obtained from NGL, GLG, and CSG photomicrographs using AxioVision LE software (CarlZeiss MicroImaging, Jena, Germany). For measurements, five different sections from each sample were used, and three different cell and tissue measurements from each section were taken. To compare NGL and GLG, adaxial surface cuticle thickness, adaxial and abaxial epidermal cell thickness, and the number of mesophyll cell layers were measured. In addition, parenchyma and abaxial and adaxial epidermal cell areas in transverse sections of both NGL and GLG were measured. To compare GLG and CSG, the number of vascular bundles, the number of parenchyma cell layers in a transverse gall section, and parenchyma tissue thickness in gall walls were measured. Parenchymatous cell area and nymphal chamber area were also measured.

Histochemical analysis

For histochemical reactions, fresh samples of mature NGL and GLG (n = 5) were fixed in 2.5% glutaraldehyde and 4.5% formaldehyde (4% Karnovsky, 0.1 mol·L^{-1}, pH 7.2) (O'Brien and McCully 1981) for 24 h, embedded in polyethylene glycol, and sectioned (20–40 μm) using a rotary microtome (Leica 2035 Biocut) (Ferreira et al. 2014, 2017*b*). Each section was subjected to histochemical tests for starch (Lugol's reagent; Johansen 1940), reducing sugars (Fehling's reagent; Sass 1951), lipids (Sudan IV and Sudan red B; Brundrett et al. 1991), and total proteins (mercuric bromophenol blue solution and acetic acid; Baker 1958). Treated sections were mounted on glass slides with 50% glycerin or water (Kraus and Arduin 1997), observed, and photographed with a light microscope (Leica DM500) coupled with a digital camera (Leica ICC50 HD), then compared with blank sections.

Statistical analysis

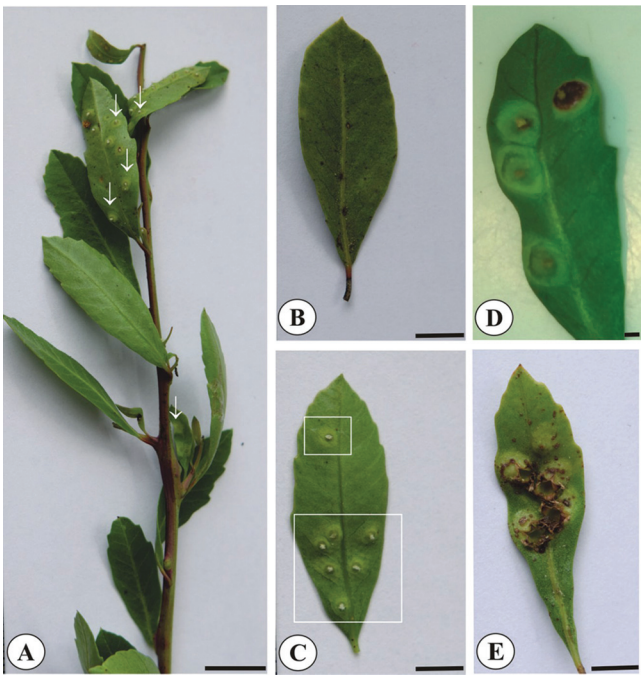
Student's t test was used to compare NGL with GLG, and GLG with CSG for each independent variable. Data normality was verified with the Shapiro–Wilk test. Differences were considered significant at a probability of 5% ($p < 0.05$). Statistical analysis was performed using InfoStat software (v.2013) (Rienzo et al. 2013).

Results

Phenology and morphological description of host organ and gall morphotype

Schinus polygama leaves are simple, elliptic, and usually glabrous with a conspicuous midrib (Figs. 1A and 1B). The first instar of *C. mammifex* (Hemiptera: Calophyidae) induces galls on the adaxial surface of the leaf lamina, generally near the midrib of young *S. polygama* leaves (Table 1). These galls are globoid, mostly located in the median and basal portions of the leaf, and less frequently in the apical region (Table 1).

Fig. 1. Nongalled leaves and *Calophya mammifex* leaf galls on *Schinus polygama*. (A) Branch with young leaves and galls at the beginning of growth and development phase (white arrows). (B) Mature nongalled leaf with conspicuous midrib. (C) Abaxial surface of leaf lamina with abundant galls at the beginning of growth and development stage closed by white trichomes (boxed areas). (D) Leaf lamina with abundant galls of different colours in the final stage of growth and development. (E) Leaf lamina with open senescent galls. Scale bars = 1 cm. [Colour online.]



At the beginning of growth and development, galling insects form a globular projection on the abaxial surface of the leaf lamina (Figs. 1A and 1C), with white trichomes that close the aperture of the gall. GLG are randomly distributed; number and colour can vary on the same leaf lamina (Fig. 1D), regardless of insect developmental stage. The abaxial surface can be red, green, or intermediate colour tones, while the adaxial surface generally remains green. Also, red and green galls are observed on the same leaf lamina (Fig. 1D). Senescent galls are dark brown and open to the abaxial surface (Fig. 1E) due to pressure exerted by adult terminalia.

Gall induction begins in mid-spring (October), and the first galls are observed in the growth and development phase at the end of spring in Southern Chile (November) (Fig. 2). From mid-summer to late winter (January to August), only immature *C. mammifex* instars (I, II, and III) are observed, indicating a diapause period in insect development. During the spring (November to December), galls open and the adults emerge, completing an annual life cycle. *Schinus polygama* is an evergreen plant with little

Table 1. *Calophya mammifex* (Hemiptera: Calophyidae) gall position on *Schinus polygama* (Anacardiaceae) leaves.

Position of galls on the leaf lamina	Total galls	%
Apex	66	14.9
Mid portion	211	47.5
Base	167	37.6
Leaf margin	100	22.5
Leaf inner	344	77.5
Total galls	**444**	—

phenophase demarcation. A leaf-flushing peak occurs during spring, and leaves with senescent galls fall from the host plant in late spring (November) (Fig. 2).

Anatomical features of nongalled leaves and leaf galls

Mature *S. polygama* leaves are amphistomatic, with a smooth cuticle and unistratified epidermis (Figs. 3A and 3B). Leaf lamina are bifacial with two layers of palisade parenchyma facing the adaxial surface and a variable number of spongy parenchyma cell layers facing the abaxial surface (Fig. 3A). The spongy parenchyma cells are loosely organized, and both palisade and spongy parenchyma have idioblasts with crystal druses (Fig. 3B). Secretory ducts associate with vascular tissues and are included in phloem parenchyma (Fig. 3A).

First instar nymph feeding activity induces a globoid gall with an ample chamber, sheltering a single gall inducer (Fig. 3C). Gall establishment occurs in the mesophyll, and a homogenous parenchyma is formed by hyperplasia and cell hypertrophy. Gall walls can be divided into an abaxial parenchymatous portion and an adaxial vascularized portion (Fig. 3D). The abaxial portion is formed of 7–10 nonvascularized cell layers with periclinal elongation (Fig. 3D). The abaxial projection terminates with the gall opening, from whence the adult emerges. The gall opening is covered by lignified multicellular uniseriate trichomes (Fig. 3C). The adaxial portion can be divided into three tissue compartments: outer, median, and inner compartments (Fig. 3D), derived from the adaxial, median, and abaxial layers of the leaf lamina ground meristem. The inner compartment is formed of 4–5 nonvascularized cell layers with periclinal elongation and a unistratified epidermis forming the nymphal chamber outline (Fig. 3E). In the median compartment, neoformed vascular bundles occur, with well-developed vascular parenchyma (Fig. 3F). Vascular bundles are numerous and the phloem is oriented towards the nymphal chamber (Fig. 3G). A single layered epidermis with a thick cuticle delimits the gall wall (Fig. 3H).

During growth and development, galls increase in size because of continuous divisions of parenchyma cells. In the maturation stage, a large number of vascular bundles are observed. The gall opening begins at the end of maturation, facilitating the adult's exit. The senescence stage begins when the gall opens fully and the insect escapes (Fig. 3I). During this phase, the abaxial portion degrades rapidly, tissues are disorganized, and in the gall opening, suberization and trichome decay occur (Fig. 3J). Druses abound in the adaxial portion (Fig. 3F) and are numerous towards the abaxial portion throughout senescence (Fig. 3K).

Histochemical profiles of leaves and galls

Starch grains were detected throughout the chlorophyllous parenchyma (Fig. 4A and 4B) in nongalled leaves, but they were not detected in mature galls. Reducing sugars were detected in the mesophyll, bundle sheath cells, radial parenchyma (Fig. 4C), outer and inner tissue compartments, and perivascular parenchyma of the galls (Figs. 4D and 4E). Lipid droplets were detected in the chlorophyllous parenchyma, vascular bundles, and secretory ducts in NGL, yet they were not as abundant in the epidermal cells (Fig. 4F). In GLG, lipids were detected both in outer and median compartments (Fig. 4G), as well as in phloem parenchyma cells (Fig. 4H). Proteins were observed in the spongy and vascular parenchyma of leaves (Fig. 4I). In GLG, proteins were mostly detected in median compartment cell walls (Fig. 4J).

Quantitative comparison

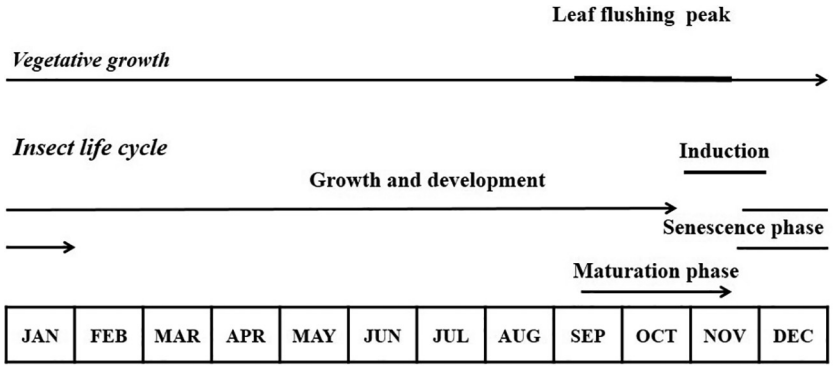
Every cytometric and histometric measurement was different between NGL and mature GLG, except for the abaxial epidermal cell area (Table 2). Adaxial cuticle thickness, adaxial and abaxial epidermal cell thickness, adaxial epidermal cell area, number of cortical cell layers, and parenchymatous cell area were all greater in mature galls than in NGL (Table 2). Abaxial epidermal cell area was similar in NLG and GLG (Table 2).

The two mature gall morphotypes differed in some variables (Table 3). The number of cell layers and the thickness of gall cortex were significantly greater in CSG than in GLG (Table 3). Parenchymatous cell area was also greater in CSG when compared with GLG (Table 3). Stem galls had more vascular units per transverse median section than GLG vascular bundles (Table 3). Nymphal chamber area was not significantly different between gall morphotypes (Table 3).

Discussion

The phenological features of *C. mammifex* induced galls follow the expected pattern for Mediterranean climatic conditions of southern Chile. Some anatomical and histochemical features follow peculiarities determined by gall-inducing calophyid feeding habits. Accordingly, some gall features are determined by developmental constraints imposed by *S. polygama* host organs. In stem galls, the increased vascular unit differentiation may be related to the physiological and anatomical potential of stems, with cambial and procambial meristematic regions.

Fig. 2. Univoltine life cycle of *Calophya mammifex* on *Schinus polygama* leaves. Gall induction occurs during the leaf-flushing peak (October to November). Growth and development stage ranges from November until mid-September, when galls enter the maturation stage, which lasts until mid-November. The senescence stage goes from October until November, when leaves with senescent galls fall from the host plant.



Diagnostic features of *C. mammifex* galls

Schinus polygama is an evergreen species with available oviposition sites throughout the year (Guedes et al. 2018). Nevertheless, *C. mammifex* induces galls only during the spring, probably because of unfavourable environmental conditions during summer, autumn, and winter in the Mediterranean climate of southern Chile. This climate has cold, wet winters and hot, dry summers (Giorgi and Lionello 2008), which can determine the diapause period and univoltine life cycle of *C. mammifex*, as previously described for *C. rubra* in the same climatic conditions (Guedes et al. 2018). Galling insect reproductive success seems to be determined by environmental conditions and host plant characteristics (Fernandes and Price 1992; Yukawa and Akimoto 2006). Multivoltine life cycles seem to occur because of subtropical and tropical climate conditions for other psylloid galls on evergreen plants (Lima 2008; Dias et al. 2013*b*) and have been associated with the constant availability of resources (Carneiro and Isaias 2015). However, in seasonal climates such as the Mediterranean climate of southern Chile, inducers such as *C. mammifex* and *C. rubra* must adapt their life cycles to periods with favourable conditions (Guedes et al. 2018).

Immediately after the rainy season, temperatures increase in Mediterranean climates, which triggers a leaf-flushing peak. Such conditions seem to favour diapause breaking, adult *C. mammifex* emergence, and the induction of a new gall cycle on young leaves. For most systems, this leaf-flushing period corresponds to the preferential time for gall induction (Abrahamson et al. 1991; Gonçalves-Alvim and Fernandes 2001), since young leaves seem to be more responsive to galling insect stimuli (Rohfritsch and Anthony 1992) and could also guarantee the quality and quantity of available resources for insects (Yukawa 2000; Yukawa and Akimoto 2006). In addition, the selection of oviposition site is important for gall development, as it can determine nutrient allocation (Price and Roininen 1993) and improve sink-strength generated by the gall (Larson and Whitham 1991). *Calophya mammifex* preferentially induce galls in the median portion of leaves, as other Psylloidea (Weis et al. 1988; Ferreira et al. 1990; Dias et al. 2013*b*) and Cecidomyiidae (Formiga et al. 2009).

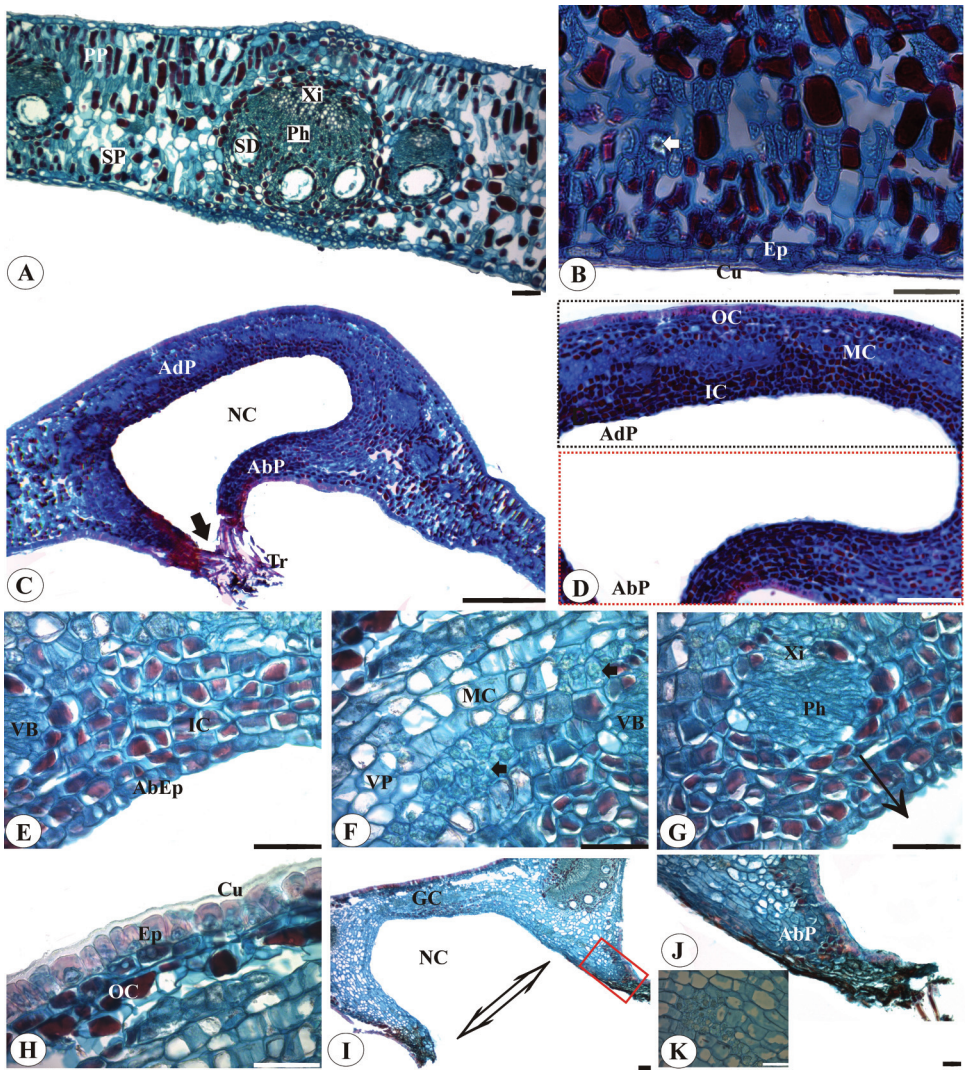
Morphogenetic constraints of host leaves on *C. mammifex* galls

The description of the leaf anatomy of *S. polygama* coincides with that of Dias et al. (2013*a*) for *Schinus engleri* (reported as *S. polygamus*), a closely related species from southern Brazil. Dorsiventral mesophyll with druses and the presence of secretory structures embedded in phloem parenchyma are common features among *Schinus* (Blanco 2004; Nascimento-Silva et al. 2011).

Regarding the dermal system, *C. mammifex* influence has been constrained to the cuticle of gall development sites and trichome differentiation in gall apertures. The thick cuticle of GLG implies a greater investment in protection against water loss because the cuticle reflects solar rays and maintains leaf tissue temperatures (Fahn and Cutler 1992), and conditions outside the gall are highly desiccating (Ramløv and Lee 2000), particularly considering that spring and summer are very dry in Mediterranean climate regions (Acevedo et al. 1999). The redifferentiation of trichomes in the gall aperture has also been associated with insect protection against natural enemies as well as unfavourable environmental conditions (Guedes et al. 2016).

The histochemical profile of GLG also reveals high constraints of host leaf metabolism, which is inferred by the similarity between the histochemical detection of the metabolites between host leaves and galls, except for the absence of starch in galls. *Calophya mammifex* induced galls probably have a weak capacity to synthesize photoassimilates and function primarily as resource sink (Weis et al. 1988; Raman et al. 2006; Álvarez et al. 2009). The accumulation of reducing sugars, proteins, and lipids in gall phloem and vascular parenchyma indicates high metabolic activity and the redifferentiation of a nutritive-like tissue (Ferreira et al. 2017*a*). Lipids and pro-

Fig. 3. Leaf and gall anatomy. (A and B) Mature *Schinus polygama* leaf. (A) Bifacial leaf lamina with palisade parenchyma facing the adaxial surface and spongy parenchyma facing the abaxial surface. (B) Abaxial epidermal surface with druses (arrow). (C–K) Leaf gall induced by *Calophya mammifex*. (C) Mature gall with large nymphal chamber (arrow indicates gall opening). (D) Gall walls divided into an abaxial (dotted red box) portion and an adaxial portion (dotted black box), divided into three compartments. (E) Inner compartment with multilayered cells of periclinal elongation and uniseriate abaxial epidermis limiting the nymphal chamber. (F) Well-developed vascular parenchyma in the median compartment with abundant druses (arrows). (G) A vascular bundle in the median compartment with phloem oriented toward the nymphal chamber (arrow). (H) Outer compartment delimited by uniseriate epidermis with thick cuticle. (I–K) Senescent gall (double arrow indicates separation of gall opening), detail of red rectangle shows in (J) degraded and suberized tissues of abaxial portion and (K) abundant druses in abaxial portion of the senescent gall. AbEp, abaxial epidermis; AbP, abaxial portion; AdEp, adaxial epidermis; AdP, adaxial portion; Cu, cuticle; Ep, epidermis; GC, gall cortex; IC, inner compartment; LL, leaf lamina; MC, median compartment; NC, nymphal chamber; OC, outer compartment; Ph, phloem; PP, palisade parenchyma; SD, secretory ducts; SP, spongy parenchyma; VB, vascular bundle; VP, vascular parenchyma; and Xi, xylem. Druses (arrows). Scale bars = 500 μm (A, C, and D); 50 μm (B and E–K). [Colour online.]



teins were also detected in gall outer tissue compartments, indicating their function as common storage tissues (Ferreira et al. 2017*a*). The accumulation of primary metabolites and druses in galls indicates a redirection of nongalled host organ patterns (Schonrogge et al. 1998), and this has been reported for Psylloidea galls in Neotropical climates (Oliveira et al. 2006; Oliveira and Isaias 2010; Isaias et al. 2011; Carneiro et al. 2014*a*, 2014*b*; Malenovský et al. 2015). In Mediterranean conditions, such accumulation is a product of the peculiar reduced gall metabolism throughout summer, autumn, and winter.

Peculiarities of *C. mamifex* induced galls vs. *C. rubra* induced galls

Cell hypertrophy and tissue hyperplasia, as observed in *C. mammifex* induced galls, are the most common cellular processes in gall development (Mani 1964). The larger number of cell layers and higher number of cells

Fig. 4. Histolocalization of metabolites in *Schinus polygama* leaves and *Calophya mammifex* galls. (A and B) Reaction of Lugol's reagent to starch in leaf tissues, (A) around vascular bundle and (B) in chlorophyllous parenchyma (arrowheads). (C–E) Reactions of Felhing's reagent to reducing sugars, (C) in leaf chlorophyllous parenchyma, endoderm, phloem, and secretory ducts and (D) in inner compartment, surrounding the vascular bundles of the gall. (E) Detail of vascular bundle in the gall, with reducing sugars in phloem, endoderm, and cortical cells. (F) Reaction of Sudan IV to lipids in leaf chlorophyllous parenchyma (arrowheads). (G and H) Reaction of Sudan red B to lipids in the gall, (G) in outer and inner compartments and (H) in neoformed vascular bundles (arrowheads). (I and J) Reaction to bromophenol blue revealing proteins, (I) in spongy leaf parenchyma (arrows) and (J) in gall median compartment (arrows). MC, median compartment; NCh, nymphal chamber; Ph, phloem; PP, palisade parenchyma; SD, secretory ducts; SP, spongy parenchyma; VB, vascular bundle; and Xy, xylem. Druses (*). Scale bars = 200 μm (A and D); 50 μm (B, C, and E–H). [Colour online.]

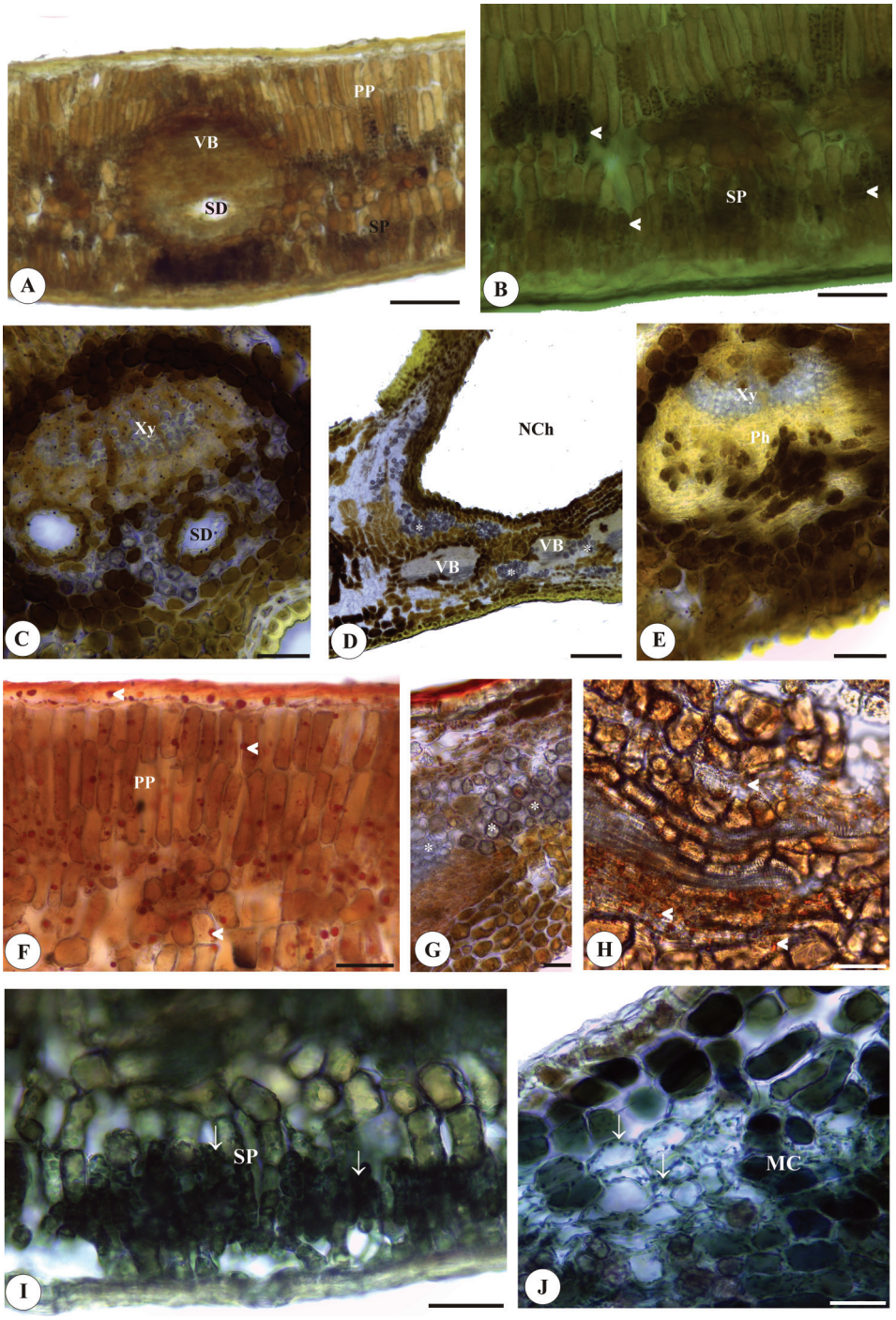
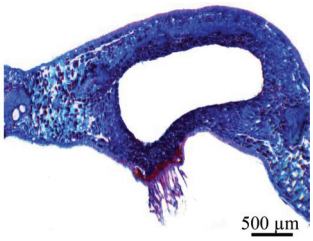
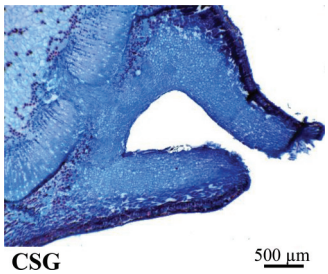


Table 2. Cytometry and histometry of nongalled leaves (NGL) and mature globoid leaf galls (GLG) induced by *Calophya mammifex* (Hemiptera: Calophyidae) on *Schinus polygama* (Anacardiaceae).

Parameter	NGL	GLG	*p* value
Adaxial cuticle thickness (μm)	6.1±1.3b	8.9±1.8a	0.0001
Adaxial epidermis thickness (μm)	16.3±2.2b	27.0±6.8a	0.0001
Adaxial epidermal cell area (μm²)	360.2±86.5b	498.4±153.9a	0.0003
Parenchyma cell layers (*n*)	9.3±1.0b	14.9±2.8a	0.0001
Parenchymatic cell area (μm²)	426.5±145.9b	535.5±133.9a	0.0194
Abaxial epidermis thickness (μm)	13.7±2.3b	16.5±2.9a	0.0014
Abaxial epidermal cell area (μm²)	338.6±98.9a	333.6±97.0a	0.8582

Note: Data presented are the mean ± SD. Different letters represent significant differences ($p < 0.05$).

Table 3. Cytohistometric comparison between globoid leaf galls (GLG) and conical stem galls (CSG) on *Schinus polygama* (Anacardiaceae).

Gall morphotype	Cell layers (*n*)	Gall cortex thickness (μm)	Parenchymatic cell area (μm²)	Vascular bundles/vascular unit (*n*) in the median region of the gall	Nymphal chamber area (mm²)
 **GLG**	14.8±2.3b	307.8±54.3b	543.7±65.9b	3.4±0.4b	0.7±0.5a
 **CSG**	29.3±2.8a	1200.7±256.3a	3217.8±1685.1a	7.4±1.1a	2.0±1.3a
p value	0.0001	0.0016	0.0239	0.0001	0.1007

Note: Data presented are the mean ± SD. Different letters represent significant differences ($p < 0.05$).

in stem galls induced by *C. rubra* than in leaf galls induced by *C. mammifex* may be explained by the activation of vascular procambium and cambium during stem gall development (Mani 1964; Guedes et al. 2018). The differential rates of hyperplasia and cell hypertrophy in distinct tissue compartments determine the distinct gall shapes (Isaias and Oliveira 2011).

The reorganization of plant vascular tissues during gall development (Oliveira et al. 2016) facilitates nutrient translocation and access to host-plant nutrients by the galling insects (Wool et al. 1999). Therefore, the neoformation of vascular bundles and a well-developed vascular parenchyma is expected for sap-feeding insects (Shorthouse and Rohfritsch 1992; Wool et al. 1999; Raman 2011), such as *C. mammifex*, which has two feeding sites, the phloem (its main food source) and the vascular parenchyma. The differentiation of new vascular tissues (mostly phloem) in GLG and CSG (Guedes et al. 2018) is directed to the nymphal chamber and grows like a mantle around the inner compartment in the adaxial portion. The orientation of the phloem portions toward the nymphal chamber is similar to the common orientation of vascular bundles in leaves (Álvarez et al. 2009; Ferreira et al. 2017*a*) and favours the feeding process, as the stylets of the inducing insect can suck phloem sap without crossing the xylem.

In woody perennials like *S. polygama*, the auxin produced by growing buds in spring stimulates procambium activation in a basipetal direction (Taiz and Zeiger 2006). This stimulus may explain the neoformation of vascular units by procambium activation in CSG, which is the most conspicuous difference between leaf and stem galls in *S. polygama*. This peculiarity is evidence that the host organ's morphogenetic potential determines gall anatomy.

Despite the quantitative differences in tissue layers and vascular tissues, there are numerous convergences between GLG and CSG. Many anatomical characteristics of the two gall morphotypes are likely conserved traits in Psylloidea, particularly in Calophyidae-induced galls (*C. mammifex*, *C. rubra,* and *C.* cf. *duvauae*), where three general features are convergent and independent of host organs. First, the three morphotypes are open galls, with overlapping trichomes covering gall apertures; second, the nymphal chambers are large, even when hosting a single small inducing insect; and third, Calophyidae-induced galls are highly parenchymatic and vascularized, with abundant development of phloem and vascular parenchyma.

Recently, gall complexity was evaluated based on the occurrence of one or several anatomical features such as cell hypertrophy, tissue hyperplasia, vascular bundle hypertrophy, cell redifferentiation, histochemical changes, and manipulation of meristematic activity (Ferreira et al. 2017*a*). Considering the anatomical and histochemical features, *C. mammifex* and *C. rubra* may be considered complex galls, contrary to the literature on Hemipteran galls (Rohfritsch 1992; Oliveira and Isaias 2010).

Conclusions

Environmental conditions in the Mediterranean climate of southern Chile probably determine a univoltine life cycle and a diapause period for *C. mammifex* development on *S. polygama,* as well as the anatomical and histochemical features of its galls. For the first time, we detected energetic metabolites in a nutritive-like tissue and in a common storage tissue in galls in Mediterranean climate conditions, which may be related to the nutritional improvement of galls. Galls induced by *C. mammifex* and *C. rubra* have sufficient anatomical and histochemical peculiarities to be diagnosed as complex structures, whose distinction in vascular system differentiation implies structural constraints imposed by host plant organs.

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