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ORIGINAL PAPER

Microscopic study of nine galls induced in *Populus nigra* by aphids of the Iberian Peninsula

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Abstract

Aphids (Hemiptera, Aphididae) feed on the phloem and some of them induce the formation of conspicuous galls on their primary hosts. Histological studies were proposed to elucidate the evolutionary history of galling habit in Pemphigini tribe, assessing if gall complexity indicates the phylogenetic relations among gall inducers. Nine Eriosomatinae galls induced on *Populus nigra* from the Iberian Peninsula were studied microscopically. The galls are induced by Pemphigini of the genera *Thecabius* (2 galls) and *Pemphigus* (7 galls). Using multiple correspondence analysis of the observed microscopic characteristics, a hierarchical cluster was obtained showing the existence of 2 groups of galls. One group consists of the 2 galls induced by *Thecabius* and, within the genus *Pemphigus*, those belonging to the subgenus *Pemphigus* (*P. populi and P. vesicarius*). The other group consists of galls of the genus *Pemphigus*, subgenus *Pemphiginus* (*P. bursarius, P. immunis, P. spyrothecae, P. protospirae* and *P. populinigrae*). The gall histological complexity is related to Pemphigini phylogeny, confirming the importance of such studies in galling aphid taxonomy and possible pathways of galling habit evolution. Similarities are established between the galls of *Pemphigini* aphids studied here with others we studied microscopically, induced by Fordini and Eriosomatini. Finally, a classification of all Eriosomatinae galls is proposed, establishing 3 groups: galls that cause severe malformations (induced by derived galling *taxa*), those that cause less severe malformations, and those that cause mild malformations (pseudogalls). It also demonstrates the importance of the strategy of waste evacuation in derived galling species.

Keywords Aphids · Galls · Eriosomatinae · Pemphigini · *Populus nigra* · Plant histology

Introduction

Aphids (Hemiptera, Aphididae) that feed on the phloem sap are one of the groups of insects that cause the formation of galls (Roskam 1992; Shorthouse and Rohfritsch 1992).

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Although most induce malformations in their hosts, only aphids of the Hormaphidinae and Eriosomatinae subfamilies induce the formation of conspicuous galls on their primary hosts (Wool 1984; Blackman and Eastop 2020).

Aphids belonging to the Eriosomatinae subfamily have a complex life cycle that have as their primary host tree or shrub species on which they induce gall formation in various organs. As secondary hosts, they use species from various botanical families, in which the colonies normally live on the roots (Blackman and Eastop 2020). In the primary host, the fundatrices that hatch from winter eggs are the gall inducers.

In most species, the fundatrix-induced gall harbors its entire offspring until the winged fundatrigeniae spread to their secondary host. However, a few species induce the formation of 2 types of galls: one type is induced by the fundatrices (henceforth "temporary gall", i.e., a gall specific for the fundatrix), in which the first generation of wingless fundatrigeniae is brought forth. These leave the temporary gall and induce the formation of galls that will come to term (henceforth "definite gall", i.e., a gall specific for the fundatrigeniae), that is, galls from which the winged fundatrigeniae will eventually spread toward their secondary hosts.

Eriosomatinae species that have been described in the Iberian Peninsula and the Balearic Islands are divided into three tribes: Eriosomatini, Fordini, and Pemphigini. These induce galls on *Ulmus*, *Pistacia*, and *Populus*, respectively (Pérez Hidalgo and Nieto Nafría 2003). There are few microscopic studies of aphid-induced galls in the literature, except for those performed by us. Regarding galls induced by Eriosomatinae in the Iberian Peninsula, studies have been carried out using both light and electron microscopy of galls induced by Fordini (Álvarez et al. 2009, 2014, 2016; Álvarez 2011, 2012; Martinez et al. 2018) and Eriosomatini species (Álvarez et al. 2013). In the present study, 9 galls from the third tribe, the Pemphigini, which induce them in the Iberian Peninsula on *P. nigra* L., are analyzed microscopically.

The aim of current study is to assess, by histological analyses, if gall complexity indicates the phylogenetic relations among gall inducers of the tribe Pemphigini. Our hypothesis is that gradual divergences in gall structures demonstrate the evolutionary history of galling aphids. Specifically, we study the two galls that *T. affinis* (Kaltenbach, 1843) induces on the leaf lamina (temporary gall and definite gall), and those induced by 7 species of the genus *Pemphigus* both on leaf Blade (*P. populinigrae* (Schrank, 1801), *P. (Pemphiginus) populi* Courchet, 1881 y *P. (Pemphiginus) vesicarius* Passerini, 1861) and on petioles (*P. bursarius* (Linnaeus, 1758), *P. protospirae* Lichtenstein, 1885 y *P. spyrothecae* Passerini, 1860) and branches (*P. immunis* Buckton, 1896) (Fig. 1). The 7 species of the genus *Pemphigus* are taxonomically grouped into two subgenera: *Pemphigus* and *Pemphiginus* (Blackman and Eastop 2020). Taking into account their shape and the opening mechanisms that allow alate fundatrigeniae to leave for their emigration flight to the secondary hosts, galls of the subgenus *Pemphiginus* (*P. vesicarius* y *P. populi*) are characterized by being leaf galls that develop as more or less irregular blisters that do not communicate with the outside. The mature gall cracks open, allowing the emigrants to leave. On the other hand, galls of the subgenus *Pemphigus* (*P. bursarius*, *P. immunis*, *P. populinigrae*, *P. protospirae* y *P. spyrothecae*) are characterized by occurring on leaf blades, petioles, or branches and always have an opening to the outside, with a variable number of trichomes.

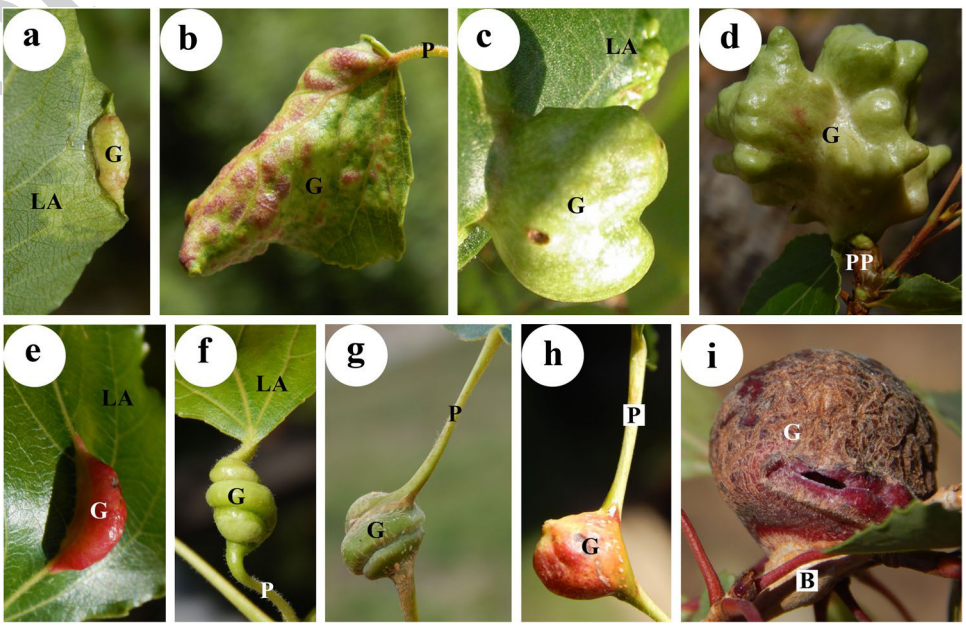

Fig. 1 Galls included in the present study. **a** Gall specific to the fundatrix (temporary gall) of *T. affinis*. **b** Gall specific to the fundatrigeniae (definite gall) of *T. affinis*. **c** *P. populi*. **d** *P. vesicarius*. **e** *P. populinigrae*. **f** *P. spyrothecae*. **g** *P. protospirae*. **h** *P. bursarius*. **i.** *P. immunis*. *B* branch, *G* gall, *LA* leaf blade, *P* leaf petiole, *PP* shortened and thickened leaf petiole

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It is through this opening that the alate will depart on their migration flight.

Material and methods

At least 5 samples of nine different galls induced on *P. nigra* were collected at various locations of the Iberian Peninsula (indicated in parentheses): *P. bursarius* (León), *P. immunis* (Teruel, Valencia), *P. populi* (León), *P. populinigrae* (León), *P. protospirae* (Teruel), *P. spyrothecae* (León), *P. vesicarius* (Teruel), and *T. affinis*—temporary gall (León) and *T. affinis*—definite gall (León). Young ungalled leaves and branches were also collected. Samples were fixed in situ in FAA (formaldehyde, acetic acid, and ethyl alcohol).

Samples taken to the laboratory, followed the procedures described by Álvarez et al. (2009), samples were dehydrated in an ethanol increasing series and embedded in paraplast, in order to make different blocks of each of them, using isoamyl acetate as an intermediate liquid. Each block was then sectioned in a paraffin rotatory microtome. Serial cuts of 12 µm thickness were obtained, which were deposited on slides. Once dewaxed, the routine stained method employed Safranin-Fast Green. Subsequently after dehydration, they were mounted permanently on microscope slides using Entellan as mounting medium. Some slides were permanently mounted without dyeing. Slides were observed with Nikon E600 under bright-field, epifluorescence and polarized light conditions, and the most representative fields photographed. For scanning electron microscopy (SEM), fixed gall fragments were passed through an increasing alcohol series, gold scattered, and observed using a Jeol JSM-6480LV SEM.

For each gall, the following was written down (see Table 1):

- General characteristics: the organ on which the gall is located and the gall's orientation.
- Wall of the gall: wall thickness, number of layers of storage parenchyma, presence of tannins, crystals and sclereids.
- Epidermis-air: presence of epidermis-air, thickness of the cuticle, presence of stomata and trichomes.
- Vascular bundles: type of vascular bundle, presence of bundle sheaths and vascular tissue (phloem or xylem) facing the lumen of the chamber.
- Epidermis-lumen: presence of epidermis-lumen, type of epidermis, presence of cuticles, stomata, trichomes, and dimples.
- Closure zone: presence of closure zone and presence of trichomes, crystals, and tannins.

To check the clustering of the galls according to the histological characteristics studied (Table 1; General characteristics not included in analysis), a multiple correspondence analysis (MCA) was performed followed by a hierarchical cluster analysis (HCPC) applied to the 3 factors that explain 80% of the variance. These analyses were carried out using the packages FactoMineR 1.42 (Le et al. 2008) and factoextra 1.0.5 (Kassambra and Mundt 2017) in R 3.6.2 (R Corre Team 2019).

Results

Statistical treatment of the microscopic characteristics studied (Table 1) established the existence of two well-defined groups of galls (Fig. 5): Group 1, consisting of the two galls of *T. affinis* and the galls of *P. populi* and *P. bursarius*; and Group 2, consisting of the galls of *P bursarius* and *P. populinigrae* on the one hand, and of *P. immunis*, *P. protospirae* and *P. spyrothecae* on the other.

Galls of Group 1 according to the hierarchical cluster (Fig. 5). Predominantly leaf galls.

Thecabius affinis—temporary gall and definite gall

The temporary gall has the appearance of a fold at the leaf edge, whereas in the definitive gall the entire lamina is folded, forming a pseudogall: modified areas closest to the midvein (Fig. 3g) and almost not modified areas in the distal portion. Tannins but not crystals are observed in the wall of the temporary gall, unlike the wall of the definite gall, in which crystals but not tannins are detected.

Both galls are oriented toward the abaxial leaf surface, and their walls are 250–450 µm thick and have less than 15 layers of parenchyma, like the ungalled leaf blade (Fig. 3c). They also present an epidermis-air with a thin cuticle and a uniseriate epidermis-lumen with stomata and without trichomes. The vascular bundles are collateral and the closure zone lacks trichomes (Fig. 4f).

Pemphigus vesicarius

The gall is a modified leaf, with a shortened and broad petiole at the basis (Figs. 1h, 2d), from which normal leaf blades without petiole (which do not form part of the gall) also emerge. This modified petiole has a hypertrophied phloem and a bundle sheath of fibers with some sclereids (Figs. 4h,i). In the non-galled petiole, the vascular bundles have a bundle sheath with only fibers (Figs. 3d,e, 4g).

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Table 1 General characteristics, Gall wall, Epidermis-air, Vascular bundles, Epidermis-lumen and Closure zone of the galls studied: *PB*: *P. bursarius*. *PI*: *P. immunis*. *PL*: *P. populinigrae*. *PP*: *P. populi*. *PR*: *P. protospirae*. *PS*: *P. spyrothecae*. *PV*: *P. vesicarius*. *TA*-d: Gall specific to the fundatrigeniae (definite gall) of *T. affinis*. *TA*-t: Gall specific to the fundatrix (temporary gall) of *T. affinis*. Wall, epidermis and vascular bundles of leaf, petiole and branch

	TA -t	*TA* -d	*PP*	*PV*	*PL*	*PB*	*PS*	*PR*	*PI*	*Leaf*	*Petiole*	*Branch*
General characteristics												
Location	L	L	L	le	L	P	P	P	B			
Orientation	ab	ab	ad	na	ad	na	na	na	na			
Gall wall										**Wall**		
Tannins	+	-	+	+	++	+	+	+	+	+	+	+
Crystals	-	+	-	-	-	+	++	+	+	+	+	+
Thickness (μm)	+	+	++	++	++	++	+++	+++	+++	(+)	(+)	+
Parenchyma (layers)	+	+	++	++	++	++	+++	+++	+++	+	+	++
Sclereids	-	-	-	-	++	-	+	++	-	-	-	-
Epidermis-air										**Epidermis**		
Epidermis	+	+	+	+	+	+	+	+	+	+	+	+
Cuticle	+	+	++	+	++	++	++	++	++	+	++	++
Stomata	+	+	+	+	+	+	+	+	-	+	+	-
Trichomes	-	-	-	-	-	+	+	+	+	-	+	+
Vascular bundles										**Vascular bundles**		
Collateral	+	+	+	+	+	+	-	-	-	+	-	-
Amphicribral	-	-	-	-	-	-	+	+	+	-	+	+
Bundle sheath	-	-	-	-	-	-	-	-	-	+	+	+
Phloem towards lumen	+	+	+	+	+	+	+	+	+			
Epidermis-lumen												
Epidermis	+	+	-	-	+	++	++	++	++			
Cuticle	+	(+)	na	na	-	(+)	(+)	(+)	(+)			
Stomata	+	+	na	na	-	-	-	-	-			
Trichomes	-	-	na	na	+	+	+	+	+			
Dimples	-	-	+	+	-	-	-	-	-			
Closure zone												
Closure zone	+	+	-	-	+	+	+	+	+			
Trichomes	-	-	na	na	+	+	+	-	+			
Accumulation of crystals	-	-	na	na	-	-	-	-	-			
Accumulation of tannins	-	-	na	na	-	-	+	+	-			

In general "−"indicates absence, "+" indicates presence, and "++" indicates greater presence. For *Wall thickness*, (+): 130-150 μm; +: 250-450 μm; ++: 650-100 μm; +++: > 1000 μm. For *Parenchyma*, +: < 15 layers of cells; ++: 20–35 layers of cells; +++: > 50 layers of cells. For *Cuticle* of *Epidermis-air*, +: thin; ++: thick. For *Epidermis* of *Epidermis-lumen*, +: uniseriate; ++: multiseriate. For *Cuticle* of *Epidermis-lumen*, +: thin; (+): thin and discontinuous

At the right, under "Leaf" the data of the *Epidermis* refer to the adaxial Epidermis, under "Petiole" and "Branch" it refers to the epidermis that surrounds the structure. *ab* abaxial, *ad* adaxial, *B* branch, *L* leaf blade, *le* leaf primordium, *na* not applicable, *P* leaf petiole

Pemphigus populi

A cellular formation toward the leaf adaxial surface is observed, leaving the midrib outside the gall itself (Fig. 2c).

Both galls (*P. vesicarius* and *P. populi*) have walls that are not reminiscent of the leaf lamina. The walls have a thickness of 650–1000 μm and 20–35 layers of parenchyma, presenting collateral vascular bundles (Figs. 4a, b). They do not present trichomes at the epidermis-air and, very characteristically, they have no epidermis-lumen but they do have dimples on the surface of the chamber (Figs. 3a, k, 4a,b). These galls do not have a closure zone.

Galls of Group 2 according to the hierarchical cluster (Fig. 5). Predominantly vascular galls.

Pemphigus populinigrae

The gall appears as a leaf fold over the adaxial leaf surface, in which the midvein is part of the gall (Fig. 2e). The wall, which is not reminiscent of the leaf blade, has no crystals but many tannins, and also abundant sclereids in an amphicribral pattern (Fig. 3j).

Pemphigus bursarius

Lateral formation on the petiole: the unaffected regions of the petiole are on one side, and the gall is on the other (Fig. 2h). The area of the petiole furthest from the lumen of the gall has a normal morphology: subepidermal collenchyma and chlorophyll parenchyma; vascular bundles

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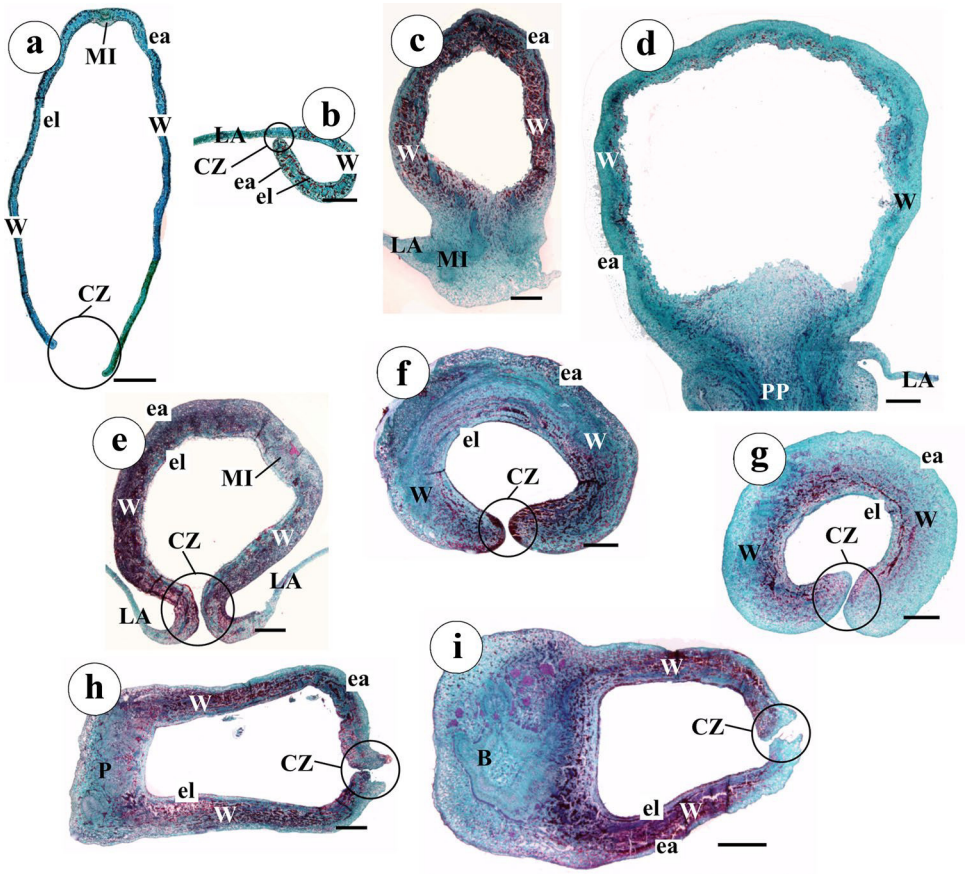

Fig. 2 Microscopic appearance of the galls included in the present study. **a** Gall specific to the fundatrigeniae (definite gall) of *T. affinis*. **b** Gall specific to the fundatrix (temporary gall) of *T. affinis*. **c** *P. populi*. **d** *P. vesicarius*. **e** *P. populinigrae*. **f** *P. spyrothecae*. Notice the red flange in the closure zone (CZ) corresponding to tannin accumulation. **g** *P. protospirae*. **h** *P. bursarius*. **i** *P. immunis*. Stainings: **a–i** Safranin-Fast Green. Microscopes: **a–i** Bright-field microscope. *B* branch, *CZ* closure zone, *ea* epidermis-air, *el* epidermis-lumen, *LA* leaf blade, *MI* midvein, *P* petiole, *PP* shortened and thickened petiole. W: wall of the gall. Scale bars: **a–i** = 1 mm

with a bundle sheath of fibers. The area of the petiole closest to the chamber is disordered, with incomplete bundle sheaths, sometimes with fibers and sometimes with sclereids. Trichomes are seen in the epidermis-air, as in the ungalled petiole.

Both galls (*P. populinigrae* and *P. bursarius*) have a wall thickness of 650–1000 µm and 20–35 layers of parenchyma. In both cases, the epidermis-air has a thick cuticle and the epidermis-lumen is multiseriate. Both in the epidermis-lumen and in the closure zone multicellular trichomes are observed (Fig. 4d). The vascular bundles are collateral.

Pemphigus immunis

Development lateral to the branch: the unaffected regions of the branch are on one side, and the gall is on the other side (Fig. 2i). The area of the branch furthest from the lumen of the gall has a normal morphology: uniseriate epidermis, storage parenchyma, and amphicribral vascular bundles with bundle sheath of fibers. The area of the branch closest to the chamber has disordered vascular bundles and accumulations of sclereids. They have unicellular and multicellular trichomes in the closure zone (Fig. 4e).

Pemphigus protospirae

Coiled petiole. The wall has many sclereids.

Pemphigus spyrothecae

Coiled petiole. The wall of the gall in the area furthest from the chamber is reminiscent of the ungalled petiole, with collenchyma and chlorophyll parenchyma in a subepidermal arrangement. The gall wall has many crystals (Figs. 3f,i). Accumulation of tannins is observed in the closure zone (Fig. 2f).

The three galls (*P. immunis*, *P. protospirae* and *P. spyrothecae*) all have amphicribral vascular bundles (Fig. 3h), very thick walls (more than 1000 µm thick and more than 50 layers of parenchyma), epidermis-air with trichomes (Fig. 3b) and multiseriate epidermis-lumen with multicellular trichomes (Fig. 4c), and a discontinuous and thin cuticle.

Discussion

Galls are induced structures in which processes of active growth and differentiation take place (Stone and Schönrogge 2003), as confirmed in the galls included in the

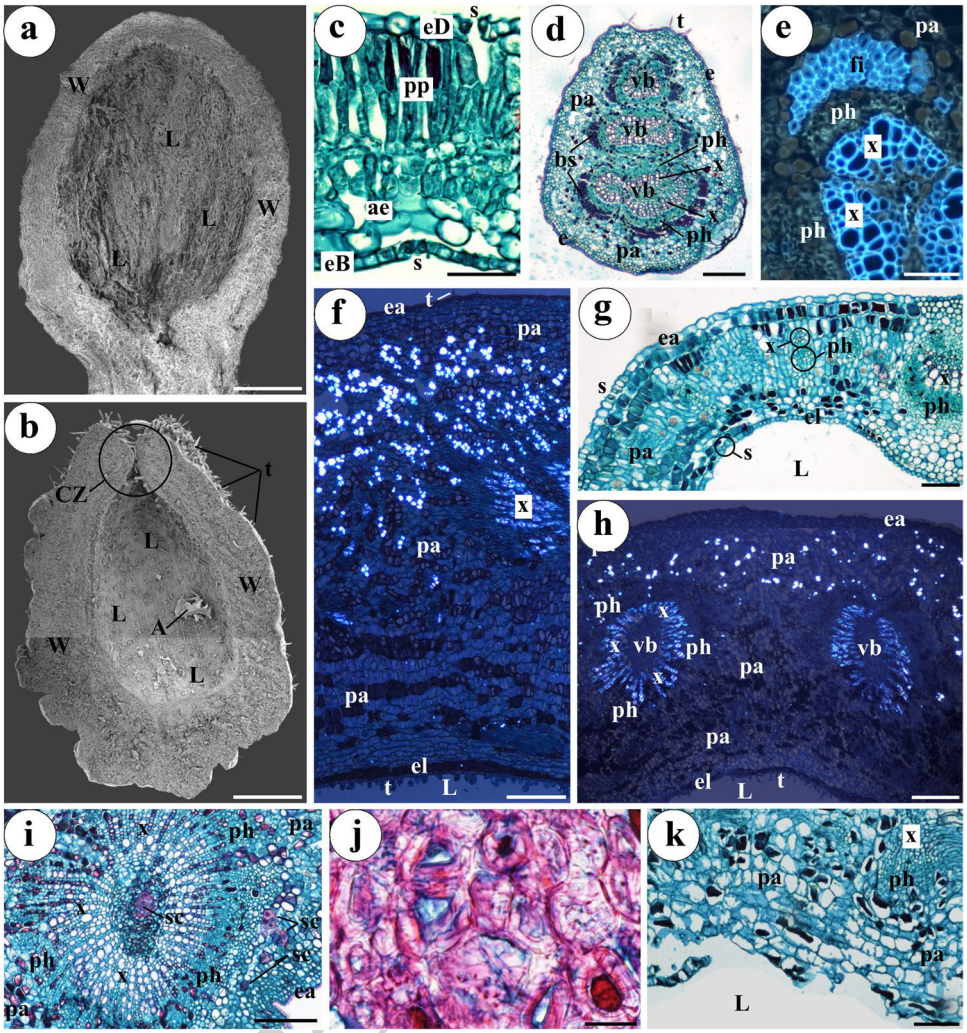

Fig. 3 **a–b** Closed gall and open gall. **a** Gall induced by *P. populi*. The inner surface of the chamber (L) has a porous appearance. **b** Gall induced by *P. immunis*. In the closure zone (CZ) and on the outer surface trichomes (t) are seen. **c–e** Normal leaf and petiole morphology of *P. nigra*. **c.** Leaf blade of *P. nigra*. Both the adaxial epidermis (eD) and the abaxial epidermis (eB) show stomata (s). Between both epidermises a palisade parenchyma (pp) and an aerenchyma (ae) are seen. **d** Leaf petiole of *P. nigra*. Three amphicribral vascular bundles (vb): xylem (x) completely surrounded by phloem (ph). On the outside they present a bundle sheath (bs). **e** Detail of (d). Amphicribral vascular bundle with fibrous (fi) bundle sheath. **f** Gall wall with many layers of parenchymal cells (pa). The bright spots are calcium oxalate crystals. Note the trichomes (t) in both the epidermis-air (ea) and the epidermis-lumen (el). Multiseriate epidermis-lumen (el). Gall induced by *P. protospirae*. **g–h** Vascular bundles in the gall walls. **g** Collateral vascular bundles. Gall specific to the fundatrigeniae (definite gall) of *T. affinis*. Note stomata in both epidermises. Uniseriate epidermis-lumen (el). **h** Amphicribral vascular bundles (vb). Galls induced by *P. protospirae*. The multiseriate epidermis-lumen (el) presents trichomes (t). The bright spots are calcium oxalate crystals. **i** Presence of sclereids in the center and outside of an amphicribral vascular bundle. Gall induced by *P. protospirae*. **j** Detail of sclereids in a gall induced by *P. populinigrae*. **k** Gall chamber (L) with dimples. The cells do not form an epidermis-lumen. Gall induced by *P. vesicarius*. Stainings: **c-d**, **g**, **i–k** Safranin-Fast Green. Microscopes: **a–b** SEM. **c–d**, **g**, **i–k** Bright-field microscope. **e** Epifluorescence microscope. **f–h** Polarized light microscope. *A* Aphid molt, *Ae* epidermis-air, *bs* bundle sheath, *CZ* closure zone, *e* epidermis, *eB* abaxial epidermis, *eD* adaxial epidermis, *el* epidermis-lumen, *fi* fiber, *L* lumen of the gall, *pa* parenchyma, *ph* phloem, *pp* palisade parenchyma, *s* stoma, *sc* sclereid, *t* trichome, *vb* vascular bundle, *W* wall of the gall, *x* xylem. Scale bars: **a–b** = 1 mm; **c, e** = 50 µm; **d, f, I** = 200 µm; **g, k** = 100 µm; **h** = 300 µm; **j** = 25 µm

present study. Additionally, the galls are considered extended phenotypes of their inducers (Stone and Schönrogge 2003), since the gall structures that give a better survivorship of their inducers should be selected. Considering this, our results indicate that structural features of galls are related to the phylogenetic relationships of galling Pemphigini.

All 9 galls have thickened walls with more or less layers of parenchyma and more or less hypertrophied vascular bundles (especially the phloem). These are common features

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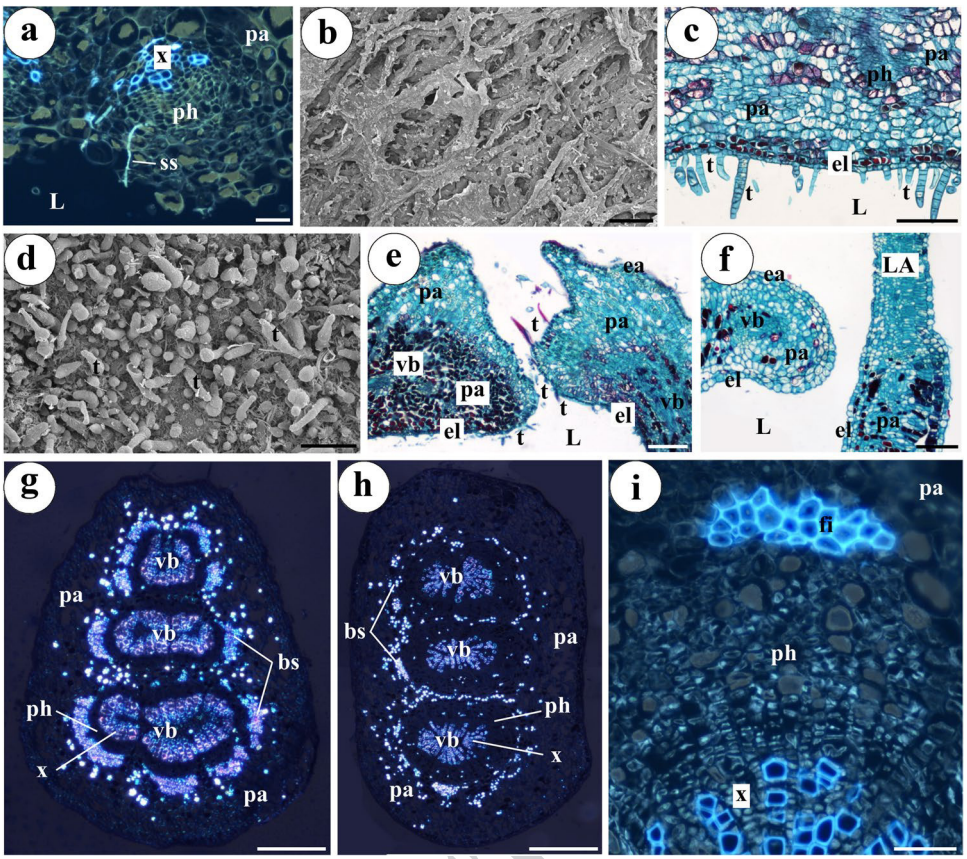

Fig. 4 **a-d** Interior surface of the gall. **a** Collateral vascular bundle in gall without epidermis-lumen. Note the stylet sheath (ss) of the aphid reaching the phloem (ph). Gall induced by *P. vesicarius*. **b** Characteristic dimples of the inside of a gall without epidermis-lumen. Gall induced by *P. vesicarius*. **c** Multicellular trichomes (t) on multiseriate epidermis-lumen (el). Gall induced by *P. protospirae*. **d** Interior of the gall with trichomes (t). Gall induced by *P. populinigrae*. **e–f** Closure zone. **e** Closure zone with trichomes (t). Gall induced by *P. immunis*. **f** Closure zone without trichomes. Gall specific to the fundatrix of *T. affinis*. Note how the normal morphology of the leaf lamina (LA) changes to form the gall. **g–i** Normal petiole and shortened and thickened petiole. **g** Fig. 3d under polarized light microscope. Three amphicribral vascular bundles (vb). Note the birefringence of the xylem (x), the fibrous bundle sheaths (bs), and the surrounding bright spots (calcium oxalate crystals). Petiole of *P. nigra*. **h** Shortened and thickened petiole characteristic of galls induced by *P. vesicarius*. As in the previous image, there are three amphicribral vascular bundles (vb), a bundle sheath (bs) of fibers (and some sclereids), and calcium oxalate crystals. **i** Detail of the foregoing image. Amphicribral vascular bundle of a petiole of *P. vesicarius*. Note the hypertrophy of the phloem (ph) with respect to the unmodified petiole of Fig. 3e. Stainings: **c**, **e**, **f** Safranin-Fast Green. Microscopes: **a, i** Epifluorescence microscope. **b, d** SEM. **c**, **e**, **f** Bright-field microscope. **g**, **h** Polarized light microscope. *bs* bundle sheath, *ea* epidermis-air, *el* epidermis-lumen, *fi* fiber, *L* lumen of the gall, *LA* leaf blade, *pa* parenchyma, *ph* phloem, *ss* stylet sheath, *t* trichome, *vb* vascular bundle, *x* xylem. Scale bars: **a, i** = 50 μm; **b, c, e–g** = 200 μm; **d** = 100 μm; **h** = 500 μm

of aphid-induced galls. We already described these characteristics in previous microscopic studies of galls induced by Fordini on species of the genus *Pistacia* (Álvarez et al. 2009, 2014, Álvarez 2011, 2012, Muñoz Viveros et al. 2014, Álvarez et al. 2016, Mellah et al. 2016, Martinez et al. 2018) and by Eriosomatini on the genus *Ulmus* (Álvarez et al. 2013). The hypertrophied vascular bundles of the galls studied in this work do not present bundle sheaths (which do exist in the leaf, petiole, and branch of *P. nigra*), and the phloem is always oriented toward the lumen of the gall. Outwardly they present a uniseriate epidermis-air and an accumulation of crystals is never observed in the closure zone in galls that have them.

The presence of a bundle sheath in the vascular bundles of the galls is exceptional in all Eriosomatinae galls we have studied. It appears only in galls caused by *Asiphonella cynodonti* on *Pistacia palaestina* (Álvarez et al. 2016) where it interacts with the support of the structure. The fact that the phloem is in all cases oriented toward the chamber in the galls induced by the studied Pemphigini may reflect a characteristic of these aphids, compared to those that induce galls in which the vascular tissue oriented toward the chamber is the xylem. Galls induced by Eriosomatini (Álvarez et al. 2013) and Fordini (Álvarez et al. 2016) also present the phloem very close to the lumen. It should be kept in mind that to feed, aphids that live in galls with the xylem

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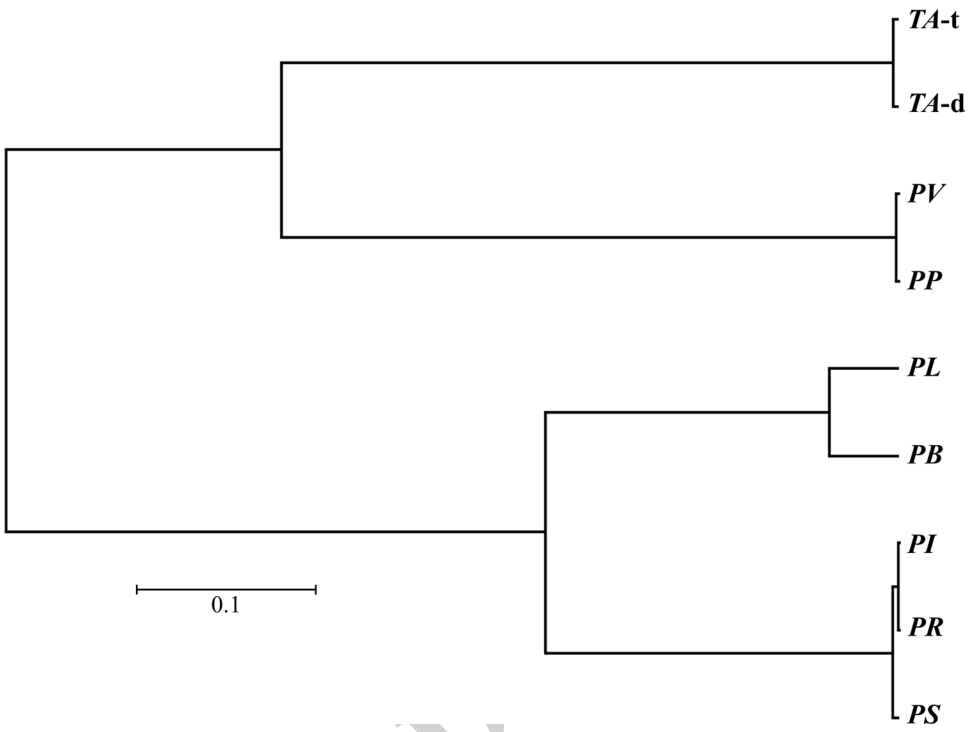

Fig. 5 Histological clustering of galls on *Populus* using the histological characteristics of the galls studied (see Table 1). *PB*: *P. bursarius*. *PI*: *P. immunis*. *PL*: *P. populinigrae*. *PP*: *P. populi*. *PR*: *P. protospirae*. *PS*: *P. spyrothecae*. *PV*: *P. vesicarius*. *TA*-d: Gall specific to the fundatrigeniae (definite gall) of *T. affinis*. *TA*-t: Gall specific to the fundatrix (temporary gall) of *T. affinis*. Scale bar represents height distance

close to the lumen must cross the cells of the xylem to reach the phloem cells. Studies addressing the characteristics of the rostra of the gallicolous aphids or the size of the stylets or some other physical or morphological characteristic may help to shed light on this issue.

The presence of a uniseriate epidermis-air is common to all galls induced by Eriosomatinae. In none of them is the epidermis-air absent or multiseriate. This may be related to the fact that the epidermis of the leaves of *Pistacia* (Álvarez et al. 2008), *Ulmus* (Álvarez et al. 2013), and *Populus* (present study) is also uniseriate. The presence of crystals in the closure zone may be related to the protection of the gall against attack by certain intruders (Fernandes et al. 1989). Their absence in the galls studied in this work may be related to the fact that there are no predators that access the gall through the closure zone or otherwise. Knowledge of the rates of predation or parasitism that affect the studied gallicolous forms may clarify this matter, as well as the presence of tannins in the closure zone of some galls but not in others. Finally, the fact that the vascular bundles are collateral in some galls and amphicribral in others is related to the location of the galls studied. In leaves and in leaf galls, the vascular bundles are collateral, while in general in petioles, branches, and in the galls settled on them the vascular bundles are amphicribral.

In predominantly leaf galls (Fig. 5), there are galls induced by 2 genera: *Thecabius* and *Pemphigus*.

The temporary gall of *T. affinis* shares structural characteristics with the galls induced by *F. riccobonii*, *F. formicaria*, *F. marginata*, *P. cimiciformis*, *S. betae*, *A. cynodonti*, and *Aplonerura lentisci* on different species of the genus *Pistacia* (Álvarez et al. 2009, 2016). They all have the appearance of modified leaf blades in which the midvein is generally not involved. Temporary galls of *F. formicaria, F. marginata, F. riccobonii*, and *S. betae* on *Pistacia* have also been described in the Iberian Peninsula, and they are generally located at the apex of the leaflets (Pérez Hidalgo and Nieto Nafría 2003). The definite gall of *T. affinis* bears a great resemblance to the gall caused by *Eriosoma ulmi* in *Ulmus minor* (Álvarez et al. 2013), in the sense that both are pseudogalls whose most notable histological characteristic is the non-severe and irregular modification of the leaf lamina: some areas are modified and others are unmodified.

Galls induced by *P. populi* and *P. vesicarius* (both of subgenus *Pemphigus)* are microscopically comparable to those induced by *Rectinasus buxtoni*, *Geoica utricularia*, *Baizongia pistaciae,* and *Slavum wertheimae* on different species of *Pistacia* (Álvarez 2012; Álvarez et al. 2014, 2016). All of them are closed galls, without epidermis-lumen and with dimples on the surface of the chamber.

The predominantly vascular galls (Group 2 according to the hierarchical cluster (Fig. 5)) are found on different locations of the host: the midvein of the leaf (*P. populinigrae*), the petiole (*P. bursarius*, *P. protospirae*, and *P. spyrothecae*), and the branch (*P. immunis*). All are characterized by being galls with a multiseriate epidermis-lumen, with multicellular trichomes and most have a thin, discontinuous cuticle.

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In addition, they have conspicuous closure zones, most of which have trichomes.

As for the genus *Pemphigus* as a whole, the statistical grouping obtained from the histological characteristics is an infrageneric taxonomic classification, in which the subgenera *Pemphigus* and *Pemphiginus* are clearly separated (Blackman and Eastop 2020). The results of the present study allow new (microscopic) contributions to be made to the knowledge of the two subgenera. Thus, the galls induced by species of the subgenus *Pemphigus* are closed galls (without a closure zone) in which mechanisms for rupturing the gall wall must be developed to allow the exit of the winged fundatrigeniae. On the other hand, galls induced by the subgenus *Pemphiginus* are galls that have a closure zone and trichomes in the multiseriate epidermis-lumen of the chamber. In these galls (see for example *P. immunis* in Fig. 2j), it is seen that the part of the plant furthest from the gall retains the characteristics of the organ. This may be related to the site where the gall is produced by the aphid: it is possible that the aphid intervenes only in a part of the vascular bundles and the parenchymatic cells closest to the induction site; this would depend on the availability of sensitive sites in the plant that react to chemical stimuli and mechanisms of gallicolous insects (Pfeffer et al. 2018). Keep in mind that the development of plants follows the morphogenetic patterns determined in their meristems, which can be manipulated by gallicolous organisms by determining the over-differentiation or inhibition of some characteristics of the organs, as well as the differentiation of different types of cells (Ferreira et al. 2019; Hearn et al. 2019). Thus, Schultz et al. (2019) studied phylloxera galls and reported that the insect intervenes in the procambium, providing meristematic tissue and redirecting the development of the leaf toward the development of the galls.

Perhaps the most notable microscopic feature of the subgenus *Pemphiginus* is the presence of multiseriate epidermis-lumen with multicellular trichomes. These attributes are not found in the leaf blade, petiole, or branch of *P. nigra*, and this clearly differentiates these galls from those induced by Fordini and Eriosomatini previously studied by us (Álvarez et al. 2009, 2013, 2014, 2016; Álvarez 2011, 2012; Martinez et al. 2018; Mellah et al. 2016; Muñoz-Viveros et al. 2014).

Related to the above, it is interesting to give a moment's thought to the waste disposal mechanisms in galls induced by species of the genus *Pemphigus* studied here. The galls induced by *P. populi* and *P. vesicarius* (of the subgenus *Pemphigus*) are the only closed galls included in this study, which as mentioned before share microscopic characteristics with galls induced by *R. buxtoni*, *G. utricularia*, *B. pistaciae*, and *S. wertheimae* on different species of *Pistacia* (Álvarez 2012; Álvarez et al. 2014, 2016). They all lack an epidermis-lumen and the interiors of their chambers show dimples. This type of inner gall wall favors the elimination of accumulated waste, which is absorbed by the wall until it reaches the vascular bundles (Álvarez et al. 2014, 2016; Kutsukake et al. 2012, 2019). On the other hand, the galls induced by *P. bursarius*, *P. immunis*, *P. spyrothecae*, *P. protospirae,* and *P. populinigrae* (those of the subgenus *Pemphiginus*) are open galls whose epidermis-lumen is multiseriate, and presents trichomes and the cuticle is discontinuous or even absent. These characteristics may be related to the increased hydrophobicity of the inner wall (Kutsukake et al. 2019). The aphids of these galls coat the honeydew with waxy secretions, forming "honeydew balls" (Pike et al. 2002; Kutsukake et al. 2012; Uematsu et al. 2018) that are pushed toward the center of the gall without getting dirty (Kutsukake et al. 2019), or are pushed toward the opening of the gall (perhaps in a similar way how the soldier aphids of *P. spyrothecae* clean the galls (Benton and Foster 1992). This could explain why it is not uncommon to see traces of honeydew and wax on the leaves or on the soil just below where these galls are located. Furthermore, the wax impregnates the surface of the trichomes that thus obtain a greater hydrophobicity, keeping the inside of the gall clean and dry (Uematsu et al. 2018). The presence of a multiseriate epidermis also contributes, because it forms a barrier that prevents water from the gall wall from entering into its lumen (Pike et al. 2002; Álvarez et al. 2009).

Álvarez et al. (2013) classified the Eriosomatinae galls studied microscopically up to that moment into 3 types of galls: 1/ closed galls that explode as they mature (for example *G. utricularia*, *T. ulmi*, and *P. populi* studied here); 2/ galls with a closure zone that opens when the time is right (for example, *F. marginata* and *P. populinigrae* described in the present study); and 3/ galls that are permanently open (e.g., *E. ulmi* and the here described definite gall of *T. affinis*). The first type of gall causes severe alterations, the second type less severe alterations, and the third type the mildest alterations. In the latter case, the arrangement of the vascular bundles in the walls of the galls was also considered. In light of the results obtained in the present study, perhaps this arrangement should not be taken into account, since it is a characteristic of the plant organ on which the gall is induced. As an example, the amphicribral vascular bundles observed in the petiole of *P. nigra* are also seen in the petiolar galls induced by *P. protospirae* and *P. spyrothecae*.

All in all, it is possible to incorporate the 9 galls induced by Pemphigini on *P. nigra* of the Iberian Peninsula into the proposed classification in which most of the galls of Eriosomatinae aphids studied by us to date fit:

– Closed galls that open when they mature and that eliminate waste through dimples in the chamber. The galls of the subgenus *Pemphigus* (*P. populi* and *P. vesicarius*) are in this group within the Pemphigini. Along with

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them are the already mentioned Fordini: *R. buxtoni*, *G. utricularia*, *B. pistaciae* y *S. wertheimae*.

- Galls with a closure zone that isolate the waste in hydrophobic chambers by covering it with wax. In this group within the Pemphigini are the galls of the subgenus *Pemphiginus* (*P. bursarius, P. immunis*, *P. spyrothecae*, *P. protospirae* y *P. populinigrae*) with trichomes in the multiseriate epidermis-lumen of the chamber. Without trichomes in the epidermis-lumen, we find the Fordini *F. riccobonii*, *S. betae*, and *P. cimiciformis*, *F. formicaria*, *F. marginata*, *A. lentisci*, *A. cynodonti*, *Geopemphigus torsus* y *G. blackmani*.
- Galls that are permanently open or pseudogalls. In this group is the definite gall of *T. affinis* and *E. ulmi* of the tribe Eriosomatini.

It is noteworthy that microscopic studies are a source of useful information when contributing to the natural history and taxonomy of groups of gallicolous aphids, as has been shown in previous studies by Álvarez et al (2014, 2016) and in the present work. This is evident if one takes into account that galls, despite being plant structures, are an extension of the phenotype of the inducing organism to the host (Stern 1995; Stone and Schönrogge 2003; Hearn et al. 2019). Future microscopic studies of other Hormaphidinae and Eriosomatinae-induced galls will be necessary to confirm this.

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