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1 ORIGINAL PAPER

2 Histological changes induced by the cynipid wasp *Dryocosmus* 3 *kuriphilus* (Hymenoptera: Cynipidae) in leaves of the chestnut 4 *Castanea sativa* (Fagaceae): Mechanisms of galling impact on host 5 vigor

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9 **Abstract**
10 *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae), the Asian chestnut gall wasp (ACGW), is an invasive pest that threatens 11 native stands and orchards of European Sweet Chestnut (*Castanea sativa* Mill.). ACGW induces galls in stems, petioles, and 12 midribs. These galls cause inhibition of tree growth and fruit production. An understanding of morphogenetic changes in 13 host organs is important to evaluate how plant resources are redirected to galls. Structural divergences in *C. sativa* petioles, 14 midribs, and respective galls were investigated. Larvae of *D. kuriphilus* are found in the central region of young petioles 15 and midribs in the spring. They are positioned in the pith region of petioles and midribs, surrounded by vascular tissues. 16 The increase in cell layers and volume is evident in the ground tissues of galls, i.e., parenchyma, collenchyma, and scle- 17 renchyma that originate from ground meristem. Gall formation causes the separation of the original vascular system into 18 several collateral and amphicribral vascular bundles. The vascular web branching likely favors the redirection of resources 19 from developing leaf blades to the galls by compensatory hydraulic mechanisms. The rapid growth rates of galls are likely 20 supported by an increased water supply to gall sites. Cytoplasmically dense and metabolically active nutritive linings of the 21 larval chambers are the sole source of food for larvae. Nutritive cells are maintained by a rich vascular supply. The redif- 22 ferentiation of mechanical tissue surrounding the nutritive tissue also requires energy and protects the *D. kuriphilus* larva 23 until pupation. These vascular alterations impact the normal formation of tissues in distal regions of the leaf, which reduces 24 the productivity of chestnut trees.

25 **Keywords** Cell hypertrophy · European Sweet Chestnut · Hyperplasia · Nutritive tissue · Vascular neoformation

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Introduction

Most cynipids (Cynipidae, Hymenoptera) are not considered economic pests (Stone et al. 2002; Pujade-Villar 2014), but a notable exception is *Dryocosmus kuriphilus* (Yasumatsu 1951), also known as Asian chestnut gall wasp (ACGW). This wasp is the most harmful pest of *Castanea* spp. (Fagaceae, Fagales), due to the severe loss of fruit yield caused by its galls on leaves and shoots (Aebi et al. 2006; Quacchia et al. 2008). 28 29 30 31 32 33 34

Galls are neoformed plant structures, induced by organisms such as insects, mites, nematodes or microrganisms (Mani 1964). Gall inducers, especially insects and mites, are usually specific to their host-plant species. The anatomy and metabolism of gall morphotypes are strongly related both to the species of gall inducer and the species of its host 35 36 37 38 39 40

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plant (Stone and Schönrogge 2003; Ferreira et al. 2019a). We still know little about the process of gall initiation and how cynipid inducers control plant growth. Some signaling molecules are known to cause initial modifications in plant cell development; however, no gene transference has been observed (Ferreira et al. 2019a; Cambier et al. 2019; Hearn et al. 2019). Galls are induced to provide both nutrients and shelter for the developing larva, and may also provide protection against natural enemies and harsh environmental conditions (Price et al. 1987; Stone and Schönrogge 2003; Álvarez et al. 2009), and the same occurs in galls of *D. kuriphilus*.

The ACGW is native to China and was accidentally introduced first into Japan (Shirakami 1951; Yasumatsu 1951) and other countries in Asia, then into the USA (Payne et al. 1975). More recently, ACGW invaded Europe (Brussino et al. 2002), reaching Catalonia (Spain) in 2012 (Pujade-Villar et al. 2013). It has now spread throughout the entire Iberian Peninsula (Jara-Chiquito et al. 2016), where it infests native stands and orchards of European Sweet Chestnut (*Castanea sativa* Mill.).

The invasive *D. kuriphilus* and its galls cause serious damage and economic loss. Up to 75% of fruit production can be lost (Payne et al. 1975; Brussino et al. 2002) because heavy galling inhibits shoot development, reduces foliage photosynthetic area, and suppresses floral sprouting. Heavy galling has even led to tree mortality (Aebi et al. 2006).

The life cycle of *Dryocosmus kuriphilus* begins when adults lay eggs inside the winter buds in May-July (Itô et al. 1962; Warmund 2013). The eggs are deposited in the youngestleaf primordia. The larvae hatch 30 to 40 days after oviposition but remain in the buds until the next spring, when the buds begin to grow (Brussino et al. 2002; Viggiani and Nugnes 2010). During the winter, the buds are covered with densely matted, small white trichomes, and infected buds cannot be detected (Warmund 2013). After budbreak in the spring, larvae develop in synchrony with the development of the buds. They feed for 20 to 30 days inside the leaf primordia or the stem apical meristem and induce the formation of visible leaf galls on the petioles and midribs of leaves or on stems (Fig. 1). Each gall consists of green or red swellings 5–40 mm in length. The larvae are fully grown by the middle of spring (Warmund 2013). The adults leave the mature galls in early summer and oviposit in new chestnut buds. By autumn, the abandoned chestnut galls senesce, but remain on the tree for one to two more years, while the tips of galled branches die. Because of this dieback, galled branches do not produce chestnuts in the following year (Quacchia et al. 2008; Warmund 2013).

The location and number of larval chambers in cynipid galls are influenced by maternal oviposition, whereas gall initiation and maintenance are influenced by oviposition and larval feeding (Folliot 1977; Reale et al. 2014; Cambier et al. 2019). Since galls are formed entirely of plant tissues, gall initiation and growth are influenced by host-plant traits and by environmental factors (Bailey et al. 2009). Normal plant development follows the morphogenetic patterns determined by plant meristems, which are changed by the galling stimuli, whereas the rearrangement of gall tissues begins in meristematic tissues such as protoderm, ground meristem and procambium, leading to overdifferentiation and/or inhibition of some anatomical structures, and sometimes to cell redifferentiation (Ferreira et al. 2019a; Hearn et al. 2019).

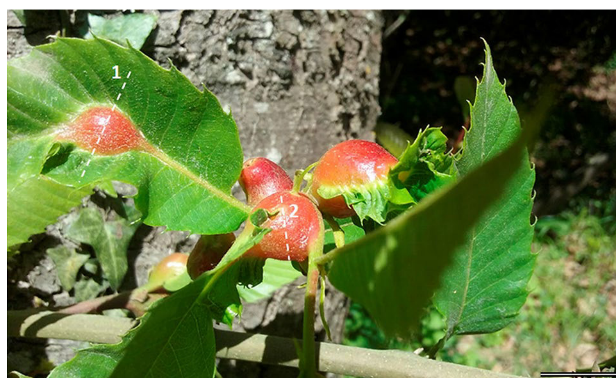

Fig. 1 Chestnut galls induced by *Dryocosmus kuriphilus* showing a midrib gall (1) and a petiole gall (2). The dashed white lines indicate transverse sectioning orientation for the histological study. **Scale bar** = 1 cm

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Layers of nutritive tissue that line the larval chamber begin forming soon after the galls are initiated. Nutritive tissues in galls are formed by specialized parenchyma, with dense cytoplasm, prominent nucleus, and accumulation of primary metabolites, near the feeding sites (Schönrogge et al. 2000; Ferreira et al. 2017). An increase in the amount of nutritive substances in ACGW gall tissues is thought to occur, even though the histological changes that lead to the loss of plant vigor are unknown (Warmund 2013; Reale et al. 2014). A sclerenchyma sheath usually develops around the nutritive layers, as larval feeding continues and the gall matures (Warmund 2013; Ferreira et al. 2019a). The innermost layers lining the larval chambers are morphologically similar in all cynipid galls. Generation- and species-specific gall structures result from variation in the development of the outer parenchyma and dermal-system tissues (epidermis and/or periderm) (Shorthouse and Rohfritsch 1992; LeBlanc and Lacroix 2001; Stone et al. 2002; Stone and Schönrogge 2003; Bragança et al. 2020).

Vascular tissues within galls are connected to those of the host organ, and the vascular web is usually increased by hypertrophy or hyperplasia in vascular tissue, and by vascular neoformation. The vascular web of galls and ungalled plant organs is located within the layers of parenchyma or sclerenchyma, since procambial strands are surrounded

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by ground meristem tissues in developing plant organs (Rohfritsch 1992). Therefore, the rearrangement of vascular tissues in gall formation may also be influenced by changes in growth rate during the phase of growth and development (Isaias et al. 2018; Ferreira et al. 2019a; Bragança et al. 2020). When the gall tissues are differentiated, the larvae continue to feed until they complete their development and leave the galls, and gall growth ceases (Folliot 1977).

Studies of gall anatomy began in earnest in the 1800s with researchers such as Lacaze-Duthiers (1853), Beijerink (1883), and Fockeu (1889), who used classical histological techniques to show changes from gall initiation to maturity. A resurgence of these techniques occurred in the late 20th Century, when researchers such as Bronner (1975, 1977), Meyer and Maresquelle (1983), Pujade-Villar (1987), Shorthouse and Rohfritsch (1992), Brooks and Shorthouse (1998), Arduin and Kraus (1995), Kraus and Tanoe (1999), and Souza et al. (2000) made important contributions to the field. Probably the first major gall studies are those of Houard (1904) and Ross (1932). Notable studies of cynipid gall histology include examinations of *Biorhiza pallida* by Rey (1966, 1967, 1969, 1971), *Plagiotrochus suberi* by Garbin et al. (2005), and gall morphotypes of several species by Harper et al. (2004).

The present study assessed changes in the histological features induced by *D. kuriphilus* in leaves of *C. sativa*, by comparing the anatomy of mature galls induced on petioles and midribs with corresponding ungalled structures. Because the anatomical and vascular rearrangement may indicate the role of gall anatomy in the redistribution of water and photoassimilates, we concentrated on changes in the vascular system. The results may be useful in future studies of source-sink relationships in *C. sativa*, which could affect leaf development and reduce plant productivity. This contribution describes histological features of ACGW galls on *C. sativa*, observations complementing those of Warmund (2013), who studied initial morphological and physiological changes during gall induction by *D. kurphilus* in *Castanea mollissima* Blume.

Materials and methods

Fresh ungalled, recently expanded leaves (midribs and petioles), along with galled petioles and midribs (n = 5 each) were randomly collected from lower branches of the host *Castanea sativa* in May 2018, when the galls contained full-grown larvae (Warmund 2013). Collected galls were mature (Fig. 1), containing full-grown larvae that were still actively feeding. Collections were made from different natural stands of chestnuts located in Catalonia (Spain). The host trees were growing close to mixed forests that contained native species of oak (Jara-Chiquito et al. 2019). Samples ($1\ cm^2$) of ungalled leaf midribs and petioles (controls), and galls were fixed in FAA formalin fixative (formaldehyde, acetic acid and 70% ethyl alcohol, 1:1:18). Following the procedures described by Álvarez et al. (2009), samples were dehydrated in an increasing ethanol series and embedded in Paraplast®, using isoamyl acetate as an intermediate liquid medium. Paraffin blocks were sectioned at 12 µm in a rotary microtome and the sections were affixed to microscope slides. After deparaffinization with xylol, sections were stained with Safranin-Fast Green, dehydrated, and mounted permanently in Entellan® on microscope slides. Additional sections were stained with Lugol to detect the presence of amyloplasts, and others were double-stained with Hematoxylin-Eosin for cytological study. Additional sections were permanently mounted without staining. Slides were examined with a Nikon E600 compound microscope under brightfield, epifluorescence, and polarized light conditions and photographed with a coupled digital camera.

Results

Histology of ungalled leaves (Fig. 2)

Midrib (from adaxial to abaxial side) (Fig. 2a, c)

The midribs of recently expanded leaves are encased with a single layer of epidermis on the adaxial (upper) surface, with small cubic cells covered externally with a thin cuticle. A layer of approximately 7–11 cortical cells is present outside the vascular system, with a mean of 8.37 layers of cells. The cortical layer of the midrib is composed of 3–6 layers of subepidermal annular collenchyma, followed by 5–8 layers of homogeneous storage parenchyma. The cortical layers encase a vascular system that appears circular in transverse section (Fig. 2a). The entire vascular system is encased externally with a bundle sheath composed of the innermost layer of storage parenchyma. Three to six cylindrical layers of developing pericycle fibers (still depositing secondary cell walls) (Fig. 2c) encase the vascular system. The vascular system is organized in three portions that appear arched in transverse section (hereafter: arcs) (Fig. 2a, c). The adaxial vascular arc is convex and is composed of, from the upper to lower layers: adaxial phloem, procambial cells, differentiating metaxylem, and protoxylem, and is considered bicollateral (the phloem portion is positioned on the adaxial and abaxial sides of the xylem portion). Four to six layers of storage parenchyma separate one arc from the next. The two abaxial vascular arcs are concave, bicollateral, and composed of, from the upper to lower layers: protoxylem, differentiating metaxylem, procambial cells, and phloem (Fig. 2c). Different stages

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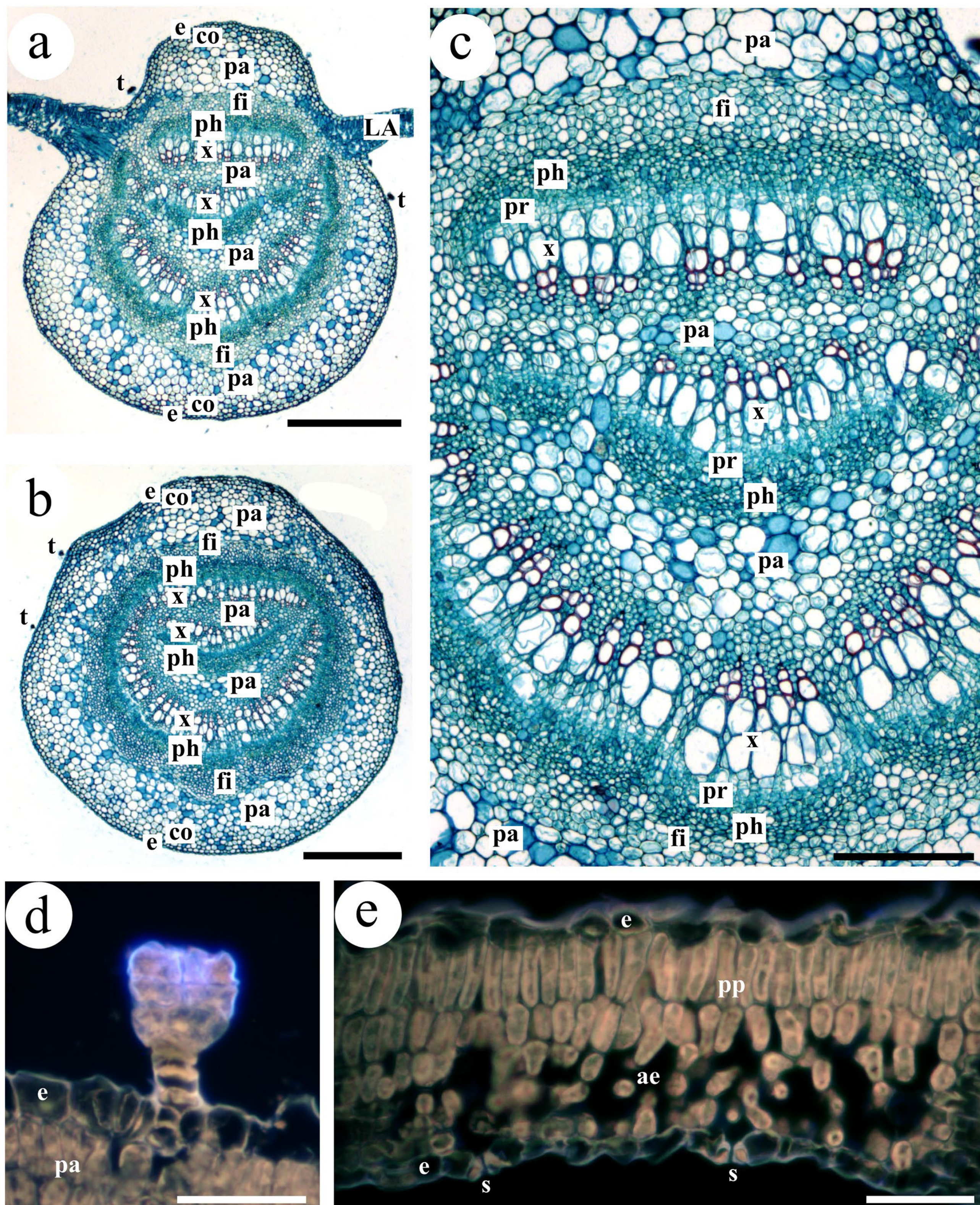

of vessel elements are found in the vascular arcs, from the procambium to the mature metaxylem cells. A remaining procambium (smallest cells without secondary wall) between the xylem and phloem differentiates additional young vessel elements (large cells without a secondary wall or with little secondary wall), and mature vessel elements already have lignified secondary walls and are dead at maturity (Fig. 2c).

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Fig. 2 Anatomy of control leaf of *Castanea sativa* Mill. (Fagaceae). a Cross section of leaf midrib. Note the three vascular arcs from the adaxial face to the abaxial face; in the first, phloem (ph)/xylem (x); in the second, xylem/phloem; and in the third, xylem/phloem. The three arcs are surrounded by bundle sheath fibers (fi). Note the parenchyma cells between the first and the second vascular arcs, and between the second and the third. b. Transverse section of petiole. As in the midrib, there are three vascular arcs surrounded by bundle sheath fibers (fi), and two sets of parenchyma cells (pa) between the vascular arcs. c. Detail of the vascular arcs. Note the procambium between the phloem (ph) and the xylem (x). d. Multicellular glandular trichome on midrib. e. Leaf lamina with chlorophyll palisade parenchyma (pp) in adaxial region and aerenchyma (ae) in abaxial region. Stains: a, b, c. Safranine-Fast Green. Microscope illumination: a, b, c. Bright-field. d, e. Epifluorescence. Abbreviations: *ae* aerenchyma, *bu* bundle sheath fibers, *co* collenchyma, *e* epidermis, *LA* leaf lamina, pa: parenchyma, *ph*:phloem, *pp* chlorophyll palisade parenchyma, *pr* procambium, *s* stoma, *t* trichome, *x*:xylem. Scale bars: a, b = 500 µm; c = 200 µm; d, e = 50 µm

234 The lower midrib cortex comprises 4–6 layers of homogeneous storage parenchyma, followed by 4–6 layers of annular collenchyma. The latter is encased with an abaxial single layer of epidermis with small cubical or papillose cells, covered with a thin lower cuticle. Sparse multicellular glandular trichomes (Fig. 2d) are present. Amyloplasts are uncommon in the cortical parenchyma cells. Druses are found in the parenchyma, in smaller numbers in the cortical cells (the largest ones) than in the parenchyma between the vascular arcs, where the smallest druses are present. 243

244 **Petiole (from adaxial to abaxial side) (Fig. 2b, c)**

245 The petiole is covered with a single layer of small cubical epidermal cells, covered with a thin cuticle. The petiole cortex is encased by the epidermis and comprises 4–6 layers of annular collenchyma, followed by 4–10 layers of homogeneous storage parenchyma. About 8–12 cell layers of collenchyma and parenchyma occur in the petiole cortex, with an average of 10.00 layers of cells. 251

252 A continuous cylinder of differentiating bundle-sheath fibers surrounds the entire vascular system. The vascular system is divided into three collateral vascular arcs, with xylem opposed to phloem cells, as seen in transverse sections, separated by the storage parenchyma (Fig. 2b). The adaxial vascular arc is convex and is sometimes fused with the abaxial vascular arc, forming a cylinder. From the upper to lower layers, the adaxial arc is composed of phloem, procambium, metaxylem and protoxylem. A storage parenchyma with homogeneous cells separates the vascular arcs. The two abaxial vascular arcs are also collateral, but the protoxylem is adaxial, followed by metaxylem, procambial cells, and abaxial phloem. The abaxial layers of the petiole are composed of 5–8 layers of homogeneous storage parenchyma, followed by 3–6 layers of annular collenchyma (Fig. 2b). The abaxial epidermis is a single layer composed of flat cells covered with a thin cuticle. Multicellular glandular trichomes (Fig. 2d) are common. 269

270 Druses are abundant in the parenchyma outside the bundle sheath, and in the innermost cells, with the largest druses occurring outside the sheath. Amyloplasts occur sparsely in the cortical parenchyma. 273

274 **Leaf blade (from adaxial to abaxial side) (Fig. 2e)**

275 The adaxial side of the leaf blade is covered with a single layer of epidermis, with large flat cells and a thin upper cuticle. The mesophyll is dorsiventral, with 2–3 layers of palisade parenchyma containing chloroplasts, followed by 3–4 layers of chlorophyllian aerenchyma with a small meatus. The abaxial epidermis is a single layer of small cubical cells, stomata, and some multicellular glandular trichomes. Numerous large druses occur in the aerenchyma cells. No amyloplasts were observed. 283

284 Galls of Dryocosmus kuriphilus (Fig. 3)

285 **Midrib gall (Figs. 1, 3a)**

286 The galls are covered with a single layer of epidermis with small flat cells on the outside, covered with a thin cuticle. Some multicellular glandular trichomes (Fig. 2d) are present. Annular or laminar collenchyma (2–3 layers) occurs in the subepidermal layers, with hypertrophied cells and slightly thickened cell walls, adjoining a homogeneous storage parenchyma with hypertrophied cells. About 26–37 layers of large cells (mean 30.14 layers) compose the cortical region of the galls. The vascular system is divided into small vascular bundles within the cortical storage parenchyma, surrounding the ovoid larval chamber (Fig. 3a). The majority are open collateral vascular bundles (Fig. 3c), i.e., with the phloem facing outward and a remaining procambium between the xylem and phloem. Some vascular bundles are amphicribral (i.e., the phloem surrounds the xylem) (Fig. 3d) and surrounded by bundle-sheath fibers on the phloem side (Fig. 3e). Storage parenchyma closest to the vascular bundles has abundant medium-sized druses, but these druses are smaller than those in the outermost storage parenchyma. Amyloplasts were not observed in gall tissues. 305

306 The innermost cell layers of the gall usually surround a single larval chamber, but sometimes more than one larval chamber is found per gall. About 15 layers of sclereids under the cortical storage parenchyma form a mechanical tissue (sclerenchyma sheath), which surrounds each larval chamber (Fig. 3f). Internally, a 5-layered nutritive tissue lines the larval chamber and is encased by the sclerenchyma sheath (Fig. 3g, h). The nutritive tissue consists of large, basophilic cells, with porous-appearing cytoplasm, a large nucleus 314

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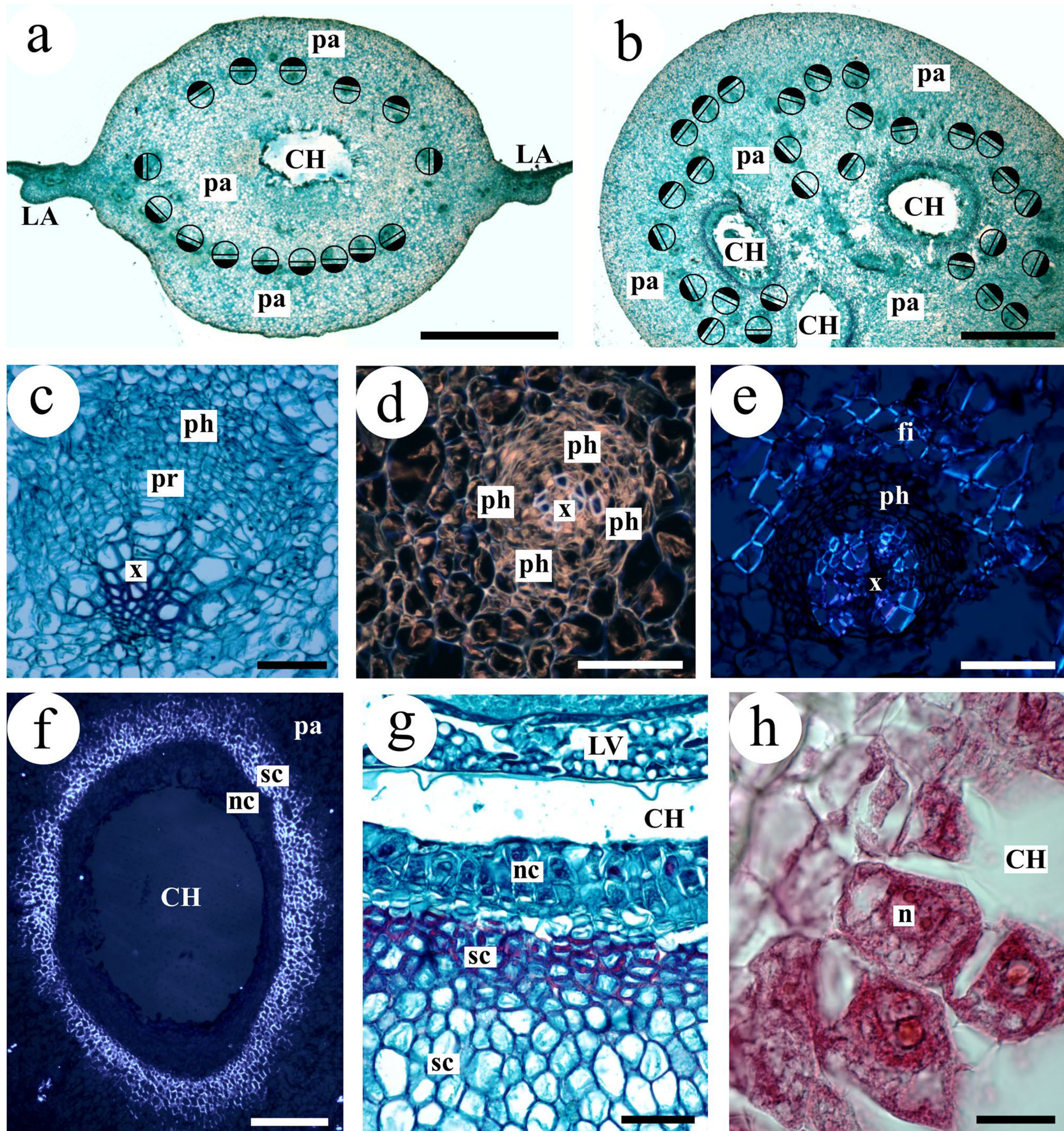

Fig. 3 Anatomy of galls induced by *Dryocosmus kuriphilus* (Yasumatsu 1951) (Hymenoptera: Cynipidae) on leaves of *Castanea sativa* Mill. (Fagaceae). a Midrib gall with a single larval chamber. Each circle indicates a vascular bundle, with the portions filled in black indicating the phloem position. Note that the larval chamber (CH) is surrounded by scattered vascular bundles, with the phloem facing outward. b Portion of multilocular gall (with three larval chambers) induced in the petiole. The three larval chambers (CH) are surrounded by scattered vascular bundles where the phloem is arranged peripherally. c Open collateral vascular bundle. d Amphicribral vascular bundle. e Collateral vascular bundle with bundle sheath fibers (fi) outside the phloem (ph). f Nutritive cells (nc) lining a larval chamber, and adjoining layers of sclerenchyma (sc). g Sclereids (sc) near the nutritive cells (nc) have thickened secondary walls. h Nutritive cells with characteristic granulose cytoplasm and prominent nuclei. Stains: a, b, c, g. Safranin-Fast Green. h Hematoxylin-Eosin. Microscope illumination: a, b, c, g, h. Brightfield. d Epifluorescence. e, f Polarizing. Abbreviations: *bu* bundle sheath fibers, *CH* larval chamber, *LA* leaf lamina, *LV* larva, *n* nucleus, *nc* nutritive cells, *pa* parenchyma, *ph* phloem, *pr* procambium, *sc* sclereids, *x* xylem. Scale bars: a, b = 2 mm; c, d, e, g = 100 µm; f = 500 µm; h = 20 µm

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with abundant euchromatin, and a conspicuous nucleolus (Fig. 3g).

Petiole gall (Fig. 3b)

Petiole galls are covered with a single layer of epidermis with periclinally elongated rectangular cells, covered with a thin cuticle. Multicellular glandular trichomes are found on the epidermis, similar to those occurring on the control petiole (Fig. 2d). A subepidermal collenchyma with 2–3 layers is sometimes present, all with slightly thickened cell walls. A homogeneous storage parenchyma is present, with hypertrophied polyhedral or obliquely elongated cells. The cortical region of the gall (collenchyma and parenchyma) has 23–40 layers of these hypertrophied cells (33.88 cell layers on average). Amyloplasts and druses were not observed in gall tissues.

A set of small vascular bundles is encased in the storage parenchyma, usually arranged in a circle, parallel to each larval chamber. Sometimes they are open (i.e., with a remaining procambium) collateral (i.e., the phloem is only on the abaxial side of the xylem) bundles (Fig. 3c) (almost always with the phloem outward), and sometimes amphicribral bundles (Fig. 3d). The vascular bundles are sometimes encompassed by fibers in the outer region (Fig. 3e). The innermost cell layers are composed of a mechanical tissue, with 5–15 layers of sclereids (Fig. 3f, g). A nutritive tissue with basophilic and granular cytoplasm, and a large nucleus and nucleoli, lines the larval chambers (Fig. 3g), surrounded by the mechanical tissue (Fig. 3h). Most petiole galls have 1–3 larval chambers.

In cases where the galls coalesce, the larval chambers are separated by several layers of storage parenchyma. When the larval chambers are close together, the mechanical layers commonly fuse, and lack vascular bundles in the fusion area. When the larval chambers are contiguous, they merge into a large lobed chamber with a continuous sclerenchyma ring.

Discussion

Impacts of galls on structures of host organs

The observations on leaf anatomy reported here agree with those of Pinto et al. (2011) and may explain how the inducer larvae change the meristematic activity in the developing petioles and midribs. A differential arrangement of the vascular system occurs in the petiolar and midrib galls, and may be explained by the intense cell hypertrophy and proliferation of ground tissues (collenchyma and parenchyma). The vascular web is increased inside the gall, due to the intense cell proliferation in meristematic tissues, leading to the segregation of the vascular arcs into separate vascular bundles. Branching of the vascular web and hypertrophy of vacuolated parenchyma likely result in diversion of water and photoassimilates to the gall rather than the ungalled distal portions of the leaf. Hydraulic compensation mechanisms would favor water accumulation in galls, and consequently their growth. Histological changes observed during gall formation likely lead to changes in source-sink strength, affecting the continuity of growth in the affected branches and the production of chestnut fruit.

The first-instar larvae induce alterations in epidermal cells after the eggs hatch. At this stage, alterations occur in surface cells, with proliferation and stratification of epidermal cells surrounding the larva, forming a cup-shaped larval chamber (Reale et al. 2014). The leaf primordia epidermis may be distinguished in this stage, with glandular trichomes already present (Reale et al. 2014). First-instar larvae overwinter in the dormant buds, and in the spring, the galls grow and mature (Itô et al. 1962).

Alterations in dermal and ground tissues in galls

Our results showed that the larvae occupy the central region of young petioles and midribs after the dormancy period, forming gall chambers encased by procambial tissues, and leading to changes in the arrangement of vascular tissues. As the epidermis is already differentiated in young leaf primordia, similar epidermal structures are found on the galls. The gall epidermis remains a single layer, but the cell expansion patterns are slightly modified, becoming periclinally elongated and non-papillose. This alteration is due to cell hypertrophy and hyperplasia in the ground-system cells, increasing the gall volume. The epidermal cells are elongated to accommodate the increased surface area, as is common in galls of other species (Álvarez et al. 2009; Isaias et al. 2011; Ferreira and Isaias 2013, 2014; Oliveira et al. 2016). Compared to the ground and vascular tissue systems, the dermal system shows fewer alterations in gall development, since the protoderm differentiates into epidermis earlier than the other primary meristems (Raman 2011; Nobrega et al. 2021), as occurs in the galls studied here. More-complex alterations in the epidermis reflect the complexity of the gall, with changes in the density, size, and morphology of trichomes and stomata, and other epidermal specializations (Ferreira et al. 2019a; Nobrega et al. 2021). Complex cynipid galls may have sticky, spiny, or resinous surfaces, due to the neoformation of emergences (indumentum formed by dermal and subepidermal tissues) in galls, and function as barriers against parasitoids and other natural enemies (Stone and Schönrogge 2003).

Although only minor changes occur in the epidermis, the tissues beneath this layer undergo substantial anatomical changes, involving the appearance of new tissues, including a layer of sclerenchyma, nutritive cells, and a

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rearrangement of the vascular system. These changes categorize the gall as the prosoplasmatic histioid type (see Ferreira et al. 2019a), as is typical for galls of other species of cynipids (Rohfritsch 1992; Brooks and Shorthouse 1998). Galls of cynipids are considered the most structurally complex (Rohfritsch 1992; Brooks and Shorthouse 1998; Ferreira et al. 2019a).

Extensive cellular hyperplasia and hypertrophy occur in the ACGW gall, as is typical for the galls of most species (Mani 1964; Rohfritsch 1992; Brooks and Shorthouse 1998; Bronner 1992; Ferreira et al. 2017, 2019a). Hyperplasia and hypertrophy are extensive in ground tissues during gall development. Hyperplasia results in an increase in the number of layers of cortical cells (from 8.37 to 30.14 in control midribs compared to midrib galls; and from 10 to 33.88 in ungalled petioles compared to petiole galls). Collenchyma cells also enlarge, with a concomitant decrease in the thickness of cell walls. Druses or other crystal types are usually more abundant in galls than in galled organs (Dias et al. 2013; Guimarães et al. 2014; Jankiewicz et al. 2017; Ferreira et al. 2019b; Álvarez et al. 2021). The galls studied here showed a reduction or absence of druses, which can be also detected in other gall morphotypes, depending not only on the host-plant species but also on the species of gall inducer (Álvarez et al. 2009).

The inner layers of the ground tissues are also altered in ACGW galls. The mechanical tissue is formed by cell redifferentiation of parenchyma into several layers of cells with thicksecondary walls, the sclereids, which lend rigidity to the structure (Ferreira et al. 2019a). Sclerenchyma layers or mechanical tissues are commonly observed in galls of cynipids (Hymenoptera) (Rohfritsch 1992; Brooks and Shorthouse 1998). Sclerenchyma differentiation occurs during the gall maturation phase, after the growth and development phase (Rohfritsch 1992), as observed by Warmund (2013) for ACGW galls. The amount of sclerenchyma in these galls was negatively correlated with the number of parasitoids per gall, as observed by Cooper and Rieske (2010), probably due to the rigid secondary cell walls, which protect larvae in mature galls from oviposition by parasitoids (Stone and Schönrogge 2003).

The innermost layers of ACGW galls have cells with a dense cytoplasm, relatively large nucleus, and nucleoli, which indicates that these are metabolically active nutritive cells (Brooks and Shorthouse 1998; Ferreira et al. 2017, 2019a). Nutritive tissues are common in galls induced by larvae with a chewing feeding habit, and usually accumulate lipids and proteins important for insect nutrition (Bronner 1975, 1977, 1992; Brooks and Shorthouse 1998; Isaias et al. 2018; Ferreira et al. 2019a). Lipid bodies, mitochondria, and fragmented vacuoles were observed by Warmund (2013) in nutritive tissues of ACGW galls on *C. mollissima*.

Changes in vascular web of ACGW galls and possible role in reducing host-plant vigor

The processes of cell proliferation and hypertrophy in the ground tissues lead to the separation of the original vascular arcs of ungalled petioles and midribs into several vascular bundles, arranged in circles parallel to larval chambers. We assume that the branching of the vascular system around the gall chambers enhances the supply of water and photoassimilates to the gall. Even though most of the vascular bundles of the galls are open (i.e., contain a remaining procambium that differentiates into new vascular elements) and collateral (adaxial xylem and abaxial phloem), some of them are amphicribral (i.e., the phloem surrounds the xylem), indicating that the ACGW has altered the pattern of procambium differentiation. The occurrence of procambial neoformation, together with the branching of the vascular system, may explain the different patterns and sizes of the vascular bundles embedded in the gall parenchyma.

Changes in hormonal balance resulting in vascular neoformation were shown in galls of *Agrobacterium tumefasciens* (Aloni et al. 1995; Dodueva et al. 2020), affecting the transport of nutrients, water, and photoassimilates. The apparent reduction of the vessel element diameter in neoformed vascular bundles of ACGW galls may be related to increased pressure in the gall xylem, reducing the hydraulic conductivity. In this situation, compensatory mechanisms may lead to transport of water to adjacent cell walls and vacuoles of the cortical parenchyma (Bragança et al. 2020). This hydraulic compensation may enhance gall growth, since increased vacuolar turgidity is essential in cell hypertrophy and proliferation. Therefore, the hydraulic compensation could increase water absorption due to the altered vascular web, affecting water distribution in ungalled portions of the leaf and petiole. The reduced hydraulic conductivity increases the hormonal supply and the sink strength in galls of *A. tumefasciens* (Aloni et al. 1995; Ullrich and Aloni 2000), which likely negatively impacts the growth of ungalled portions of the galled branches.

Conclusion

Dryocosmus kuriphilus induces changes mainly in the ground and vascular system tissues of leaf primordia, leading to alterations in the arrangement of vascular tissues. Changes in the organization of the vascular system are caused by the intense hyperplasia and hypertrophy in the gall parenchyma and collenchyma, leading to a branched vascular web around the larval chamber. The histological changes reported here will help to decipher the possible metabolic developmental pathways, supporting future investigations

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of the signal patterns and gene expression manipulated by inducers to redirect resources to their own benefit.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate All listed authors have approved the manuscript before submission, including the names and order of authors.

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