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RESEARCH ARTICLE

Histological Study of Leaf Galls Induced by Phylloxera in *Vitis* (*Vitaceae*) Leaves

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Abstract The galls induced by hemipterans generally show hypertrophy of the phloem; these insects usually feed on the sap in the sieve tube elements, occasioning phloem bundle hypertrophy. However, there are some exceptions; for example, the phylloxerids feed on the gall wall parenchyma. It has remained unknown, however, whether *Daktulosphaira vitifoliae* (the vine phylloxera) also causes hypertrophy of the phloem bundles. The galls induced by *D. vitifoliae* in leaves of the rootstock variety Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) were examined microscopically. *D. vitifoliae* was found capable of inducing vascular bundle hypertrophy, as well as the nutritional enrichment of the gall wall parenchyma cells upon which the insect feeds. The hypertrophy of the phloem bundles commonly seen in hemipteran-induced leaf galls also occurs in those induced by *D. vitifoliae*, even though these do not feed on the phloem contents, but rather on the gall wall parenchyma. The appearance of phloem bundle hypertrophy in hemipteran-induced leaf galls requires the remobilization of photoassimilates that might affect the productivity of the affected plant.

Keywords *Daktulosphaira vitifoliae* · Galls · Phylloxeridae · Hemiptera · Richter-110

Significance Statement Even though *D. Vitifoliae* do not feed on vascular bundles, they are capable of inducing their hypertrophy, indicating that direct feeding activity is not the only stimulus responsible for alterations in host plant development. The hypertrophy of vascular bundles means a major flow of photoassimilates to hemipteran-induced leaf galls, which are competitive sinks, affecting the growth and productivity of affected vines.

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Introduction

Some organisms, including bacteria, viruses, fungi, nematodes, mites and insects [1] are able to alter the growth of plant tissues to induce galls. Galls originate via the redifferentiation of distinct cell types and/or the hypertrophy and hyperplasia of the host organs [1]. The most complex galls are produced by insects, and show constant morphological characteristics depending on the inducing species; indeed, galls are used in their taxonomic study [2]. The adaptive value of galls is mainly associated with the feeding of the inducing organism, although the shelter they provide from both the environment and natural enemies may also be important [3–5]

The feeding behaviour of gall-inducing insects is a determining factor in the neoformations induced in the host tissue [6]. For example, the galls induced by phloem-sucking insects are different from all others. The phloem sap is a common food source for gall-inducing hemipterans, and the galls they produce show hypertrophy of the phloem bundles [6–8]. In contrast, insects that feed by chewing and scraping plant material induce the differentiation of typical nutritive cells in their galls, upon which they actively feed [6, 9]. The nutritive cells of these galls typically have a dense cytoplasm, one or more small vacuoles, one or more prominent nuclei, and they accumulate nutritive substances such as lipids, soluble sugars and/or proteins [9] but they do not usually possess amyloplasts. "Nutritive-like tissues" containing starch may, however, be observed in hemipteran-induced galls, although these are not the direct food source sought [6].

Amongst the hemipterans, the superfamilies Psylloidea and Aphidoidea are very diverse [1]. The galls they induce form through leaf rolling, folding of the leaf margin, and the appearance of globose structures on the leaf blade [2, 10, 11], involving an organization of the tissues different to that seen in the gall-free areas of leaves [2, 7, 10–13]. However, the galls induced are structurally similar and show hypertrophy of entire vascular bundles with accumulations of soluble sugars in the phloem cells and those close to the gall chamber [6, 14]. The members of Adelgidae and Phylloxeridae, however, feed on the gall wall parenchyma [15–18]. Raman et al. [19] indicate that during the early stages of the growth of galls produced by *Daktulosphaira vitifoliae* (vine phylloxera), cells with cytological features of nutritive cells differentiate. These authors also reported them to show increased concentrations of starch and lipids.

It has remained unsure, however, whether, in addition to inducing the differentiation of nutritive tissues, phylloxera also causes the hypertrophy of the phloem bundles seen in other hemipteran-induced galls. The fact that phylloxera does not feed directly from the sieve tubes suggests such hypertrophy may not occur. The present work reports a histological examination of the leaf galls induced by this organism in Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) rootstock leaves and compares them with those made by sap-feeding insects.

Material and Methods

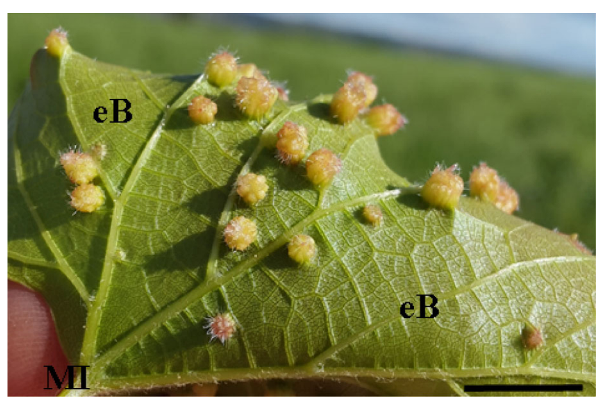
Plant Material and Plot Characteristics

Leaves (n = 4) of Richter-110 rootstock plants (n = 4) showing galls induced by *D. vitifoliae* (Fig. 1) were collected in the summer of 2015 from the La Rioja wine-producing region in Spain. Control leaves (n = 4) without galls were obtained from the same plants.

Sampling Methods and Variables Measured

Fragments of plant material were fixed in formaldehyde-acetic acid-ethyl alcohol (1:1:18), embedded in paraffin wax, and the blocks transversally sectioned to 12 μm in a rotary microtome. Sections were stained with safranin O/fast green for general viewing, or with lugol for the detection of starch. Yet others were mounted unstained for epifluorescence microscopy. All slides were examined using a Nikon E600 microscope in light field, polarizing or epifluorescence mode. Samples were also prepared for scanning electron microscopy (SEM) by dehydration in an increasing alcohol series followed by air-drying and gold coating. Observations were made using a JEOL JSM-6480LV SEM.

Images were taken of the different preparations. AxioVision® software was used to measure the diameter of



eB - abaxial epidermis; *MI* - midrib (Scale bar = 1 cm).

Fig. 1 Galls induced by phylloxera on the abaxial face of the leaf of a Richter-100 rootstock leaf the phloem and xylem bundles at least four times per gall and 4–5 times per control sample.

Statistical Analysis

Differences were compared using the Student t-test, employing SigmaStat® software. Significance was set at $P < 0.05$.

Results and Discussion

Control Leaves

Figure 2a shows a cross-section of a control leaf. The adaxial surface shows a single layer of large, cuboid cells with a thin cuticle. No stomata or trichomes are visible. Below the adaxial epidermis, the mesophyll is dorsoventral and divided into palisade and spongy chloroplast-containing parenchyma. Two layers of palisade parenchyma cells can be seen, the upper one showing tannin inclusions. These layers are followed by six layers of spongy parenchyma cells, either aeriferous with small spaces between

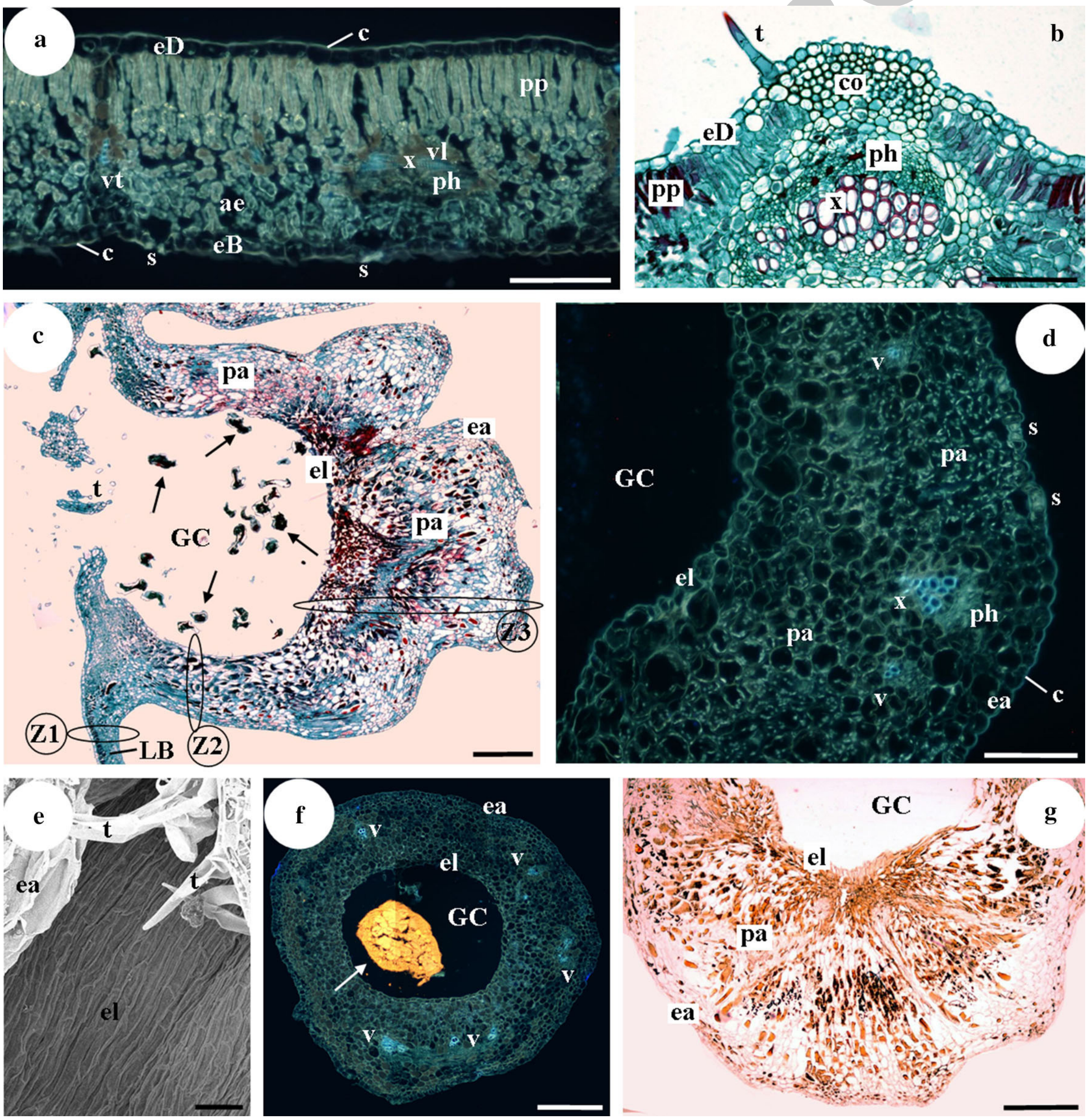


Fig. 2 Histological observations of the leaf galls induced by phylloxera in Richter-110 rootstock leaves

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them, or homogeneous with no spaces. All these cells show chloroplasts. Isolated cells in the palisade and spongy mesophyll show large accumulations of raphides, especially in the adaxial parenchyma. Druses appear in some abaxial cells, sometimes with styloids. The abaxial surface is composed of small, cuboid cells with a thin cuticle. No trichomes are present. Stomata are present and show conspicuous substomatal chambers.

The leaf veins (Fig. 2b) show an upper, single-layered epidermis with a cuticle, and sparse multicellular, non-glandular trichomes. Annular collenchyma lies subjacent, followed by storage parenchyma. Four or five vascular bundles are arranged concentrically in the veins, with the xylem in the interior and the phloem towards both epidermal surfaces. Some phloem parenchyma cells contain small druses. Storage parenchyma and collenchyma are also observed in the abaxial layers, coated by an abaxial epidermis similar to the adaxial one.

Phylloxera Galls

D. vitifoliae induces globose galls on the leaf abaxial surface. These galls are open on the adaxial surface, and their ostioles covered by living, multicellular trichomes (Fig. 2c). From the outside inward, the wall of the gall (Fig. 2d) shows a single-layered external epidermis with a thin cuticle, stomata, and sparse, living, multicellular trichomes. The region of the gall near the gall-free mesophyll (Z2 in Fig. 2c) shows 16 layers of chloroplast-containing parenchyma cells. The distal region of the galls (Z3 in Fig. 2c) shows many more cells than the previous area, with some 30 layers of parenchyma cells, in which amyloplasts are observed (Fig. 2g). This parenchyma is more or less homogeneous, composed of polyhedral cells, with no spaces, and few raphides or druses. The gall as a whole shows abundant vascular bundles, arranged with a certain periodicity (Fig. 2f). Their xylem is orientated towards the gall chamber (Fig. 2d). The chamber is lined with a single-layer internal epidermis, with an indistinct cuticle in the part of the gall furthest from the ostiole. No stomata or hollows are visible (Fig. 2e).

Histometric Comparison of the Vascular Bundles

The phloem bundles of the galls were found to be significantly hypertrophied compared to those of the leaf veins (82.68 ± 13.56 vs. 42.96 ± 9.55 μm^2). No significant differences were seen between the cross-sectional area of the gall xylem bundle compared to those of control leaf veins (109.54 ± 24.80 vs. 85.21 ± 17.55 μm^2) (Table 1).

The majority of the features observed in the present hemipteran-induced galls were similar to those seen in galls induced by arthropods in general [1, 6, 20]. These include parenchyma homogenization, cell hypertrophy, and hyperplasia (an increase in the number of cell layers). However, the phloem in the vascular bundles of the inspected galls showed significant hypertrophy compared to the control leaf sections. Hypertrophy of the phloem is associated with galls induced by phloem-sucking hemipterans [2, 6, 8, 10, 11, 14], but gall-inducing phylloxerids feed by sucking the contents of nutritive parenchyma cells, as reported by Sterling [15] and Raman et al. [19]. The vascular bundle hypertrophy observed in the present work was therefore unexpected, but the results suggest that it may be a common feature of all hemipteran-induced galls. The observed accumulation of starch in the parenchyma of the phylloxera-induced galls agrees with that reported by Sterling [15] and Raman et al. [19], who observed larger numbers of amyloplasts in gall nutritive tissues.

Table 1 Diameter of the phloem and xylem in the vascular bundles of Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) leaves, and galls induced by phylloxera

	Leaf	Gall
Xylem	85.21 ± 17.55^a	109.54 ± 24.80^a
Phloem	42.96 ± 9.55^b	82.68 ± 13.56^a

All values are in μm. Different letters in the same row indicate a significant difference ($P < 0.05$)

Phylloxera-induced galls form on the underside of vine leaves, which determines that the xylem is closer to the gall chamber than the phloem. If the inhabitants of these galls fed on phloem contents, they would have to find a way around the xylem. This, plus the fact that the vascular cells lay at six or more cells' distance from the chamber, tends to confirm that phylloxera feeds on gall parenchyma cells; indeed, the stylets of *D. vitifoliae* nymphs are 60–65 μm log and can easily reach the 5th of 6th parenchyma layer, but no further [17, 19].

The internal epidermis of the inspected galls showed a lack of hollows, a feature commonly seen, however, in aphid-induced galls. In general, aphids (including gall-inducing aphids) feed on the phloem contents, which enter at pressure into their gut, and, following the absorption of nutrients, flow out as sugary faeces [21]. To prevent the inhabitants of globose galls drowning in these faeces, the internal epidermis of the gall has hollows via which these faeces are evacuated towards the plant's vascular tissues [22]. In the present work, however, no such arrangement was seen.

Some studies report the presence of starch in galls induced by phloem-sucking hemipterans. These starch grains are reserves that guarantee the gall tissues' energy requirements are met [8, 10]. In these galls, these starch-storing tissues are called "nutritive-like tissues" since they have a dense cytoplasm and a voluminous nucleus – similar to the typical nutritive cells seen in nematode-, mite-, and chewing insect-

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induced galls [6], but they bear no direct relationship with the feeding of the gall-inducing insect. In phylloxera-induced galls, typical nutritive tissues are observed that contain starch—whereas the nutritive tissues of galls induced by chewing insects, nematodes and mites do not contain amyloplasts [9, 10]. Galls generally behave physiologically as sinks for photoassimilates [23, 24], and the accumulation of amyloplasts would appear related to the energy balance and metabolism of the structure [6, 9]. In summary, the hypertrophy seen in the vascular bundles of galls induced by the phloem-sucking members of Aphididae and Psyllidae is also seen in the galls induced by phylloxera in *Vitis*. This hypertrophy suggests that the gall-inducers go about forming strong sinks for photoassimilates. The latter are then used in gall growth and in the accumulation of reserves in the nutritive tissue fed on by the insects.

Conclusion

The hypertrophy of the vascular bundles of hemipteran-induced galls is a general characteristic also seen in galls produced by phylloxera, even though it is not a phloem-sucking gall inducer; rather, it feeds on gall parenchyma cells. The hypertrophy of the phloem bundles in hemipteran-induced galls may not be solely related to the sap-feeding habit, but provide an additional supply of photoassimilates used in gall growth, development and energy storage. Evidently, this must affect the productivity of the host plant.

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