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REGULAR PAPER

Spatiotemporal variation in phenolic levels in galls of calophyids on *Schinus polygama* (Anacardiaceae)

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Abstract

The expression of plant secondary metabolism is strongly controlled by plant both in time and space. Although the variation of secondary metabolites, such as soluble and structural phenolics (e.g., lignins), has been largely observed in gall-inducing insects, and compared to their non-galled host organs, only a few datasets recording such variation are available. Accordingly, the relative importance of spatiotemporal variability in phenolic contents, and the influence of gall developmental stages on the original composition of host organs are poorly discussed. To address this knowledge gap, we histochemically determined the sites of polyphenol and lignin accumulation, and the polyphenol contents in three developmental stages of two calophyid galls and their correspondent host organs. Current results indicate that the compartmentalization of phenolics and lignins on *Schinus polygama* (Cav.) Cabrera follows a similar pattern in the two-calophyid galls, accumulating in the outer (the external tissue layers) and in the inner tissue compartments (the cell layers in contact with the gall chamber). The non-accumulation in the median compartment (median parenchyma layers of gall wall with vascular bundles, where gall inducer feeds) is important for the inducer, because its mouth apparatus enter in contact with the cells of this compartment. Also, the concentration of phenolics has opposite dynamics, decreasing in leaf galls and increasing in stem galls, in temporal scale, i.e., from maturation toward senescence. The concentration of phenolics in non-galled host organs, and in both galls indicated the extended phenotype of *Calophya rubra* (Blanchard) and *C. mammifex* Burckhardt & Basset (Hemiptera: Sternorrhyncha: Psylloidea: Calophyidae) over the same host plant metabolic potentiality.

Keywords Calophyidae · Compartmentalization · Gall · Lignins · Polyphenols · *Schinus polygama*

Introduction

Plants, as sessile organisms, have acquired physical and chemical plastic features that provide protection against abiotic and biotic stresses (Wink 1997). Usually, plants synthesize a wide spectrum of secondary metabolites,

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particularly phenolic compounds, in response to the attack of herbivorous insects (Tooker and Helms 2014). The function of polyphenols was traditionally supposed to be the defense against herbivores and pathogens, since they may act as growth inhibitors or toxins for these natural enemies (Close and McArthur 2002; Lattanzio et al. 2006; Nyman and Julkunen-Tiitto 2000). More recently, the primary role of polyphenols was questioned, as these metabolites have been associated with antioxidant and photoprotective mechanisms in plants, which explains their increase in plant tissues under abiotic stresses and attack by pathogens and predators (Carmona et al. 2011; Close and McArthur 2002).

The development of insect galls cause biotic stress to their host plant organs, and result in an abnormal plant organ (Meyer and Maresquelle 1983), which develops under the influence of the insect and the plant genotypes (Stone and Schonrögge 2003). Moreover, galling insects have the ability to manipulate the morphogenesis of the host plant organs (Mani 1964) for their nutrition and protection (Price et al. 1987). In galls, the occurrence of phenolics and flavonoid derivatives has been related to reactive oxygen species dissipation in high oxidative stressfull conditions induced by the gall-inducing organisms (Ferreira et al. 2018; Isaias et al. 2015; Oliveira et al. 2017). Also, phenolics may be involved in indol-3-acetic acid (IAA) metabolism, and consequently influence cell hypertrophy at gall developmental sites (Bedetti et al. 2014a; Hori 1992).

The increase in phenol concentrations is usually restricted to the outer tissue compartments (Nyman and Julkunen-Tiitto 2000), and has been observed in galls induced by a diverse taxa of galling insects (Abrahamson et al. 1991; Agudelo et al. 2013; Cornell 1983; Formiga et al. 2009; Hartley 1998; Motta et al. 2005; Nyman and Julkunen-Tiitto 2000). On the other hand, a few galls may have lower concentrations of phenolic compounds than their host organs (Hartley 1998; Stone and Schonrögge 2003). Accordingly, the levels of phenolic contents may vary in spatiotemporal perspectives. This variation in phenolic levels may be a consequence of plant responses to environmental factors, to the activity of galling species, to plant intrinsic physiologal features, as well as to the plant and the gall developmental stages (Hartley 1998; Herms and Mattson 1992; Kause et al. 1999; Wink 2003, 2013). For example, in galls of some *Pontania* spp. (Hymenoptera: Tenthredinidae), phenolic levels may decrease (Hartley 1998), or increase during maturation (Nyman and Julkunen-Tiitto 2000). More than a matter of a linear variation in levels of concentration, the expression of plant secondary metabolism may be controlled both in spatial and temporal scales (Moore et al. 2014), which has been observed in galls induced by taxonomically diverse groups of inducers (Bragança et al. 2017; Dias et al. 2013a; Guedes et al. 2018a, b; Hartley 1998; Isaias and Oliveira 2014; Isaias et al. 2018; Nyman and Julkunen-Tiitto 2000).

Current model of study belongs to the genus *Schinus* L. (Sapindales: Anacardiaceae), which is often attacked by galling calophyids (Hemiptera: Sternorrhyncha: Psylloidea: Calophyidae) (Burckhardt and Basset 2000). The *Schinus* spp.–*Calophya* spp. systems have been previously studied from the anatomical and phenological perspective in Brazil and Chile (Dias et al. 2013a, b; Guedes et al. 2016, 2018a, b). In Brazil, *Calophya* cf. *duvauae* Scott is the inducer of leaf galls on *Schinus engleri* Barkley (formerly named *S. polygamus*); where a progressive accumulation of phenolics was observed (Dias et al. 2013a). *Schinus polygama* (Cav.) Cabrera is a common species in the flora of Chile (Rodríguez 2011), where it hosts at least two gall morphotypes: one induced on stems by *Calophya rubra* Blanchard, and one induced on leaves by *C. mammifex* Burckhardt and Basset (Guedes et al. 2018a, b). Both galling calophyids manipulate the morphogenesis of their host-plant organs and the general chemical composition of gall tissues, which was investigated by chromatographic profiles (Guedes et al. 2016). Such investigations of stem and leaf galls revealed that the most abundant compound in both galls is the tannin pyrogallol (Guedes et al. 2016).

Recent studies have documented the formation of tissue compartments (tissue layers), stimulated by the gall-inducing insect, at gall developmental sites. These tissue compartments store primary and secondary metabolites, related to nutritional functions, energy maintenance, chemical defense, and antioxidant protection (Bragança et al. 2017; Guedes et al. 2018b; Isaias et al. 2018; Teixeira et al. 2017). Calophyids feed on vascular tissues, mostly phloematic bundles, which are neoformed in the median tissue compartment of gall walls (Guedes et al. 2018a, b). In addition, in both leaf and stem gall morphotypes the compartmentalization of nutritional primary metabolites has been observed in the median tissue compartments, where calophyids feed (Guedes et al. 2018a, b). Based on previous studies, it is expected that phenolic compounds should accumulate in tissue compartments not involved in gall nutritional profile, which could prevent the contact of the insects feeding apparatus with toxic phenolics. Also, the levels of phenolic compounds in leaf and stem galls could be associated with the phenolics composition and concentration in the related host organs, and by the physiological developmental stage of the galls. Therefore, taking the superhost *S. polygama* and galls induced by *C. rubra* and *C. mammifex* as models of study, we expect to advance on the knowledge of the little explored context of spatiotemporal variation of phenolics in galls. We assume that once the phenotypes of gall inducers extend to the physiological level in gall developmental sites (Ferreira et al. 2018), insights on the extended phenotype of gall inducers over the same host plant may be discussed by the investigation of specific peculiarities of the influences of two congeneric galling insects over the same host plant

secondary metabolism. Our main questions are: (1) is the compartmentalization of secondary metabolites similar or different regarding the influence of the different species of galling insects? (2) Are there variations in the levels of phenolics linked to gall developmental stage or to the gall-inducing calophyid species? And (3) is there any convergence in spatiotemporal variation in the levels of phenolics in the cogeneric calophyid galls?

Materials and methods

Collection of plant material

Trees of *S. polygama* ($n = 5$) were marked in an area of sclerophyllous forest in southern Chile (36°39′32″S 72°16′43″W at 152 m.a.s.l). On October and November 2016, samples ($n \geq 5$) of non-galled leaves, globoid leaf galls, non-galled stems and conical stem galls (Fig. S1) were collected from each plant. The host organs were collected at the same physiological stage and the galls at three developmental stages (Table 1).

Histochemical analyses

Fresh fragments of mature non-galled leaves and non-galled stems, and of globoid leaf galls and conical stem galls at the three developmental stages were fixed in ferrous sulfate and formalin (37% formaldehyde and 0.1 g L^{-1} iron(II) sulfate heptahydrate) (Johansen 1940) for 72 h for polyphenol detection (black precipitates), and in Karnovsky's solution (2.5% glutaraldehyde and 4.5% formaldehyde in 0.1 mM phosphate buffer) (O'Brien and McCully 1981) for 24 h for lignin detection (Ferreira et al. 2017). The samples were washed in distilled water, embedded in polyethylene glycol 6,000, and sectioned in a rotary microtome (30–40 µm) (Ferreira et al. 2017). For detection of lignins, the sections were submitted to Mäule's reagent (Patten et al. 2005). Mäule's reagent reacts to syringyl propane lignins by producing a red or pink color, and to coniferyl and *p*-coumaryl lignins by producing a brown color (Patten et al. 2007). The orange or brownish-orange reactions indicate intermediary levels of syringyl and coniferyl/coumaryl alcohol lignins (Ferreira et al. 2017; Van Cutsem et al. 2011). The sections were observed under light microscope (Leica DM500, Wetzlar, Germany), photographed (Leica ICC50 HD) and compared to its respective blank section, obtained from fresh material and from material fixed in Karnovsky's solution.

Sample preparation and extraction of soluble polyphenols

Samples of mature non-galled stems and non-galled leaves, and of conical stem galls and globoid leaf galls in three developmental stages, previously dissected to remove the insects, were oven dried at 30 °C for 1 week. 200 mg of each sample from each plant ($n = 5$) was pulverized and macerated in 10 mL of 80% methanol (8:2 v/v) at room temperature in darkness. After 24 h, the extracts were filtered through the Whatman® No. 1 filter paper, and stored at 4 °C for spectrophotometric analysis (Agudelo et al. 2013). Before the analyses, the extracts were concentrated at a reduced pressure with a rotary evaporator until completely dry. Subsequently, the crude extracts were resuspended in 10 mL 80% methanol (8:2 v/v), and analyzed for total soluble polyphenol contents using spectrophotometric methods.

Quantification of total soluble polyphenol contents

Total soluble polyphenol contents were determined with the Folin–Ciocalteu reagent, according to Attard (2013), with slight modifications. An aliquote (10 µL) of extracts diluted in distilled water were pipetted into wells of a microtitre plate (MTP), 100 µL of Folin-Ciocalteu reagent was added, and then the plate was shaken for 30 s, and incubated for 5 min. Subsequently, 80 µL of sodium carbonate was added. The plate was shaken on the MTP reader for 30 s, incubated at 40 °C for 30 min, and then read at 765 nm. Solutions between 0 and 500 mg L^{-1} of gallic acid were used as standards to generate the calibration curve. The results were expressed as gallic acid equivalent (GAE) per milligrams of dry weight (µg GAE mg dry weight^{-1}). All samples and standards were measured in triplicate against water blank.

Table 1 Classification of the three developmental stages of non-galled leaves, non-galled stems, globoid leaf galls and conical stem galls

Developmental stages	Organs		
	Non-galled leaves	Non-galled stems	Leaf and stem galls[a]
Stage I	Young fully expanded leaves, before turning into dark green	Young stems, before turning brown	Growth and development phase (GD): galls with first, second, third and fourth instar nymphs
Stage II	Mature leaves, about 8 months old	Mature brown stems, about 8 months old	Maturation phase (MP): galls with fifth instar nymph
Stage III	Old leaves, about 1 year old	Old dark brown stems about 1-year old	Senescent phase (SP): empty galls

[a]The three developmental stages corresponded to the nymphal instars (Guedes et al. 2018a)

Statistical analyses

A two-way ANOVA was performed to evaluate the interaction between the two factors (organs and developmental stages). Significance levels for multiple tests were adjusted by using the sequential Bonferroni correction. Data normality was verified with the Shapiro–Wilk test and homogeneity of variances with the Levene test. Levels of significance for all statistical analyses were carried out using InfoStat (v. 2013) (Rienzo et al. 2013), considering $P \leq 0.05$.

Results

Sites of lignin deposition

Lignins were detected in light brown in epidermal cells and in xylem of non-galled leaves, indicating the presence of coniferyl and *p*-coumaryl lignins (Fig. 1a, b). In globoid leaf galls in growth and development phase (GD), lignins were not detected. In globoid leaf galls in maturation phase (MP), lignins stained orange in trichomes, and in the adaxial and abaxial epidermal cells, indicating intermediary levels of syringyl and coniferyl/coumaryl alcohol lignins (Fig. 1c), and light brown in vessel elements, indicating the presence of coniferyl and *p*-coumaryl lignins (Fig. 1d). In globoid leaf galls in senescent phase (SP), lignins were detected in brownish-orange in the adaxial and abaxial epidermal cells, revealing intermediary levels of syringyl and coniferyl/coumaryl alcohol lignins (Fig. 1e) and in light brown color in vessel elements, indicating the presence of coniferyl and *p*-coumaryl lignins. In non-galled stems, lignins stained orange in phelloderm, revealing intermediary levels of syringyl and coniferyl/coumaryl alcohol lignins. In addition, the lignins stained magenta in perivascular fibers, secondary xylem, and pith parenchyma cells, denoting the presence of syringyl lignin (Fig. 2a). In conical stem galls in GD, lignins were not detected. However, in conical stem galls in MP, lignins stained orange in phellem cells, revealing intermediary levels of syringyl and hydroxyl-coumaroyl lignins (Fig. 2b) and magenta/orange in the scarce differentiated

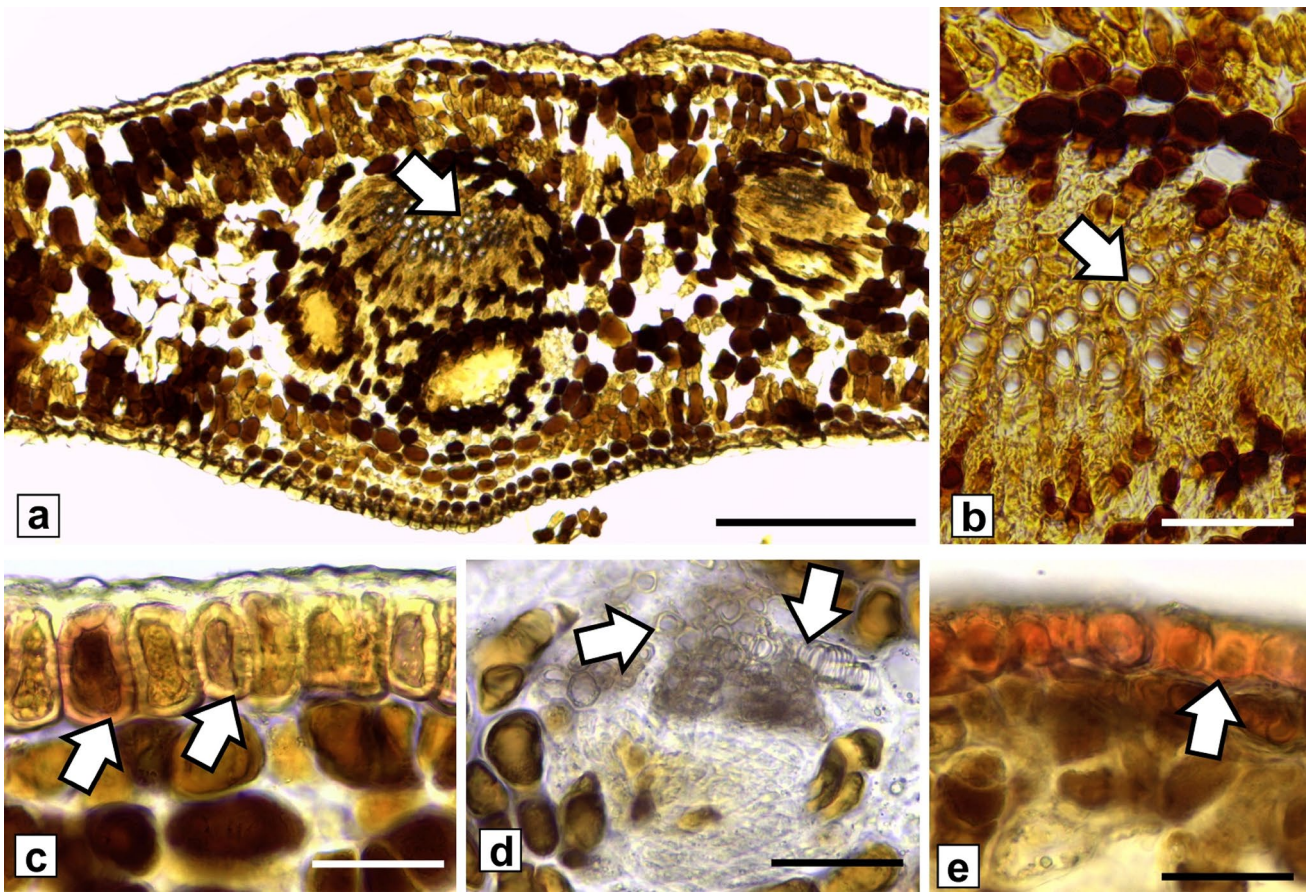

Fig. 1 Histolocalization of lignins with Mäule's reagent in non-galled leaves and globoid leaf galls induced by *Calophya mammifex* on *Schinus polygama* at three developmental stages. Arrows indicated sites of lignins accumulation: **a** leaf midrib; **b** xylem portion of vascular bundle (detail, light brown); **c–e** leaf gall: **c** adaxial epidermis (orange); **d** in vessel elements of the xylem of mature galls (light brown), and **e** in the adaxial epidermis of senescent gall (brownish-orange color). Bars: 50 µm (**b–e**), 200 µm (**a**) (color figure online)

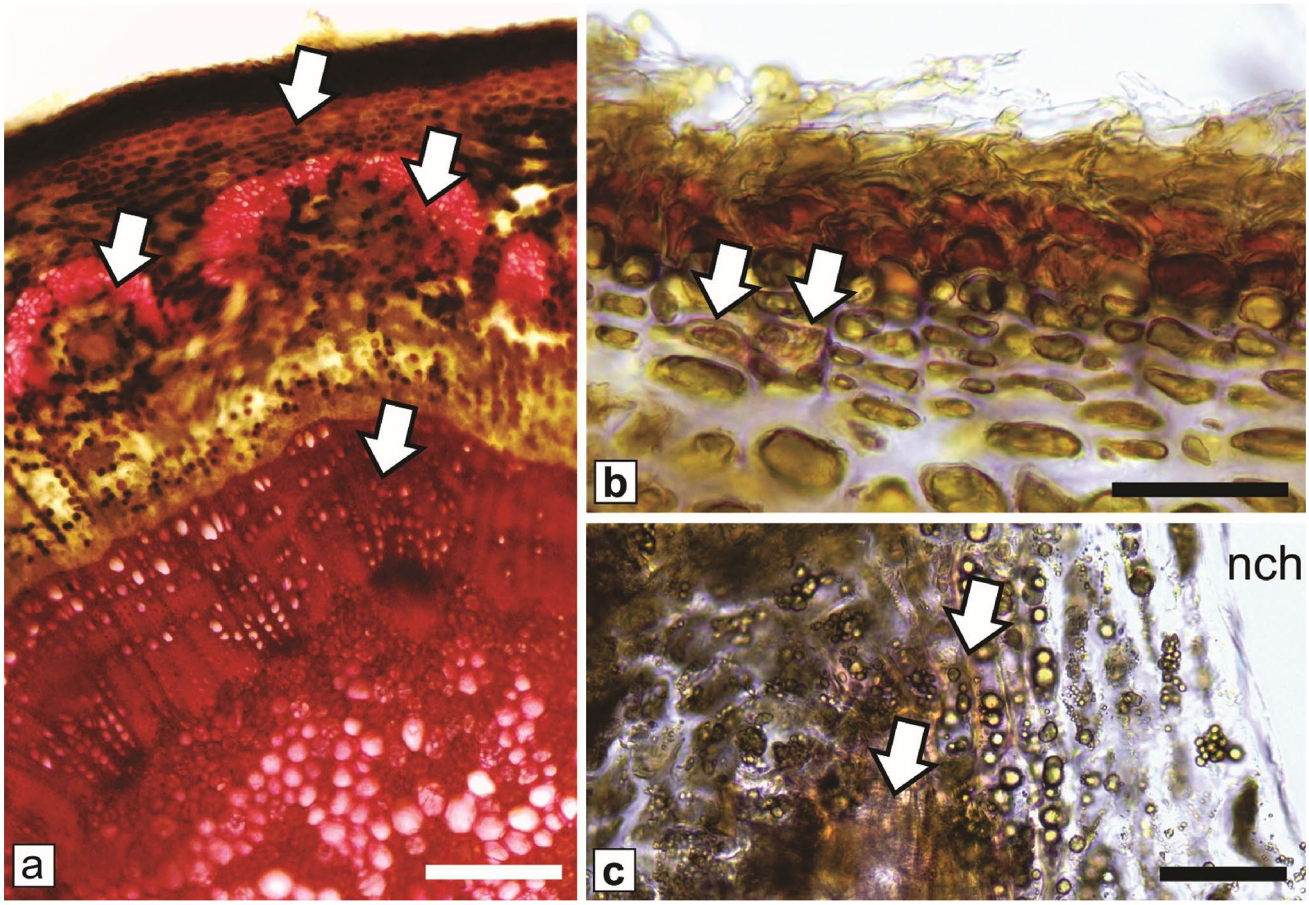

Fig. 2 Histolocalization of lignins with Mäule's reagent in non-galled stems and conical stem galls induced by *Calophya rubra* on *Schinus polygama* at three developmental stages. Arrows indicated sites of lignin accumulation: **a** phelloderm, perivascular fibers, secondary xylem, and pith parenchyma cells of mature stem (magenta); **b** phellem cells (orange), and **c** xylem portion of the vascular units of mature stem gall (magenta/orange). *nch* nymphal chamber. Bars: 50 µm (**b**, **e**, **g**, **h**), 200 µm (**a**, **f**) (color figure online)

xylem cells, denoting the predominance of syringyl lignin (Fig. 2c). In conical stem galls in SP, lignins were scarcely detected in xylem and phelloderm.

Sites of accumulation of polyphenols

Polyphenols were detected in the epidermis, chlorophyll parenchyma (Fig. 3a), vascular parenchyma, and in the epithelial and sheath cells of the secretory ducts of non-galled leaves (Fig. 3b). In GD galls, the polyphenols accumulated in the outer epidermis (of abaxial and adaxial gall walls), and both in the outer and inner tissue compartments, in the abaxial epidermal ordinary cells and trichomes (Fig. 3c). Polyphenols accumulated more scarcely in the median tissue compartment (Fig. 3c). During the maturation phase, phenolics strongly accumulated in the outer and inner tissue compartments, abaxial epidermal ordinary cells, trichomes, and more scarcely in the outer epidermal cells (of adaxial and abaxial gall walls) and in the median tissue compartment (Fig. 3d), but they were undetectable in vascular cells and idioblasts with druses (Fig. 3e). In the senescent phase, the accumulation of phenolics followed the same patterns of compartmentalization of the maturation phase, except for the tissues in the abaxial portion of gall wall and the trichomes, which degraded during senescence (Fig. 3f).

In the non-galled stems, phenolics strongly accumulated in the epidermal and cortical parenchyma cells, and in the cells of phloem parenchyma, sheath cells of the duct, xylem radial parenchyma, and in some idioblasts in pith parenchyma (Fig. 4a). In the conical stem galls in the growth and development phase, polyphenols accumulated moderately in cells of the epidermis, outer and median tissue compartments, but more intensely in cells of the inner tissue compartment (Fig. 4b, c). In the maturation phase, phenolics strongly accumulated in the phellem, outer and inner tissue compartments, and moderately accumulated in some parenchyma cells in the median tissue compartment (Fig. 4d, e).

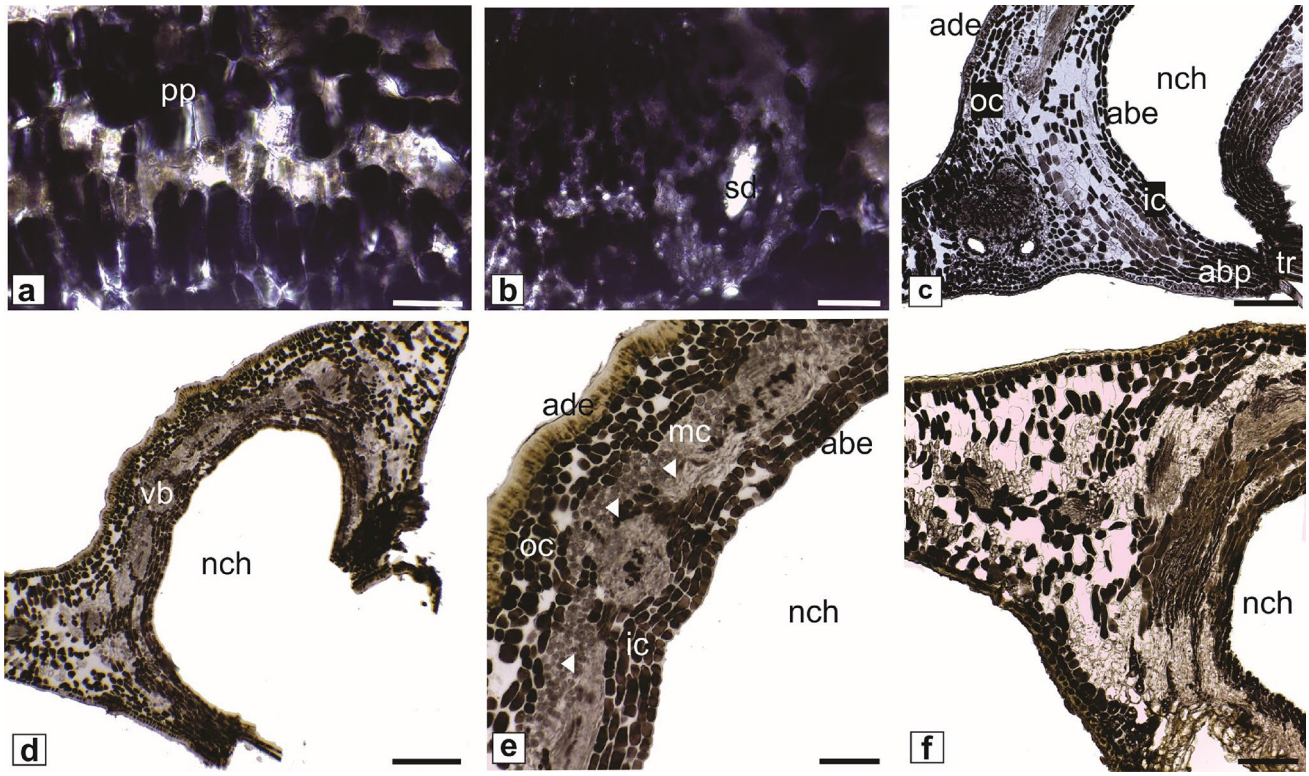

Fig. 3 Histolocalization of polyphenols with ferrous sulfate and formalin in non-galled leaves and globoid leaf galls induced by *Calophya mammifex* on *Schinus polygama*. Black and dark brown coloration indicates positive reaction to polyphenols. **a**, **b** Non-galled leaf, **a** leaf mesophyll, **b** midrib; **c**–**f** Detection of polyphenols along gall developmental stages: **c** young leaf gall (Stage I), **d** mature leaf gall (Stage II), **e** detail of the adaxial region of a mature gall (arrowheads indicate idioblasts with druses), **f** senescent gall (Stage III) with degraded, disorganized and suberized tissues in the inner tissue compartment and abaxial portion. *abe* abaxial epidermis, *ade* adaxial epidermis, *abp* abaxial portion, *ic* inner compartment, *mc* median compartment, *nch* nymphal chamber, *oc* outer compartment, *pp* palisade parenchyma, *sd* secretory ducts, *tr* trichomes, *vb* vascular bundle. Bars: 50 µm (**a**, **b**), 200 µm (**c**, **e**, **f**), 500 µm (**d**)

During the senescent phase, phenolics accumulated in the cells of phellem (not shown), outer and median tissue compartments (Fig. 4f).

Total soluble polyphenol contents

The total soluble polyphenol contents were affected by the developmental stage, the organ, and the interaction between these two factors (Table 2). Temporally, the total soluble polyphenol contents decreased toward senescence in the non-galled leaves, globoid leaf galls and non-galled stems; however, it increased during senescence in the conical stem galls (Fig. 5). The soluble phenolic contents did not show significant differences between young and mature galls (in both cases, the *P* value was higher than 0.05), but decreased significantly in senescent globoid leaf galls ($P=0.02$), and increased significantly in senescent conical stem galls ($P<0.001$). In both host organs, the non-galled leaves and the non-galled stems, the concentration of phenolics increased significantly between stage I and stage II ($P<0.001$). In the non-galled leaves, the concentration of phenolics increased significantly from stage I to stage II ($P<0.001$), and decreased in stage III to a value similar to stage I ($P=1.0$). For the non-galled stems, the concentration of phenols showed significant differences between the three stages of development ($P<0.001$), increasing in stage II and decreasing in stage III.

The total soluble polyphenol contents of the non-galled leaves were significantly lower than those of the non-galled stems in stage I ($P=0.007$), but they increased, not significantly, in both host organs in stage II ($P=0.6$), and decreased with significant differences in stage III ($P=0.02$) (Fig. 5). The phenolic concentration in the globoid leaf galls was similar to that of the non-galled leaves in stage I and III ($P=1.0$), but it was significantly lower than that of the non-galled leaves in stage II ($P<0.001$) (Fig. 5). The concentration of phenolics in the conical stem galls in relation to the non-galled stems was significantly lower in stage I and II (in both cases, the *P*-value was lower than 0.001) (Fig. 5). In stage III, there was an increment of the total soluble polyphenol contents in the conical stem galls, and a decrease in the non-galled stems, leading to significantly higher

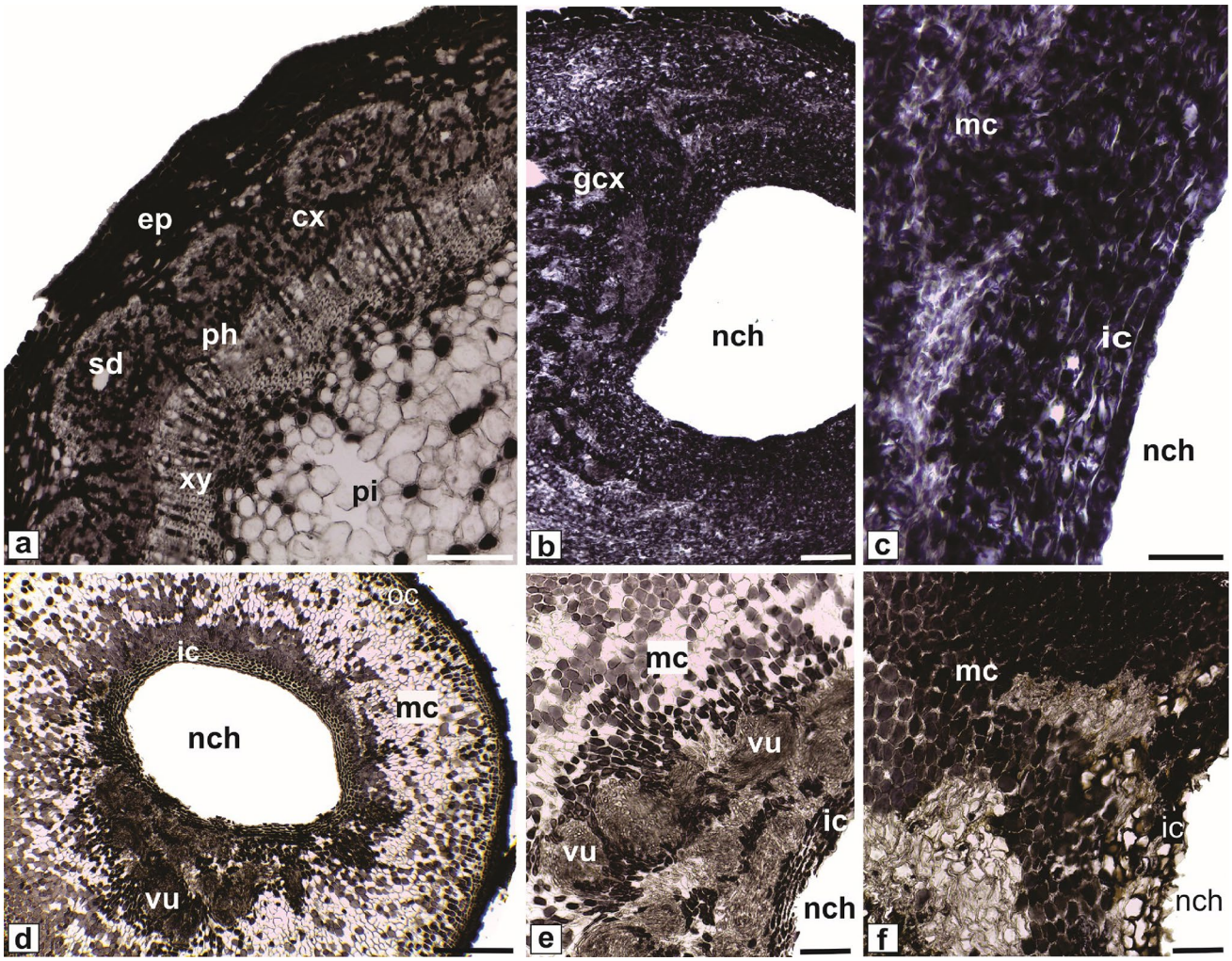

Fig. 4 Histolocalization of polyphenols with ferrous sulfate and formalin in non-galled stems and conical stem galls induced by *Calophya rubra* on *Schinus polygama*. Black and dark brown coloration indicates positive reaction to polyphenols. **a** Non-galled stem; **b–f** Detection of polyphenols along gall developmental stages: **b** young stem gall (Stage I), **c** detail of the adaxial region of a young gall, **d** mature stem gall (Stage II), **e** detail of the inner and median tissue compartments of a mature gall, **f** senescent gall with degraded, disorganized and suberized tissues in the inner tissue compartment. *ep* epidermis, *cx* cortex, *gcx* gall cortex, *ic* inner compartment, *mc* median compartment, *nch* nymphal chamber, *oc* outer compartment, *ph* phloem, *pi* pith, *sd* secretory ducts, *vu* vascular unit, *xy* xylem. Bars: 50 µm (**c**), 200 µm (**a**, **b**, **e**, **f**), 500 µm (**d**)

Table 2 Statistical results (two-way ANOVA) for comparison of polyphenol contents in galls, related to effects of developmental stages and host organs

Source	*df*	Mean-square	*F*	*P*
Developmental stages	2	99.62	36.78	< 0.0001
Organs	3	63.26	23.36	< 0.0001
Developmental stages × organs	6	137.81	50.88	< 0.0001
Error	23	2.70		

contents in the conical stem galls than in the non-galled stems ($P < 0.001$) (Fig. 5). Leaf and stem galls had different concentrations of phenolics in the three stages of development (Fig. 5). The conical stem galls had significantly lower concentrations of phenolics than the globoid leaf galls in stages I ($P = 0.009$) and II ($P < 0.001$), but significantly higher concentrations of the total soluble polyphenol contents in stage III ($P < 0.001$) (Fig. 5).

Discussion

The compartmentalization of phenolics follows similar patterns

The staining of lignins with Mäule`s reagent showed different colors in non-galled host organs and galls, indicating lignins with different composition in cell walls (Van Cutsem

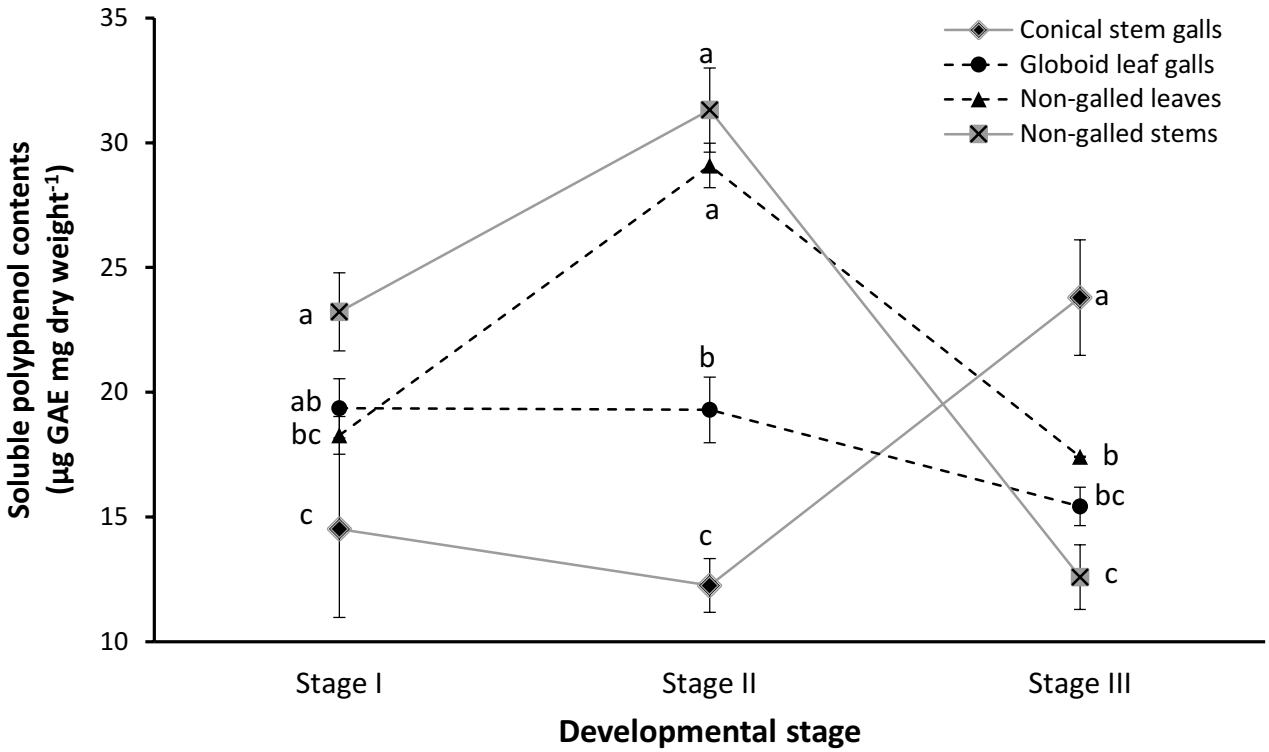

Fig. 5 Polyphenol concentration in host organs and calophyid-induced galls on *Schinus polygama* in three developmental stages. Each point represents the means and standard deviations of five individual trees, analyzed by two-way ANOVA followed by Bonferroni post-test. Different letters mean differences between treatments at $P < 0.05$

et al. 2011). The guaiacyl lignin predominates in the xylem both in the non-galled leaves and in the globoid leaf galls, but the distinct orange and brownish-orange coloration in the globoid leaf galls indicates the possible reallocation or de novo synthesis of syringyl and hydroxyl–coumaroyl lignin precursors toward gall developmental site. The typical lignin of angiosperms is guaiacyl–syringyl lignin (Higuchi 1985), however the metabolism of lignins is very plastic (Lu et al. 2010), and the amount and type of lignin normally present in tissues and organs can change, when plants are subjected to biotic or abiotic stresses (Moura et al. 2010; Neutelings 2011).

Comparing both studied galls, similar lignin profiles were observed with the detection of syringyl and coumaroyl-coniferyl lignins (orange and brownish-orange reactions). The presence of syringyl lignin was detected in the globoid leaf galls, non-galled stems and in the conical stem galls, but it was not detected in the non-galled leaves. Therefore, the galling stimuli of *C. rubra* and *C. mammifex* influence, in the same way, their host tissues, in spite of the host organs distinct potentialities. The induction of syringyl lignin deposition in gall tissues may be related to increased oxidative stress, as the increase of syringyl lignins in cell walls has been previously observed in plants stressed by pathogens (Bishop 2002).

As phloem suckers, *C. mammifex* and *C. rubra* feed in the gall median tissue compartment, a peculiarity of the *S. polygama*–calophyid systems, where new vascular tissues and well-developed vascular parenchyma cells rich in primary metabolites differentiate (Guedes et al. 2018a, b). Therefore, the absence of polyphenols in the phloem and vascular parenchyma relates to the function of their cells as nutritive-like tissues (Ferreira et al. 2016; Guedes et al. 2018a, b), ensuring the requirements for water and nutrients (Guedes et al. 2016) to *C. rubra* and *C. mammifex*. These two-calophyid species induce galls where the deposition of phenolics and lignins follows similar patterns of compartmentalization, with polyphenols accumulating similarly in the outer and inner tissue compartments.

The temporal dynamics of phenolics differs in host organs and galls

The secondary metabolism can change considerably during ontogenesis and plant growth (Donaldson et al. 2006; Neilson et al. 2013). During periods of intense plant growth, such as from young to maturation stage, there is a high demand for resources (Cipollini and Redman 1999; Patrick 1988; Van Dam et al. 2001), and consequently, plant investment in defence should be low. Currently, the

low levels of phenolics in young non-galled host organs indicated a probable tradeoff between growth and defence (Herms and Mattson 1992). This tradeoff seems to have worked out for gall establishment, as it is fundamental for the establishment of the nymphs, which are less adapted to lower levels of toxic chemicals (Berenbaum and Zangerl 1999; Harborne and Grayer 2013). During organ maturation, the conversion of low-molecular-weight phenolics into defense compounds, such as lignin or tannin occurs (Dawra et al. 1988; Herms and Mattson 1992). Therefore, probably, the increase of polyphenol contents in stage II of the non-galled host organs could be a consequence of an increased concentration of high-molecular-weight polymers, such as lignin and other polyphenols. The low concentration of phenolics in both gall morphotypes compared to their host organs indicated a phenolic-reduced environment for the development of the calophyid-induced galls.

Contrary to the expected pattern, the highest polyphenol accumulation was detected in the senescent conical stem galls. During senescence, the gall developmental sites remain attached to the host plant, and cell wall suberisation occurs in the abaxial portion, in the ostiolar opening, and in the inner tissue compartment, as protective reactions (Guedes et al. 2018a), and an impermeable and defensive barrier develops (Kolattukudy 2011). Suberization involves the deposition of various lipid polymers and lignin-like phenolics in cell walls, being considered important apoplastic barriers against infections and water loss (Borg-Olivier and Monties 1993). This feature should be indicative of the role of phenolics in protecting the site of stem galls from pathogen invasion.

The content of phenolic compounds usually increases in gall tissues (Abrahamson et al. 1991; Agudelo et al. 2013; Cornell 1983; Formiga et al. 2009; Hartley 1998; Motta et al. 2005; Nyman and Julkunen-Tiitto 2000). However, similary to the galls induced by *Pontania proxima* and *P. pedunculi* (Hymenoptera: Tenthredinidae) on *Salix* spp. (Hartley 1998), the polyphenol levels decreased in the developmental sites of both gall mophotypes on *S. polygama* in comparison to their host organs. The similar levels of polyphenol in leaf galls and non-galled leaves, as well as the lower levels of polyphenols in stem galls than in non-galled stems, except during gall senescence, may be indicative of metabolic constraints imposed by the host organ. Another explanation may be a greater ability of *C. rubra* than of *C. mammifex* to manipulate the levels of phenolics. So, levels of phenols were determined both by the gall-inducing calophyid species, and by the metabolic constraints imposed by the distinct host plant organs.

Current analyses indicate that on a temporal perspective, polyphenols may have independent chemical quantitative profiles in host organs and galls, as observed in the *Pontania* spp.–*Salix* spp. systems (Hartley 1998). Along gall development, the secondary metabolism of *S. polygama* should be restrictive due to substrate and/or energy limitation, and the synthesis of phenolic compounds should demand high energetic costs for the host plants (Gulmon and Mooney 1986). Accordingly, on temporal bases, the concentration of phenolics is distinct, both in non-galled host stems and leaves, and has opposite dynamics, decreasing in leaf galls and increasing in stem galls from maturation toward senescence.

The biological significance of spatiotemporal variation of phenolics in galls

Lignification is important to reinforce cell walls for water conduction, mechanical support and plant defence (Wang et al. 2013). Generally, cells with lignified walls, such as fibers, sclereids and vessel elements, are related to mechanical support in gall developmental sites. These cells may also act as a defensive mechanical barrier, and also provide a favorable microenvironment for the galling insect development (Rohfritsch 1992; Motta et al. 2005). However, the small amounts of lignins in cell walls of tracheary elements and dermal system in calophyid galls indicates a reduction in lignin deposition, when compared to the host leaf and stem. Such features induced by the calophyid-sucking insects are common for other calophyid-induced galls, such as *C. duvauae* (Dias et al. 2013a). Currently, the sites of lignin accumulation indicate that the synthesis of lignins is related more to water conduction and defence than to mechanical support in both calophyid galls. Finally, the non-lignification of cell layers in the gall inner and median tissue compartments may favour the insertion of the stylets of *C. rubra* and *C. mammifex* toward their feeding sites (Guedes et al. 2018a, b).

Despite the secondary role attributed to the defensive function of phenolics in gall systems (Bedetti et al. 2014b; Close and McArthur 2002), the detection of phenolics and lignins in the outer cell layers in both gall morphotypes, led us to assume their relation not only to gall development, but also to the mechanical defence in *S. polygama-Calophya* spp. systems. In addition, the detection of pyrogallol both in the globoid leaf galls and in the conical stem galls has been related to insecticide properties (Guedes et al. 2016), which supports that phenolics in *C. rubra* and *C. mammifex*-induced galls can enhance their chemical protection against natural enemies.

In the Neotropics, the variations in phenolic contents during gall development has been associated to water and light radiation stresses (Detoni et al. 2011; Formiga et al. 2009). However, current analyses have indicated that polyphenol contents in galls also depend on the developmental stage of

the calophyids, and on the metabolic peculiarities of host organs. The synthesis of phenols, as a potential chemical defence, is neither constant throughout a plant's lifetime nor is expressed evenly throughout the plant's body (Zangerl and Rutledge 1996), which can be extended to gall development, as it has been demonstrated in this study.

The detection of phenolics and lignins was spatially similar between both studied gall morphotypes, being reduced in the median tissue compartment, which has a nutritional profile. The distinct concentrations of phenolics in the calophyid-induced galls indicated both the influence of the gall-inducing species and of their associated non-galled host organs on the secondary metabolism. The influence of the galling insect species reinforces the concept of galls as the extended phenotypes of the galling herbivores, in which even closely related gall-inducing species have their own way to manipulate their host organs, producing specific and peculiar galls. Nevertheless, the influence of the host organ potentialities is also important for the determination of the levels of polyphenols in galls and their functional implications.

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Compliance with ethical standards

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

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