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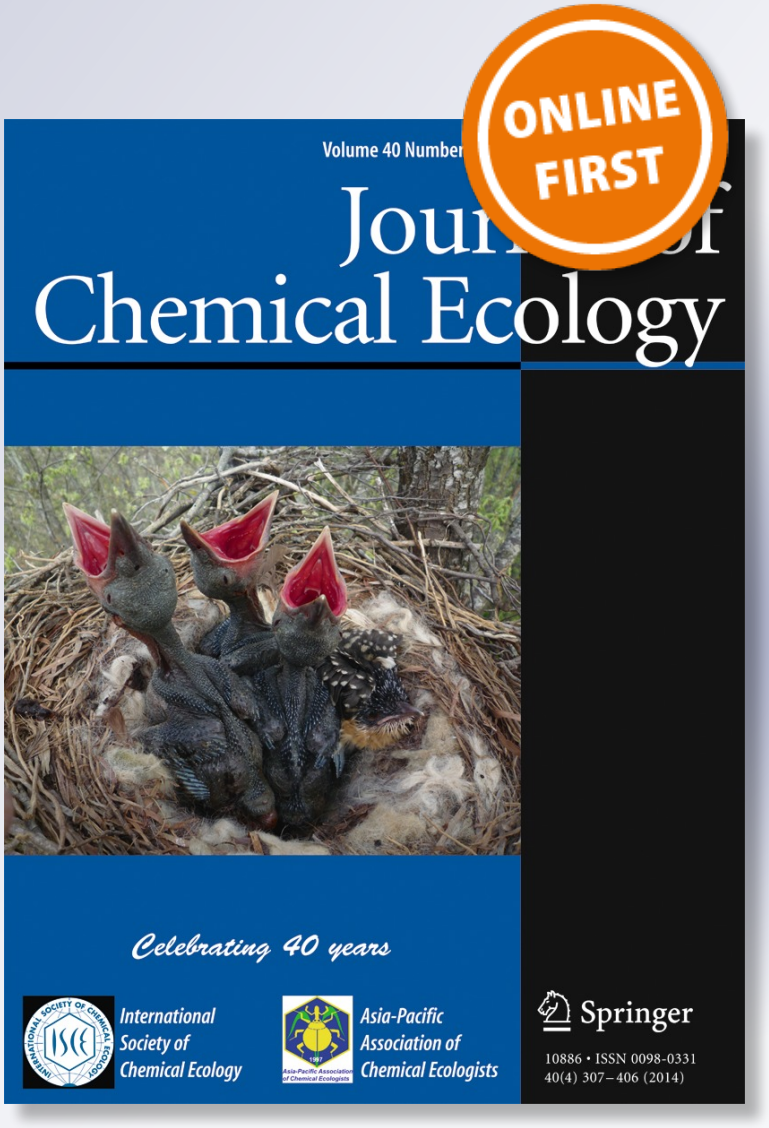

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Specific Polyphenols and Tannins are Associated with Defense Against Insect Herbivores in the Tropical Oak *Quercus oleoides*

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Abstract The role of plant polyphenols as defenses against insect herbivores is controversial. We combined correlative field studies across three geographic regions (Northern Mexico, Southern Mexico, and Costa Rica) with induction experiments under controlled conditions to search for candidate compounds that might play a defensive role in the foliage of the tropical oak, *Quercus oleoides*. We quantified leaf damage caused by four herbivore guilds (chewers, skeletonizers, leaf miners, and gall forming insects) and analyzed the content of 18 polyphenols (including hydrolyzable tannins, flavan-3-ols, and flavonol glycosides) in the same set of leaves using high performance liquid chromatography and mass spectrometry. Foliar damage ranged from two to eight percent per region, and nearly 90% of all the damage was caused by chewing herbivores. Damage due to chewing herbivores was positively correlated with acutissimin B, catechin, and catechin dimer, and damage by mining herbivores was positively correlated with mongolinin A. By contrast, gall presence was negatively correlated with vescalagin and acutissimin B. By using redundancy analysis, we searched for the combinations of polyphenols that were associated to natural herbivory: the combination of mongolinin A and acutissimin B had the highest association to herbivory. In a common garden experiment with oak saplings, artificial damage increased the content of acutissimin B, mongolinin A, and vescalagin, whereas the content of catechin decreased. Specific polyphenols, either individually or in combination, rather than total polyphenols, were associated with standing leaf damage in this tropical oak. Future studies aimed at understanding the ecological role of polyphenols can use similar correlative studies to identify candidate compounds that could be used individually and in biologically meaningful combinations in tests with herbivores and pathogens.

Keywords Flavan-3-ols · Flavonol glycosides · Herbivory · Hydrolyzable tannins · Plant-insect interactions · Polyphenols

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Introduction

It is widely assumed that polyphenols, and specifically tannins, are plant defense compounds against insect herbivores (Agrawal et al. 2012; Barbehenn et al. 2006; Feeny 1970; Forkner et al. 2004). Polyphenols (*sensu* Quideau et al. 2011) are "plant secondary metabolites derived exclusively from the shikimate derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression". From this ample group of

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compounds, certain polyphenols, known as vegetable tannins, present the capacity to interact and precipitate a wide array of molecules, including proteins (Haslam 2007). This capacity to interact with proteins was the basis for suggesting that tannins affected insect herbivores by inactivating insect enzymes as well as dietary proteins (Feeny 1969; Robbins et al. 1987). However, this idea has long been questioned since tannins seem to have contrasting effects in different plant-insect interactions (Ayres et al. 1997; Bernays 1981; Bernays and Chamberlain 1980; Heil et al. 2002). For example, tannins can function as deterrents or toxins for insect herbivores, but also act as phagostimulants (Barbehenn et al. 2008a; Bernays et al. 1980, 1991; Rey et al. 1999). Of course, specialist herbivores have adapted to use virtually all plant defensive compounds for host detection or even to integrate them in their own defensive system (sequestering) (Duffey 1980; Nishida 2002). Therefore, observing an enhanced susceptibility to specialist herbivore does not necessarily question the general defensive effects of a compound. More importantly, recent meta-analyses and studies that encompass many species in a geographic scale, suggest that there is no association between foliar damage and polyphenol content (including total phenolics and total hydrolyzable and condensed tannins) as none of them present a clear latitudinal trend (Adams et al. 2008; Moles et al. 2011a, b).

Most previous studies on the ecological roles of tannins did not focus on individual chemical compounds, but rather used general precipitation-based or colorimetric assays to quantify 'total phenolics' or 'total hydrolyzable/condensed tannins' (Appel et al. 2001; Faeth 1985). Given the enormous chemical diversity of plant polyphenols, the limiting factor might have been that researchers tried to find one single (major) effect and define this effect as the 'raison d'être' of the entire class of polyphenols. In fact, it appears to be more likely that different compounds play different roles in the interactions of plants with their environment. Even single compounds can have more than one effect, *e.g.*, on herbivores and on pathogens, or may serve as a defense against generalist herbivores (Roslin and Salminen 2008) but as a phagostimulant by adapted specialists (Barrett and Heil 2012). Polyphenols also have been proposed to act as photoprotective agents (Close and McArthur 2002), as antimicrobials (Daglia 2012), or as agents that control the mobilization of nutrients and the diversity of soil microbiota (Baptist et al. 2008). However, their chief role always has been thought to be defense, a proposition that is well-supported by recent work on hydrolyzable tannins. Ellagitannins, a class of hydrolyzable tannins, seem to have negative effects on insect herbivores *via* their oxidation in the insect midgut (Barbehenn and Constabel 2011; Salminen and Karonen 2011). As a result of tannin oxidation, toxic reactive oxygen species are released, damaging the midgut epithelium of insect herbivores (Barbehenn et al. 2008b).

Even when hydrolyzable tannins seem to be specifically involved in defense, there is a dearth of studies that relate the amount of individual polyphenols with foliar damage produced by different types of herbivores, in part due to the instability of hydrolyzable tannins and the difficulties involved in their precise determination (Mueller-Harvey 2001; Salminen and Karonen 2011; Waterman and Mole 1994). In addition, to determine the possible contribution of polyphenols as defenses, it would be helpful to evaluate if the compounds are induced in response to foliar damage, as has been suggested by the enhancement of transcriptional activity of genes involved in polyphenol biosynthesis following damage (Mellway et al. 2009; Peters and Constabel 2002). Therefore, our present study aimed at identifying candidate compounds in a widespread tropical oak for further research into the defensive roles of polyphenols. We argue that important first steps are to measure the content of individual polyphenols and tannins in plants growing in their natural habitat, determine their correlative association with the levels of damage caused by different types of herbivores, and investigate their putative inducibility in response to damage, in order to focus additional studies on the most promising candidate compounds.

Specifically, we measured the foliar damage on *Quercus oleoides* trees growing in three geographic regions (Northern Mexico, Southern Mexico, and Costa Rica) and applied modern sensitive analytical techniques for quantifying specific polyphenols (including hydrolyzable tannins, flavan-3-ols, and flavonol glycosides). This tropical oak is widely distributed and encompasses a wide variety of phenotypes (Cavender-Bares et al. 2011). We hypothesized that only *a subset* of the identified compounds would be associated with certain types of herbivory. Therefore, we considered four scenarios to explain the directionality of the correlations between individual polyphenol content and herbivory: (1) polyphenols are neutral constitutive compounds, that is, herbivory is not correlated with polyphenol content; (2) polyphenols are constitutive and defensive compounds, which would be visible as a negative correlation with herbivory; (3) polyphenols enhance plant susceptibility (perhaps by lowering the food quality and thus increasing herbivory or by making the leaves more suitable for specialist insects adapted to high polyphenol/tannin content), which would become visible as a positive correlation with herbivory; or that (4) polyphenols are inducible defenses, which also would be evidenced by a positive correlation with herbivory. Given that both scenarios 3 and 4 could result in the same type of correlation, we also performed experiments using artificially simulated herbivory to test whether the production of polyphenols is inducible in response to damage. Finally, we used multivariate analyses to determine if overall herbivory is significantly associated to all polyphenols and tannins, to just the hydrolyzable tannins, or to only a subset of individual polyphenols.

Methods And Materials

Study Species and Sampling Quercus oleoides (Fagaceae, subgenus *Quercus*, section *Quercus;* Schltdl. et Cham.) is distributed mainly in coastal zones, from the northern part of the Gulf of Mexico (state of Tamaulipas) to Costa Rica, between 0 and 500 m above sea level. The distribution of *Q. oleoides* encompasses a wide range of mean annual temperatures (23–28 °C) and precipitation (700–3200 mm) (Valencia 2004). Nine populations were selected, three in Northern Mexico, three in Southern Mexico, and three in Costa Rica (Table S1). To avoid confounding effects due to variation in elevation and possible hybridization with other oaks, only populations occurring between 0 and 300 m above sea level were selected. Only trees that were scattered on the edges of forests were sampled. At each site, the geographic coordinates and elevation of the sampled individuals were recorded, and a minimum distance between populations of 10 km and a minimum distance between trees of 10 m was established to avoid pseudoreplication. The sampling was conducted at the end of the rainy season, from November to December 2010. To standardize the sampling, we selected trees in the same phenological stage (at the onset of acorn maturation, which coincides with the end of leaf growth).

Quantification of Leaf Damage Foliar damage produced by four herbivore guilds was quantified on six trees per population. From each individual, approximately 80 randomly selected leaves were collected from sun-exposed, south-facing apical branches at a height of 4.0 m. Branches were defoliated, and leaves were placed immediately into dark plastic bags. After shaking the bag, 30 leaves were randomly selected, transported in coolers, and scanned on the same day using an Epson Perfection V700 Photo scanner (Seiko Epson Co. Long Beach, CA, USA, 2008) at a resolution of 300 dpi.

The amount of foliar damage caused by each type of herbivore was calculated using WinFOLIAPro software (2009 Regent Instruments Inc, Quebec, Canada.), and the percentage of damaged leaf area was classified according to four herbivore guilds: chewers, skeletonizers, miners, and gall-forming insects (Adams and Zhang 2009; Cranshaw 2004). Damage produced by gall-forming insects also was calculated as the ratio between the dry weight of the gall and the dry weight of the leaf.

Extraction of Polyphenols and Tannins To quantify the concentration of phenolic compounds, ten fully expanded leaves were randomly selected from the same branch used to assess foliar damage. In all cases, leaves were placed in liquid nitrogen immediately after cutting. Samples were lyophilized (Alpha 1–4 LD Plus, Martin Christ, Niedersachsen, Germany) within 4 d of collection, pulverized to powder using a ball mill (Mixer Mill MM400 Retsch Co., Düsseldorf, Germany), and stored at −20 °C in vacuum-sealed bags.

Polyphenols (hydrolyzable tannins, flavan-3-ols, and flavonols) from each sampled tree were extracted using 100 mg of leaf powder suspended in 1 ml of 70% aqueous acetone using constant agitation for 3 h at 4 °C. Samples were centrifuged, the supernatant was transferred to a vial, and acetone was evaporated under N_2 flow. A second extraction was performed with 1 ml fresh solvent for 30 min. Finally, the combined dried extracts were re-suspended in 1 ml of methanol using an ultrasonic water bath. The final product was stored at −20 °C until HPLC analysis were performed.

Analysis of Hydrolyzable Tannins by Normal Phase Liquid Chromatography-Fluorescence Detection and Tandem Mass Spectrometry (LC-FLD and LC-ESI-MS) Hydrolyzable tannins were analyzed using a method modified from Kelm et al. (2006). Separation was achieved using a 250 x 4 mm LiChrosphere diol column with a particle size of 5 μm (Merck, Darmstadt, Germany) with an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA). Acetonitrile: acetic acid (98:2) and methanol: water: acetic acid (95:3:2) were used as mobile phases A and B, respectively, with the following elution profile: 0–35 min, 0–40 % B; 35–40 min, 40 % B; 40–45 min, 40–0 % B; and 45.1-50, min 100 % A. The eluent was monitored by fluorescence detection with excitation at 280 nm and emission at 450 nm. The total mobile phase flow rate for chromatographic separation was 1 ml min^{-1}. The column temperature was maintained at 30 °C. Compound mass determination and fragmentation was accomplished with an Esquire 6000 electrospray ion-trap mass spectrometer (ESI-MS, Brucker Daltronics, Bremen, Germany). The flow from the column was diverted in a ratio of 4:1 before entering the ESI-MS chamber. To enhance ionization, 10 mmol l^{-1} of ammonium acetate in methanol were added to the column eluent at a flow rate of 0.1 ml min^{-1} with an infusion pump. The ESI-MS was operated in negative mode, scanning *m/z* between 50 and 2000, and with an optimal target mass adjusted to *m/z* 500, 700, 900, 1100, 1300, 1500, or 1800. The mass spectrometer was operated at the following specifications: skimmer voltage, 60 V; capillary voltage, 4200 V; nebulizer pressure, 35 psi; drying gas flow, 11.0 l min^{-1}; gas temperature, 330ºC. The capillary exit potential was kept at −121 V. The two most abundant ions per scan were selected for MS-MS fragmentation.

Analysis of Flavan-3-ols and Flavonols by Reversed Phase Liquid Chromatography-Tandem Mass Spectrometry (LC-ESI-MS-MS) Flavan-3-ols and flavonols were identified by fragmentation spectra on an Esquire 6000 ESI ion-trap mass spectrometer and subdivided into functional groups according to their parent mass and neutral loss spectra (Table 1). Liquid chromatography on an Agilent 1200 HPLC system (Agilent

Table 1 Polyphenols identified in *Quercus oleoides* leaf extracts

Polyphenol	Abb.	S. I.	*m/z*	RT	MS2 (*m/z*)
Hydrolyzable tannins					
Hexahydroxydiphenoyl-glucose	Hglu	—	481	13	301, 275
Di-hexahydroxydiphenoyl-glucose	DHglu	—	783	17.3	481, 301, 275
Vescalagin	Ve	—	933	21	915, 631
Castalagin	Ca	—	933	22.2	915,631
Stenophyllanin	St	—	1055	23.7	1011, 933, 765, 721
Vescavalonic acid	VA	—	1101	25.3	1057, 933
Acutissimin B	AB	—	1205	25.9	915, 872, 507
Mongolinin A	MA	—	1373	28.7	1329, 1201, 1083, 1039
Cocciferin D3	CD3	—	783	30.2	1264, 1059, 932
Cocciferin D2	CD2	—	933	31.4	1565, 1503, 1083, 924, 897
Flavan-3-ols and Flavonols					
Catechin	Cat	2	289	4.09; 4.33	109
Kaempferol glucoside	Kglu	2	447	4.88; 4.95	285
Quercetin rhamnoside	Qrh	2	447	4.55; 4.99	301
Quercetin glucoside	Qglu	5	463	3.81; 4.23; 4.29;4.62; 4.75	301
Quercetin glucuronide	Qgln	1	477	4.2	301
Catechin dimer	Cdi	4	577	3.85; 4; 4.19; 4.46	289
Quercetin rutinoside	Qru	5	609	4.19; 4.6; 5.38;5.53; 5.69	301
Unknown	Un	2	679	5.5; 5.57	517

Abb, polyphenol abbreviation; SI, structural isomers; RT, retention time (min); MS2, fragment masses used in identification

Technologies; Santa Clara, CA, USA) was performed to separate the target compounds. These compounds were separated on a 50 x 4.6 mm XDB C18 column with a particle size of 1.8 μm (Agilent). Formic acid in water (0.05 %) and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–1 min, 100 % A; 1–7 min, 0–65 % B; 7–8 min 65–100 % B; 8–9 min 100 % B; and 9–10 min 100 % A. The total mobile phase flow was 1.1 ml min^{-1}. The column temperature was maintained at 25 °C. To quantify flavan-3-ols and flavonols, an API 3200 tandem mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) that was equipped with a turbospray ion source and operated in negative ionization mode was used. The instrument parameters were optimized by infusion with pure standards of catechin, quercetin, quercetin-3-O-glucoside, and procyanidin B1. The ion-spray voltage was maintained at −4500 V. The turbo gas temperature was set at 700 °C. The nebulizing gas pressure was set at 70 psi, curtain gas pressure at 25 psi, heating gas pressure at 60 psi, and collision gas pressure at 10 psi. Multiple reaction monitoring (MRM) was used to monitor the decay of analyte parent ions into product ions, as follows: for catechin, *m/z* 289.9→109.1 (collision energy (CE) -34 V; declustering potential (DP) -30 V); for procyanidin B1, *m/z* 576.9→289.1 (CE −30 V; DP −50 V); for quercetin, *m/z* 300.8→179 (CE −28 V; DP −55 V); for quercetin glucoside, *m/z* 462.9→301 (CE -40 V; DP -390 V); for kaempferol glucoside, *m/z* 446.9→285 (CE -40 V; DP -390 V); for quercetin rhamnoside, *m/z* 446.9→301 (CE -40 V; DP -390 V); for quercetin glucuronide, *m/z* 476.9→301 (CE -40 V; DP -390 V); for quercetin rutinoside, *m/z* 608.9→301 (CE -40 V; DP -390 V); and finally *m/z* 678.9→517 (CE -40 V; DP -390 V) for an unidentified glucoside. Both the Q1 and Q3 quadrupoles were maintained at unit resolution. For data acquisition and processing, the software Analyst 1.5 (Applied Biosystems, Carlsbad, CA, USA) was used, and the compound quantification was performed using external calibration curves for catechin, quercetin, and quercetin-3-O-glucoside. The flavan-3-ol concentrations were determined relative to the catechin calibration curve, and the flavonol concentrations were determined relative to the quercetin-3-O-glucoside calibration curve. Structural isomers of the same flavonol (*e.g.*, quercetin-3-O-glucoside and quercetin-7-O-glucoside) were quantified together.

Simulation of Herbivory To test if the biosynthesis of tannins could be induced by herbivory, we established a common garden experiment using saplings from acorns that belonged to the same trees used to study natural herbivory. Acorns were collected in December 2010 (at the same time that leaves were sampled), stored at 4 °C in plastic bags, and then randomly

planted in April 2011, using 50 % peat moss, 25 % vermiculite, and 25 % agrolite as substrate. Due to the low percentage of viable saplings from Mexico, only saplings from Costa Rica were used. Saplings were grown for 1 yr, until height and leaf number reached approximately 35 cm and 8 leaves, respectively. To avoid variation in tannin content due to leaf age (Close et al. 2005; Yarnes et al. 2008b), only the two youngest apical pairs of fully expanded leaves (excluding buds and young small red leaves) were used for the experiment.

To assess general induction events, independent of any putative inducing or inhibitory effects of specific herbivore-derived elicitors, we simulated herbivory by applying leaf homogenates to mechanically damaged leaves. This method simulates cell disruption after herbivore attack and promotes the release of plant metabolites, such as glucose, sucrose, or ATP that may trigger defense related responses (*i.e.*, damaged-self recognition; Heil 2009; Heil et al. 2012). Leaf homogenate consisted in the supernatant of a suspension of ground fresh leaves of *Q. oleoides* (ball mill Mixer Mill MM400 Retsch Co., Düsseldorf, Germany) in a proportion of 0.5 g leaf powder per 3 ml of distilled water. Simulation of herbivory was produced on 10 % of the leaf area by puncturing with a metal brush and then immediately immersing the damaged leaves in the leaf homogenate. The brush also damaged the cuticle, and this was evident after several days after applying the treatment (the final damaged area was around 30 %). Two additional treatments were established: mechanical damage, in which leaves were damaged with a metal brush but immersed in distilled water, and a control, in which leaves had no damage. In all cases (*i.e.*, factors=treatment×time), five independent plants were used as replicates. The initial concentration of polyphenols was quantified before damage (day zero) and 2, 7, and 21 d after damage. Treated leaves were transported under liquid nitrogen, ground into powder, and lyophilized before chemical analysis.

Statistical Analyses Prior to the analyses, data was natural log-transformed to improve normality and homogeneity of variance, with the exception of gall damage per area, gall weight, and leaf damage by skeletonizers, which were natural log +1 transformed. Across regions, herbivory rates were compared using one way- nested ANOVA (populations nested within regions), followed by Tukey's honest significant tests. The concentration of each compound was correlated with each type of natural herbivory using Pearson's correlations, including all individuals in the field study (N=54). The simulated herbivory experiment was analyzed using a two-way ANOVA per compound, with time and type of damage as factors, using JMP 8.0 (SAS Institute Inc., Cary, NC, USA).

To explore the possible dependence of foliar damage on specific polyphenol combinations, a canonical redundancy analysis (RDA) was performed, followed by permutation tests to assess statistical significance. RDA is a multivariate extension of multiple regression analysis that quantifies the proportion of the variance in one set of variables that is explained by another set of variables (Makarenkov and Legendre 2002). For our RDA, the four herbivory types (% damage) were considered as our response matrix (leaf gall per unit leaf area, chewers, skeletonizers, leaf miners; we excluded the gall weight/leaf weight ratio due to its high correlation with leaf gall per unit leaf area, R=0.97, P<0.001). To establish our explanatory matrix, the individual concentrations of all the detected compounds were considered. To detect the best combinations of compounds that predict leaf damage among several significant models, we employed the Akaike's information criterion (AIC). The RDA and AIC were carried out in R 2.11 (R Development Core Team 2013) using the Vegan package (Oksanen et al. 2010).

Results

Total leaf damage per region ranged between 2 to 8 %, and from 0.3 to 10.7 % per population. Chewers were responsible for nearly 90 % of total foliar damage (Fig. 1, Table S2). Among the notable trends, damage by chewers was significantly higher in Southern Mexico and Costa Rica (F=11.2, P<0.001), damage due to leaf miners was highest in Southern Mexico (F=4.03, P=0.02), and galls, although not significant, were most frequent in northern Mexico and absent from oaks in Costa Rica (F=2.59, P=0.09) (Fig. 1, Table S2).

We successfully quantified 18 polyphenols, which included two flavan-3-ols (with 6 structural isomers), 6 flavonols (with 17 structural isomers), and ten hydrolyzable tannins in *Q. oleoides* using reversed and normal phase HPLC and mass spectrometry (Table 1, Fig. S1, Fig. S2). Individual polyphenols were sometimes absent from some individuals, but all each were present in every population and region. In this dataset, we found a significant positive correlation between the damage produced by chewers and the quantities of the hydrolyzable tannin acutissimin B (R=0.33, P=0.01), the flavan-3-ol catechin (R=0.27, P=0.05), and the flavan-3-ol catechin dimer (R=0.28, P=0.04). There was a significant positive correlation between leaf miners-inflicted damage and the quantity of the hydrolyzable tannin mongolinin A (R=0.34, P=0.01). By contrast, negative correlations were found between damage produced by galls and the quantities of the hydrolyzable tannins acutissimin B (R=−0.31, P=0.02) and vescalagin (R=−0.27, P=0.04) (Fig. 2, Table S3).

Subsequent experiments in which herbivory of oak sapling leaves was mimicked by mechanical damage and the application of leaf homogenate revealed that the concentration of acutissimin B increased significantly by day seven (F=11.54, P<0.001). For the other two hydrolyzable tannins tested, vescalagin and mongolinin A, the concentration

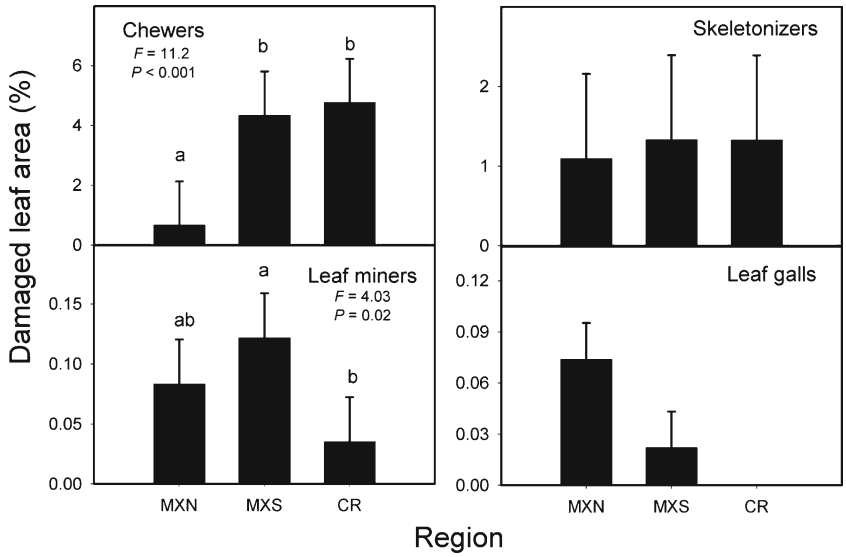

Fig 1 Damaged leaf area (%) produced by four herbivore guilds in three regions encompassing the distribution of *Quercus oleoides*. MXN=Northern Mexico, MXS=Southern Mexico, CR=Costa Rica

significantly increased following mechanical damage and application of leaf homogenate, and these changes were conspicuous also after day seven (interaction between treatment × time: vescalagin, F=2.59, P=0.02; and mongolinin A: F=6.15, P<0.001; Fig. 3). Levels of catechin and catechin dimers decreased after wounding and leaf homogenate treatment (interaction terms treatment × time: F =6.57, P<0.001; Fig. 3).

To search for specific combinations of compounds that show particularly strong association with herbivory rates, we performed canonical redundancy analysis (RDA). There was no association between herbivory and total polyphenol content (F=0.98, P=0.50, AIC=90.0), and neither total hydrolyzable tannins (F=1.34, P=0.16, AIC=94.9) nor total flavonols (F=1.14, P=0.31, AIC=96.0) were associated with herbivory. However, three models with different combinations of polyphenols were significantly associated with herbivory (F ranged from 2.33 - 3.43; P ranged from 0.03 - 0.01; AICs ranged from 88.6 - 81.2, Table 2). The best model was the combination of the hydrolyzable tannins mongolinin A and acutissimin B (F=3.43, P=0.01, AIC=81.2; Table 2).

Discussion

Our study aimed at identifying polyphenols and tannins that might significantly contribute to the anti-herbivore defense of

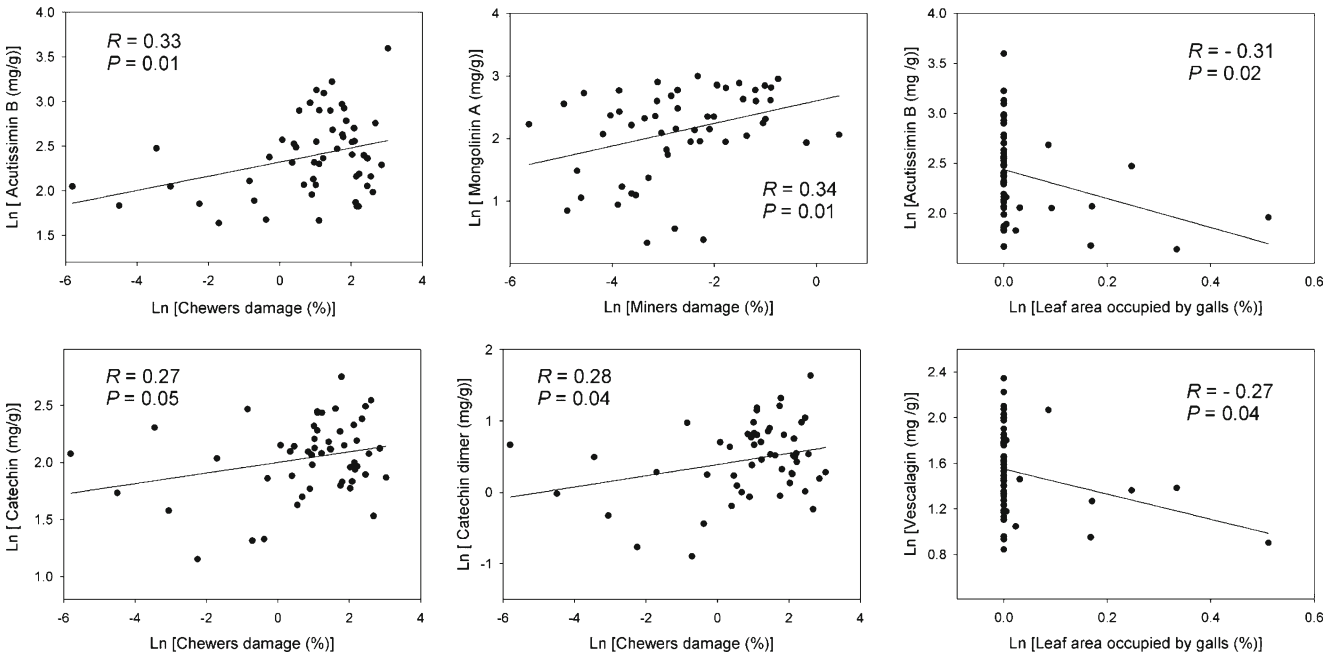

Fig 2 Pearson correlations between the concentration of specific polyphenols and tannins (mg/g) and foliar damage (%) in 54 individuals from 9 populations of the tropical oak *Quercus oleoides*

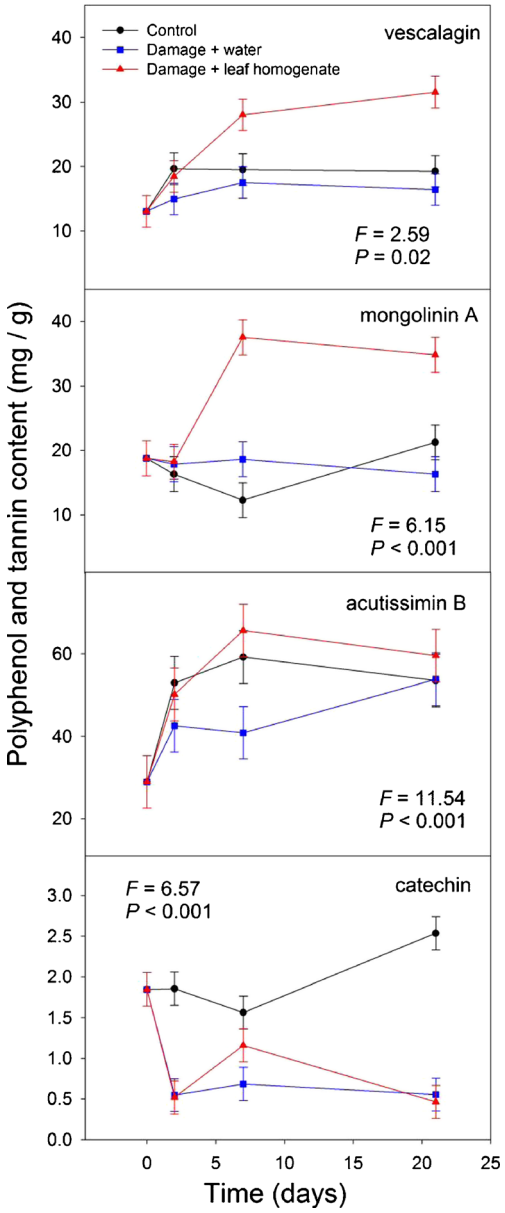

Fig 3 Polyphenol and tannin content in 1–yr-old *Quercus oleoides* saplings in response to mechanical damage (Damage+water) and simulated herbivore attack (Damage+leaf homogenate)

the tropical oak, *Quercus oleoides*. Our field survey demonstrated that only certain types of polyphenols and, in particular, certain combinations of hydrolyzable tannins were associated with the patterns that we found in standing plant damage caused by herbivores. After mimicking herbivory in saplings, an increase in the production of some polyphenols (acutissimin B, mongolinin A, and vescalagin) was observed, which also supports their putative role in plant defense against herbivores. However, the wide majority of compounds were not correlated with herbivory and did not respond to simulated herbivory, which makes their direct function in plant defense against herbivores less likely and rather suggests their role in the interaction with other environmental

Table 2 Comparison of the significance of different combinations of polyphenols and tannins when associating to total foliar damage by using redundancy analysis (RDA) and Akaike's information criteria (AIC) for 54 individuals of *Quercus oleiodes*

Polyphenols and tannins	AIC	*F*	*P*
Acutissimin B+Mongolinin A	81.2	3.43	0.01
Acutissimin B+Catechin+Mongolinin A	87.7	2.78	0.03
Acutissimin B+Catechin+Catechin dimer+ Mongolinin A	88.6	2.33	0.03
Vescalagin+Acutissimin B+Catechin+Catechin dimer+Mongolinin A	90.11	1.93	0.06
Hydrolyzable tannins	94.9	1.34	0.16
Flavan-3-ols and Flavonols	96.0	1.14	0.31
All compounds	90.0	0.98	0.50

factors, such as pathogens or light and other abiotic stresses (Arámbula-Salazar et al. 2010; Close and McArthur 2002; Close et al. 2003; Harborne and Williams 2000; Ryan et al. 2002).

Polyphenol Content and Herbivory We found significant correlations between the concentration of individual polyphenols and natural levels of foliar damage (see also Ayres et al. 1997; Yarnes et al. 2008a). Five individual compounds were significantly correlated to foliar damage caused by chewers, leaf miners, and galls. When exploring which polyphenols and tannins were most associated to herbivory, a combination of two hydrolyzable tannins (mongolinin A and acutissimin B) was most highly related to herbivory. This is in line with previous reports suggesting that hydrolyzable tannins are an important defense against insect herbivores (Barbehenn and Constabel 2011). For example, herbivory in *Oenothera biennis* (Onagraceae), mostly due to the beetle *Popillia japonica*, was positively associated with the content of many ellagitannins (hydrolyzable tannins) and flavonoids, and negatively associated with the content of the polyphenol quercetin glucuronide (Johnson et al. 2009). Vescalagin, a hydrolyzable tannin, was correlated negatively with gall presence, and was inducible in our study. This compound is considered to have a high oxidative capacity and is also known to affect the performance of insects, as it drastically reduces the growth of generalist insects and is considered critical for plant defense in other oak species (Roslin and Salminen 2008). Acutissimin B, in contrast, is better known for its possible role in human therapeutics (Kashiwada et al. 1992; Khennouf et al. 2010; König et al. 1994), and its importance in reducing the performance of insects has not been tested. In addition, we found that two flavan-3-ols (catechin and catechin dimer), were also related to herbivory. This is in agreement with previous reports showing that these compounds are involved in plant-insect interactions (Thelen et al. 2005). Moreover, the expression of dihydroflavonol reductase (DFR), an enzyme involved

in the synthesis of flavan-3-ols, increased after damage in *Populus tremuloides* (Peters and Constabel 2002). Previous studies have suggested that there are particular polyphenols and tannins involved in plant defense against insect herbivores (Barbehenn and Constabel 2011). In our study, we considered the significant correlations between foliar damage and the concentration of specific hydrolyzable tannins and polyphenols as a first indication for their possible role as defense agents. We obtained significant positive correlations, where scenario 3 (plant susceptibility) and scenario 4 (induction of defense) could be equally feasible; however, we aimed to test for inducibility in our controlled experiment, as there is evidence at the molecular level that, at specific stages, the phenylpropanoid pathway is activated after damage (Mellway et al. 2009).

Induction of Polyphenols and Tannins We recorded a progressive increase in the content of three hydrolyzable tannins and a significant reduction in the concentration of the flavan-3-ols catechin, and catechin dimer in leaves that were wounded and treated with leaf homogenate. The reduction of the catechin monomers and dimers could be due to their role as building blocks in the biosynthesis of higher order condensed tannins (Haslam 1998), a process that may be triggered by damage.

There was a clear increase in the production of the hydrolyzable tannin acutissimin B following damage, and for two compounds (vescalagin and mongolinin A) the capability of induction was higher when using leaf homogenate than when using mechanical damage alone. Mechanical damage is less effective in triggering defense responses of plants than damage produced by actual herbivores or simulated herbivory with leaf homogenate (Bricchi et al. 2010; Heil 2009). In our experimental treatments, the damaged leaves were immersed in the leaf homogenate; hence damaged tissue came into direct contact with the array of 'damage-associated molecular patterns' (DAMPs) released from its own disrupted cells and the cells from the prepared homogenate. The presence of DAMPs might lead to recognition of damage and therefore trigger increased polyphenol biosynthesis that could be responsible for resistance against herbivores. In other species, the simple addition of peptides derived from intracellular contents or the exposure to herbivore-induced volatiles from other individuals was enough to induce a defensive reaction (Heil and Karban 2010; Karban and Shiojiri 2009; Pearce et al. 2010; Scala et al. 2013). In *Q. oleoides*, the addition of leaf homogenate actively increased the production of hydrolyzable tannins. The positive correlation between the amounts of these compounds and damage levels could be due to their induction after herbivory and supports our proposed scenario 4. The nature of the elicitors and the signaling cascade that induces the production of hydrolyzable tannins in *Quercus* upon leaf damage deserves further study, but given that the mechanisms of damaged-self recognition have been proved to be highly conserved throughout the angiosperms (Heil et al. 2012), it is probable that this species also shares a common recognition pathway.

Key Combinations of Polyphenols Associated with Herbivory in Quercus oleoides We found that there were combinations of certain compounds that had a higher degree of association with overall levels of natural herbivory than total polyphenols, total hydrolyzable tannins, or individual compounds. Our results differ from those of previous meta-analyses that revealed phenolic contents, in particular tannins, to be independent of herbivory (Moles et al. 2011a, b). We consider that the cause of this discrepancy lies in the chemical approach employed. In fact, using our study species as an example, we did not find a significant correlation between total polyphenol and total tannin content with the total levels of natural herbivory. As indicated by our Table 2, only certain combinations of compounds were highly associated to herbivory. Thus, it is imperative to use precise analytic techniques to investigate the ecological role of tannins and other polyphenols in plant-insect interactions, as has been proposed elsewhere (Salminen and Karonen 2011; Scioneaux et al. 2011). Even more, given the large diversity of polyphenol chemical structures, it seems likely that various compounds may function synergistically as defense compounds, as occurs in other secondary metabolites (Courtois et al. 2012; Gershenzon and Dudareva 2007). The exploration of these roles and the involvement of these compounds in plant defense should still provide a fruitful area for future research.

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