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Compuestos polifenólicos relacionados con la defensa contra herbívoros y patógenos en el encino tropical *Quercus oleoides*

TESIS

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P r e s e n t e

Por medio de la presente me permito informar a usted que en la reunión ordinaria del Subcomité de (Ecología y Manejo Integral de Ecosistemas), del Posgrado en Ciencias Biológicas, celebrada el día 31 de octubre de 2016, se acordó poner a su consideración el siguiente jurado para el examen **de DOCTORA EN CIENCIAS** de la alumna **MOCTEZUMA MARTIÑON CORAL** con número de cuenta **510007788**, con la tesis titulada: **"Compuestos polifenólicos relacionados con la defensa contra herbívoros y patógenos en el encino tropical *Quercus oleoides*"**, bajo la dirección del **Dr. Alberto Ken Oyama Nakagawa.**

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A T E N T A M E N T E
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VARIATION IN POLYPHENOL CONTENT ACROSS THE DISTRIBUTION RANGE OF THE TROPICAL OAK *QUERCUS OLEOIDES*

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I. RESUMEN GENERAL

Los polifenoles son un grupo de metabolitos secundarios que se encuentran en varias familias de plantas. Estos compuestos presentan diversas funciones fisiológicas y ecológicas; por ejemplo, algunos dan soporte mecánico a las plantas (lignina) y participan en la conducción del agua, mientras que otros, están involucrados en interacciones bióticas como la defensa. Dentro de los compuestos fenólicos relacionados con la defensa y la resistencia, se encuentran los taninos y los flavonoides (ambos considerados como polifenoles); estos compuestos comúnmente han sido considerados como agentes tóxicos contra insectos y patógenos. Sin embargo, debido a que la síntesis de varios de estos compuestos es influenciada por factores abióticos, como la incidencia solar y los nutrientes del suelo, su papel como compuestos de defensa se ha cuestionado y reanalizado en los últimos años.

Dado el creciente interés sobre el modo en que actúan los polifenoles en los organismos y la falta de consenso sobre su función como agentes de defensa, el objetivo principal de esta tesis fue estudiar la relación de varios compuestos polifenólicos con diferentes tipos de daño foliar (tanto natural como artificial) en el encino tropical *Quercus oleoides*. Con el apoyo de herramientas de química analítica, este trabajo aumenta el entendimiento sobre la función y los mecanismos que llevan a la producción de los taninos y otros polifenoles. Se presentan cuatro apartados (IV-VII), específicamente en el apartado IV se presenta una revisión que aborda el conocimiento actual sobre los polifenoles relacionados con la defensa y resistencia en plantas. Se describen las rutas metabólicas involucradas para su síntesis (los genes implicados y enzimas claves), los mecanismos de regulación, sitios de almacenaje y el efecto que ejercen sobre los insectos y patógenos. En el apartado V, se hace un estudio detallado sobre los compuestos polifenólicos, incluyendo taninos y flavonoides, y su relación con diferentes tipos de herbivoría en hojas del encino *Q. oleoides*. En ese mismo apartado también se estudia la capacidad de inducción de estos compuestos en respuesta al daño mecánico en plántulas de la misma especie. La respuesta al daño mecánico y a la acción del quitosán (componente de la pared celular de patógenos), se estudia en el apartado VI. Por último, en el apartado VII se analiza la variación geográfica de taninos y flavonoides en varias poblaciones que comprenden la distribución de *Q. oleoides* y su relación con factores abióticos para determinar qué compuestos polifenólicos están relacionados con defensa (factores bióticos) y cuáles con factores abióticos.

De manera general, este trabajo concluye que no se les puede atribuir una única función a toda esta familia de compuestos. En *Q. oleoides*, sólo un subconjunto de estos compuestos está críticamente involucrado en la defensa de las plantas, mientras que otros compuestos están relacionados con factores abióticos. Los resultados interrelacionados de esta tesis se engloban en una discusión general donde se pone en relieve la importancia de la identificación y cuantificación de la concentración de los taninos y otros polifenoles para el entendimiento de su función.

II. ABSTRACT

Polyphenolic compounds comprise a diverse group of secondary metabolites that are present in several plant families. These compounds have different physiological and ecological functions; for example, some give mechanical support to plants and participate in water conduction (lignin) while others are involved in biotic interactions, such as defense. The phenolic compounds related to plant defense and resistance include tannins and flavonoids (both considered as polyphenols), a set of compounds commonly considered as insect deterrents and as agents for combating pathogens. However, owing to the influence of abiotic factors such as solar incidence and soil nutrients in the synthesis of several of these compounds, their role as defensive compounds has been questioned and revisited in recent years.

Given the growing interest to understand the effects of polyphenols on organisms, and the lack of consensus on their role as agents of defense, the main objective of this thesis was to study the relationship of various phenolic compounds with different types of leaf damage (both natural and artificial), in the tropical oak *Quercus oleoides*. With support of state-of-the-art analytical chemistry tools, this study increases the understanding of the role and timing of the production of tannins and other polyphenols. Four sections in this thesis (IV-VII) are included to address the role of tannins and other polyphenols. Specifically, section IV is a review that describes the metabolic pathways for the synthesis of the phenolic compounds related with plant defense and resistance, the regulatory mechanisms, storage sites of these compounds in the plants, and finally, their effect on insects and pathogens. In section V, a detailed study of polyphenolic compounds, including tannins and flavonoids, and their relation to different types of herbivory in leaves of oak *Q. oleoides* is presented. In the same section, the inducibility of these compounds is also studied in response to mechanical damage in seedlings of the same species of oak. Response to mechanical damage and the action of chitosan, which is a cell wall component of pathogens, are presented and discussed in Section VI. Finally, in section VII the geographic variation of the concentration of tannins and flavonoids in various populations comprising the distribution of *Q. oleoides* and its relation to abiotic factors is studied in order to understand which phenolic compounds are related to defense (biotic factors) and which are related with abiotic factors.

In general, this study increases our knowledge concerning the function of various polyphenols including tannins. I conclude that a single function cannot be attributed to this category of compounds. In *Q. oleoides* only a subset of these compounds is critically involved in plant defense, while the other tannins and polyphenols present in this oak are related to other factors rather than herbivory. The function of some tannins as agents of defense was supported by induction experiments conducted on seedlings, whereas other polyphenolic compounds are related to abiotic factors. The interrelated results of this thesis are encompassed in a general discussion where the importance of the individual determination of tannins and other polyphenols is highlighted for the understanding of the function of the polyphenolic compounds.

III. INTRODUCCIÓN GENERAL

Polifenoles: diversidad de estructuras y funciones

Cuando hablamos acerca de los polifenoles y los taninos llega a nosotros la imagen de los beneficios que producen estos compuestos en nuestro organismo; del vino tinto, del té verde y los frutos llenos de antioxidantes que mejoran nuestra salud y del potencial que tienen para prevenir enfermedades degenerativas (Chung et al. 1998, Corder et al. 2001, Vita 2005). Sin embargo, estos compuestos que son conocidos por tener efectos positivos en la salud de los humanos, presentan otra faceta en el mundo de los insectos herbívoros y en el mundo de los patógenos (bacterias, hongos y virus). Aunque en los humanos algunos polifenoles (e.g. elagitaninos) actúan como agentes antioxidantes que secuestran radicales libres y evitan el daño celular (Sies et al. 2005), en algunos insectos parecen actuar de manera opuesta, es decir, como agentes prooxidantes que originan estrés oxidativo y daño celular (Barbehenn y Martin 1994). En cuanto a los patógenos, generalmente los polifenoles y los taninos inhiben el crecimiento de hongos y bacterias, e inactivan varios tipos de virus. ¿A qué se deben los efectos tan contrastantes de los polifenoles? Hay varios factores involucrados para que se den estas diferentes respuestas, entre ellos está la estructura molecular de cada compuesto, el pH del medio donde se encuentran, la cantidad ingerida y las características de los herbívoros y los patógenos, así como también el momento de la síntesis de estos compuestos, es decir, si se producen independientemente de algún daño o en respuesta a este (Bernays 1981).

Tomando en consideración diversos criterios, se han propuesto varias definiciones para los polifenoles y los taninos a lo largo del tiempo, las cuales, han ido cambiando conforme aumenta el entendimiento sobre su estructura, síntesis y propiedades bioquímicas. En un principio a estos compuestos se les denominaba taninos por la acción que tenían en el curtimiento de pieles y ninguno de estos compuestos era llamado polifenol. Theodore White inicialmente definió a los taninos como compuestos capaces de unirse al colágeno y curtir pieles. Posteriormente Anthony Swain, E-C Bate Smith y Jeffrey Harborne definieron a los polifenoles en cuanto a su solubilidad en agua, peso molecular (500-3000Da), capacidad para reaccionar con sales de hierro III, permanganato y precipitar otros compuestos como alcaloides, gelatina (forma desnaturalizada del colágeno) y otras proteínas, en esta definición se no consideró la estricta unión al colágeno, la cual hace posible el curtimiento de pieles. Posteriormente, Edwin Haslam adoptó esta definición y la extendió haciendo énfasis en la estructura molecular y usó los términos “polifenol” y “tanino” como sinónimos. Con el paso del tiempo, cada definición fue refinando la anterior, y se fueron incluyendo varios aspectos además de su capacidad de unión con otras moléculas. No obstante, otras definiciones surgieron y han ido creando confusión a tal punto que algunos compuestos fenólicos muy simples, como el resorcinol, son considerados como polifenoles (Fig. 1). La definición del término “polifenol” más actual y la que será considerada en esta tesis es la de Quideau y cols. (2011) quienes consideran principalmente el número de anillos fenólicos (Fig. 1) y el origen biosintético de los compuestos.

a) b)

Figura 1. a) Fenol, compuesto aromático con fórmula molecular C_6H_5OH. b) Resorcinol, compuesto fenólico, a veces considerado polifenólico cuando en realidad es un fenol simple de acuerdo con la nomenclatura IUPAC.

De acuerdo con Quideau y cols. (2011) "los polifenoles son metabolitos secundarios que se derivan exclusivamente de la vía del ácido shikímico" (ruta metabólica que origina los aminoácidos arómaticos) "y de la vía de los policétidos" (también conocida como la vía del acetato-malonato); "poseen más de un anillo fenólico y carecen de cualquier grupo funcional con nitrógeno en su estructura más básica" (Fig. 2). Bajo esta definición los taninos, por lo tanto, forman parte del grupo de los polifenoles, el cual es un grupo más grande e incluyente que abarca a los taninos y a otros compuestos. Los taninos se caracterizan por tener un peso molecular de 500-3000 Da, aunque hay reportes de taninos con pesos moleculares de hasta 20,000 Da. Los taninos se consideran solubles en agua, aunque conforme aumenta el peso molecular, su extracción y solubilidad en agua y en otros solventes disminuye (Hagerman y Butler, 1991).

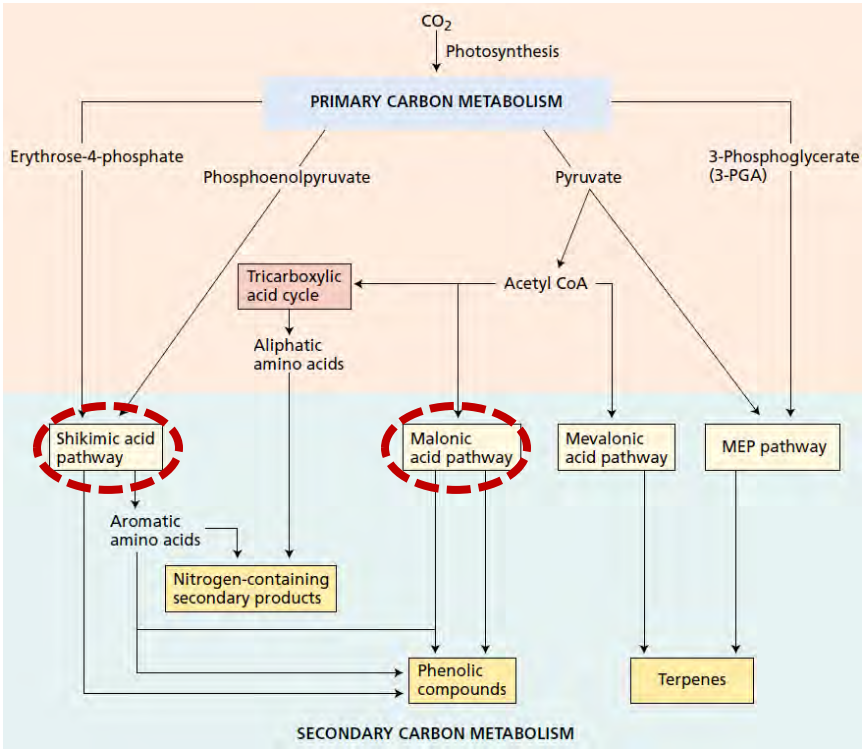

Figura 2. Representación esquemática del metabolismo primario (parte superior rosa) y secundario en plantas (parte inferior azul). Los círculos con línea punteada roja muestran las dos rutas que originan a los polifenoles: la ruta del ácido shikimico y la ruta de acetato-malato. Tomado de Taiz y Zeiger, 2006.

Se reconocen básicamente dos tipos de taninos: los condensados (o proantocianidinas) y los hidrolizables (Haslam 2007), aunque cabe mencionar que existe un tercer grupo de taninos denominados florotaninos, los cuales son menos conocidos y su distribución se restringe únicamente a las algas cafés y en menor medida a las algas rojas (Chkhikvishvili y Ramazanov 2000). A diferencia de los florotaninos, los taninos condensados e hidrolizables son muy comunes en el reino vegetal. Se encuentran en plantas con una amplia variedad de formas de vida; en pteridofitas, gimnospermas, monocotiledóneas y en 80 familias de plantas dicotiledóneas (Haslam 1998). Generalmente, las plantas leñosas sintetizan más taninos que las plantas herbáceas aunque hay algunas excepciones. Se pueden encontrar en todos los órganos de la planta: en raíces, tallos, corteza, hojas, frutos, semillas y agallas vegetales (Haslam 2002, Khanbabaee y van Ree 2001).

El contenido de taninos es un carácter variable que está en función del tipo de tejido vegetal, del genotipo y de la etapa de desarrollo de la planta. Por ejemplo, las hojas contienen entre 5 y 10% del peso seco (Barbehenn y Constabel 2011) mientras que en los granos y semillas están ausentes o en menor cantidad (0.2-5%) aunque hay excepciones como la semilla de la palma *Areca catechu* que puede contener hasta el 26% de su peso en taninos (Chung et al 1998). En cuanto al genotipo, en el género *Populus* el contenido de taninos oscila entre 0.4% y 25% del peso seco, dependiendo de la especie y genotipo del individuo (Hwang y Lindroth 1997, Osier y Lindroth 2001, Rehill et al. 2006). A lo largo del desarrollo, el contenido total y/o la proporción de condensados respecto a los hidrolizables también puede variar. Por ejemplo, en *P. tremuloides* y en *Quercus robur*, las hojas maduras almacenan el doble de taninos condensados en comparación con las hojas jóvenes (Donaldson et al. 2006) mientras que los niveles de los taninos hidrolizables se mantienen constantes o disminuyen durante el desarrollo de la hoja (Feeny y Bostock 1968, Salminen et al. 2001, Salminen et al. 2004).

Como se mencionó anteriormente, una de las propiedades más conocidas de los taninos es su alta capacidad para unirse y precipitar varios tipos de moléculas como carbohidratos, alcaloides, iones metálicos y proteínas (Haslam 1974, Barbehenn y Salminen 2011). Pueden precipitar varias veces su propio peso molecular; por ejemplo, los taninos de *Sorghum sp.* pueden precipitar hasta 12 veces su propio peso en proteínas. Esta alta capacidad de unirse a moléculas, y específicamente a proteínas, fue considerada como el principal mecanismo fisicoquímico del efecto biológico de los taninos sobre los herbívoros. Por ejemplo, cuando los taninos se unen a mucoproteínas de la cavidad oral de los herbívoros producen una sensación de sequedad y astringencia que pueden afectar la palatabilidad del tejido vegetal y, por lo tanto, actuar como agentes repelentes (Hagerman y Butler 1991). En otros casos, la unión de taninos a proteínas dietéticas y a enzimas digestivas resulta nociva para el desarrollo de los herbívoros. No obstante, a pesar de que los estudios bioquímicos sugieren un evidente efecto negativo, estudios posteriores con diversos herbívoros han dado resultados contradictorios y han puesto en relieve dos aspectos importantes. Primero, que los herbívoros, particularmente los insectos, presentan una variedad en la sensibilidad a los taninos y a otros polifenoles y, en segundo lugar, que hay otro mecanismo diferente a la unión a proteínas y otras moléculas biológicas que puede dañar severamente los insectos. Este

mecanismo se refiere al efecto prooxidante de los polifenoles e involucra la producción de agentes de estrés oxidativo (formación de radicales libres y formas reactivas del oxígeno) y daño celular. Esta variedad de efectos que producen los polifenoles y los taninos en los insectos, ha llevado a cuestionar su papel como metabolitos de defensa en las plantas e incluso se han propuesto otras funciones alternativas donde varios factores abióticos entran en juego para su producción.

¿Agentes de defensa en plantas o producto de factores ambientales?

Evidencia a favor de la defensa

El papel ecológico de los polifenoles comenzó a estudiarse a finales de la década de los 60 a partir de las observaciones de Feeny sobre el efecto que producen en el desarrollo de la polilla *Operophtera brumata* (L.) cuando era alimentada por hojas del encino *Quecus robur* de diferentes edades (Feeny 1970, Bernays 1981, Barbehenn et al. 2001). Fenny observó que las hojas viejas, con mayores contenidos de polifenoles reducían el peso de las polillas (o aumentaban el tiempo de desarrollo). Este efecto negativo era explicado por la capacidad de los taninos de precipitar proteínas dietéticas y enzimas que afectaban a la polilla. Otra explicación alterna era que los taninos condensados endurecían las hojas y disminuían su palatabilidad, por lo que las hojas en vez de ser comidas eran evitadas.

La idea de los efectos nutricionales negativos de los taninos fue considerada durante varios años como el único mecanismo que servía como defensa. Bajo esta perspectiva, los taninos eran considerados defensas cuantitativas cuyo efecto dosis-dependiente llevaba a la disminución en la palatabilidad del tejido vegetal (medido por una reducción en consumo), a la disminución en la eficiencia en la utilización del tejido consumido y, por lo tanto, a la disminución de la ganancia de peso de los insectos y prolongación del tiempo de desarrollo (Hagerman y Butler 1991, Fig. 3). El retraso en el desarrollo hace que los insectos sean más vulnerables a depredadores y/o parásitos (Haslam, 2002, Barbehenn y Martin 1994, Rey et al. 1999, Heil et al. 2002a, Heil et al. 2002b).

Figura. 3. Larva y crisálidas de *Spodoptera* frugiperda, "gusano cogollero". A) Larva alimentándose de hojas vivas del encino *Quercus oleoides*. B) Comparación de tamaño entre una larva alimentada con la dieta habitual

de *S. frugiperda* (hojas de maíz, parte superior) y una la larva de la misma especie alimentada con hojas de *Q. oleoides* (parte inferior).

Hay otras evidencias que apuntan a que los polifenoles también pueden actuar como agentes tóxicos (defensas cualitativas) y no solamente como defensas cuantitativas (dosis-dpendientes). Bajo condiciones de pH alcalino, como ocurre en el intestino medio de muchas larvas de lepidópteros, los polifenoles pueden causar un alto estrés oxidativo (sobre todo los taninos hidrolizables). Estos taninos se auto-oxidan y forman quinonas altamente reactivas que forman enlaces covalentes (en vez de puentes de hidrógeno) con proteínas ya sean de origen vegetal o animal y promueven la producción de formas reactivas de oxígeno (Barbehenn et al. 2006, Barbehenn et al. 2008a, Quideau 2009). Al parecer, la toxicidad de los taninos está en función del número de grupos hidroxilo de su estructura, siendo mayor en los taninos con mayor número de estos grupos funcionales (Wink, 2001). El estrés oxidativo (o producción de formas reactivas del oxígeno) que generan puede producir necrosis en el epitelio del intestino medio de los lepidópteros. Estas lesiones pueden ocasionar la muerte de las larvas ya que el tejido dañado puede ser una vía de comunicación entre la flora natural del intestino, las enzimas digestivas y el hemocele del insecto (Steinly y Berenbaum 1985, Barbehenn et al. 2005, Barbehenn et al. 2008b).

Otra evidencia a favor del papel de defensa de los polifenoles es la acción de los elagitaninos (taninos hidrolizables) sobre los patógenos. Estos compuestos combaten una gran variedad de bacterias y hongos al dañar su pared celular (Quideau 2009) y combaten virus ya sea evitando su entrada a las células (Fukuchi et al. 1989, Nocchi et al. 2016) o inactivando enzimas como la transcriptasa reversa en el caso de los retrovirus (Kakiuchi et al. 1985). Este tipo de estudios se han llevado a cabo principalmente en patógenos que afectan al ser humano, como el virus de la inmunodeficiencia humana y en bacterias y hongos como *Helicobacter pylori* y *Candida sp*., respectivamente (Taguri et al 2004, Buzzini et al. 2008). Pero en cuanto a su rol de defensa contra fitopatógenos, los elagitaninos han sido poco explorados, no obstante, su función ecológica pudiera ser importante. Uno de los pocos estudios en plantas se llevó a cabo en *Solidago altissima* y los resultados fueron contrarios a lo esperado. En *S. altissima* se analizó si la producción de taninos aumentaba durante el desencadenamiento de una reacción hipersensitiva (RH) ocasionada por un díptero formador de agallas (Tephritidae, mosca de la fruta); comúnmente la RH ocurre cuando hay una invasión de patógenos en las plantas y es una reacción que conlleva a la muerte de las células infectadas, sin embargo, en *S. altissima* se encontró que la producción de taninos aumentaba con el tiempo tanto en los clones de *S. altissima* susceptibles como en los clones resistentes fueran atacados o no por la mosca de la fruta. No obstante cabe destacar que el contenido fue mayor en los clones susceptibles y en el tejido de las agallas (Abrahamson et al. 1991). El efecto de los taninos, en especial de los elagitaninos, sobre los fitopatógenos es un tema que no ha sido profundizado dentro de la ecología química y que merece más atención para esclarecer si presentan alguna función antimicrobiana y/o antifúngica**.**

Como se mencionó anteriormente, uno de los factores que ha llevado a la confusión en cuanto al rol defensivo de los taninos es el efecto diferencial que producen sobre los herbívoros. El efecto de los taninos depende tanto de su estructura química como del herbívoro que los

consume. Se ha visto que un mismo tanino puede producir diferentes efectos en diferentes herbívoros, o bien, que taninos con estructuras similares producen efectos diferentes en una misma especie de herbívoro (Ayres et al. 1997). Este fenómeno se ha observado en insectos folívoros como los lepidópteros y escarabajos, y en herbívoros vertebrados como las ardillas. Por ejemplo, se ha encontrado que los taninos de las bellotas de *Quercus sp* afectan de manera sustancial a ardillas rojas (*Sciurus vulgaris*) pero no a las grises (*S. carolinensis*). Al parecer, en las ardillas grises se han desarrollado mecanismos conductuales, digestivos y enzimáticos para evitar los efectos dañinos de estos compuestos (Lurz et al. 1995, Haslam 2002). Este tipo de resultados ha llevado a que en algunos trabajos se cuestione la función de los taninos y ha llevado a replanteamientos sobre su función general.

Factores ambientales relacionados con la producción de polifenoles

En escalas geográficas se ha tratado de determinar si existe una relación entre la concentración de polifenoles en las plantas y la latitud. Esta relación descansa sobre la premisa de que las plantas de los trópicos poseen niveles altos de resistencia por estar sujetas a mayor herbivoría (Moles et al. 2011a, Moles et al. 2011b). En la mayoría de estos trabajos no se encuentró una tendencia clara. No siempre las plantas sujetas a mayor presión de herbivoría son las que presentan una mayor concentración de polifenoles (Adams et al. 2008). Bajo esta amplia aproximación, una de las explicaciones de la variación en la concentración de los compuestos fenólicos, es la incidencia de luz (Dudt y Shure 1994, Close y McArthur 2002). En este sentido, la incidencia de luz UV-B se ha estudiado en combinación con la presión de herbivoría para determinar el peso que tienen los factores bióticos y abióticos sobre la producción de polifenoles. En *Betula pubescens*, por ejemplo, se encontró que la incidencia de luz UV-B no es suficiente para aumentar la concentración de fenoles, pero en cambio, la exposición de *B. pubescens* a larvas del lepidóptero *Epirrita autumnata* si es un factor suficiente para activar la vía metabólica de los polifenoles. El daño producido por *E. autumnata* incrementa el nivel de la enzima polifenol-oxidasa en el tejido vegetal de *B. pubescens*. Esta enzima juega un papel importante en la conversión de di-fenoles a quinonas reactivas, lo que permite que las quinonas se unan a proteínas y aminoácidos covalentemente (Constabel y Ryan 1996). Agrawal y cols. (2009) estudiando el aspecto y composición química de la cutícula foliar de varias especies de *Asclepias sp.,* encontraron una relación clara entre el contenido de polifenoles y el tipo de hábitat de las especies, es decir, relacionaron el contenido de estos compuestos de manera indirecta con factores abióticos como la incidencia solar. Encontraron que el contenido total de fenoles era 25 % mayor en las especies con hojas glabras en comparación con las especies con hojas pubescentes y glaucas (verde claro, tono polvoso, Fig. 4). Pero cuando se analizaron específicamente los glucósidos de quercetina, los cuales son flavonoides que actúan como protectores solares, las especies con hojas pubescentes y glaucas tuvieron una concentración 50% mayor que las especies glabras, lo que concuerda con la alta exposición de luz a la cual están expuestas este tipo de especies en los ambientes áridos.

Figura. 4 Superficie abaxial foliar de tres especies de *Asclepias* que representan los tres tipos de hojas: glabras, pubescentes y glaucas. Tomado y modificado de Agrawal et al. 2009.

Los resultados derivados de estudios sobre la incidencia de luz y la producción de polifenoles deben tomarse con cautela, ya que la incidencia de luz promueve principalmente la producción de flavonoides y no la de los taninos. Los flavonoides y los taninos poseen propiedades diferentes. Por un lado, los flavonoides son agentes antioxidantes capaces de secuestrar radicales libres y proteger a la planta del fotodaño y por el otro, los taninos hidrolizables, contrariamente a los flavonoides, actúan como agentes pro-oxidantes en los insectos y son capaces de producir radicales libres y formas reactivas de oxígeno. Por lo tanto, las cuantificaciones de fenoles o polifenoles totales no siempre son un indicador adecuado para sacar conclusiones sobre el papel de estos compuestos en la defensa de plantas. La identificación y cuantificación individual es necesaria para poder entender su función.

Otros factores abióticos relacionados con la producción de polifenoles son los nutrimentos del suelo (Coley et al. 1985) y la concentración de CO_2 atmosférico (Veteli et al. 2007). En algunos sistemas, los nutrimentos del suelo afectan la composición química de las plantas; bajo esta perspectiva, por ejemplo, se ha estudiado el efecto que tienen los incendios sobre la fitoquímica de las plantas. Este tipo de disturbios aumentan la disponibilidad de nutrientes en el suelo y aumentan la incidencia de luz al abrir espacio en la cobertura vegetal, lo que, en consecuencia, puede llevar a cambios químicos en las plantas que se establecen posteriormente y también aumentar la presión de herbívoría en comparación con sitios intactos. No obstante, no se ha encontrado una clara relación de estos factores (aumento en disponibilidad de nutrimentos) con la producción de polifenoles. Rieske (2002) estudió el efecto de los incendios en la producción de taninos en plántulas de *Quercus prinus*, y aunque al principio de la temporada de crecimiento las plántulas de sitios incendiados mostraron un mayor porcentaje de taninos, esa tendencia se perdió al final del estudio. En otro caso similar, Adams y Rieske (2003) no encontraron diferencias entre plántulas de *Quercus alba* establecidas después de incendios y plántulas de sitios intactos. Otras características del suelo, como la retención de agua, han resultado tener una mayor relación con el contenido de polifenoles en plantas en comparación con los nutrimentos (Arámbula et al. 2010). Por lo tanto, al no haber un patrón claro, una de las conclusiones derivadas respecto a la disponibilidad de nutrimentos del suelo y los polifenoles es que no necesariamente la cantidad de nutrimentos del suelo es lo que determina la cantidad de compuestos polifenólicos sino más bien la tasa de crecimiento intrínseca de cada especie.

Aparentemente, las especies de lento crecimiento son las que acumulan mayor cantidad de estén tipo de compuestos constitutivos (Endara y Coley 2011).

En cuanto a la concentración de CO_2 atmosférico, estudios indican que el aumento de este gas incrementa el contenido de compuestos fenólicos en las plantas. Este tipo de estudios se han llevado a cabo con la finalidad de predecir cambios químicos bajo escenarios de cambio climático global. Dado que el aumento de CO_2 es acompañado por un incremento en la temperatura media global, ambas variables han sido consideradas. Debido a que el aumento de temperatura se relaciona comúnmente con una disminución en el contenido de fenoles, cuando se analizan en conjunto estas dos variables (CO_2 y temperatura) el efecto de ambas sobre la producción de fenoles se anula (Veteli 2007). Sin embargo, hasta la fecha son pocos los estudios que han abordado la composición química de fenoles en especies con ámbitos de distribución que incluyan zonas tropicales y no tropicales con gradientes de temperatura y precipitación contrastantes, lo cual permitiría realizar mejores inferencias sobre el papel de diferentes factores ambientales sobre la producción de polifenoles foliares particulares.

Evidencias de inducción de polifenoles por daño vegetal

Uno de los argumentos más sólidos para considerar a los polifenoles como compuestos de defensa contra herbívoros y patógenos es el incremento en su síntesis tras daño vegetal, así como su capacidad de acumularse en las zonas dañadas de las plantas (Bernards et al. 2006). En algunas plantas, se ha encontrado que el daño producido mecánicamente o por herbívoros, promueve la síntesis de taninos condensados e hidrolizables (Baldwin y Schultz 1983, Barbehenn y Constabel 2011), y más aún, se ha encontrado que se promueve la síntesis de enzimas capaces de oxidar a estos compuestos y volverlos perjudiciales para los insectos. Este hecho puede ser la explicación de por qué, en algunos casos, las plantas más atacadas tienen una mayor cantidad de polifenoles, es decir, que estas plantas en vez de ser vulnerables a la herbivoría (o contrariamente más adaptadas para soportar una alta presión de herbivoría) más bien pudieran estar respondiendo a ataques específicos y el investigador pudiera estar tomando la muestra después de ocurrir el fenómeno de inducción. Por lo tanto, el momento del muestreo, previo o posterior al daño puede ser la clave. Se pueden encontrar especies leñosas de lento crecimiento con un alto contenido de polifenoles cuyo nivel pudiera aumentar aún más a causa de un daño producido por herbívoros o patógenos (inducción).

Algunos polifenoles, específicamente los flavonoles y los taninos, tienen acción repelente contra insectos y su producción puede desencadenarse a partir del daño vegetal. Por ejemplo, el aumento en el contenido catequina puede resultar del daño producido por larvas de lepidópteros y curculiónidos donde los genes relacionados con la enzima Antocianidina sintasa se pueden activar en respuesta al daño (Ralph et al. 2006). Otro ejemplo de inducción ocurre en *Glycine max* (soya) cuando es atacada por su herbívoro, el lepidóptero *Helicoverpa zea.* En este estudio, Bi y Felton (1995) encontraron que cuando *G. max* era dañada por larvas de *H. zea*, la actividad de la enzima Fenilalanina amonia liasa (PAL) aumentaba más del doble (2.35 veces). Además, encontraron que los individuos de *H. zea* alimentados con plantas previamente dañadas sufrían de mayor estrés oxidativo que los

individuos alimentados con hojas intactas. Esto sugiere que un aumento de la actividad de la enzima PAL conlleva al aumento de compuestos pro-oxidantes (taninos) y la disminución de compuestos antioxidantes en el intestino medio de las larvas. Otro ejemplo, ocurre en el álamo (*Populus tremuloides*) en el cual se genera una cascada de respuestas de defensa tras daño (Lawrence and Novak 2001, 2006). Constabel y Barbehenn (2006) realizaron una revisión de los casos de probable inducción de polifenoles a partir de reportes en los cuales la concentración de la enzima Polifenol oxidasa incrementó en respuesta a daños producidos por herbívoros. Estos autores encontraron que, en al menos 12 interacciones planta-herbívoro, se pueden generar respuestas de inducción. Las especies de plantas involucradas incluyen siete familias, incluyendo familias de importancia ecológica y económica como la Solanaceae y Poaceae. Con base en estos antecedentes cabría suponer que este mecanismo es común en el reino vegetal.

Tomando en consideración los puntos antes mencionados, el propósito fundamental de esta tesis fue determinar la relación de varios compuestos polifenólicos con diferentes tipos de daño foliar (tanto natural como artificial) y con diferentes factores abióticos como la temperatura y precipitación en el encino tropical *Quercus oleoides*. Para determinar estas relaciones, en esta tesis se estudiaron tanto en condiciones de campo como de laboratorio a individuos de *Q. oleoides* provenientes de diferentes poblaciones, abarcando los sitios más contrastantes del rango de distribución de la especie, para poder poner a prueba si la variación en la concentración de diferentes polifenoles se modifica en respuesta a factores bióticos y/o abióticos. Esta tesis incluye cuatro apartados (capítulo IV a VII). En el capítulo IV se presenta una revisión sobre los mecanismos de producción de estos compuestos y su estructura. En los siguientes capítulos se evaluó la relación entre el contenido de polifenoles y el daño producido por diferentes gremios de insectos, así como su inducción en plántulas por daño mecánico (capítulo V) y por la acción del quitosán (Capítulo VI) el cual es un compuesto de la pared celular de los patógenos, se estudió también la variación en la concentración de compuestos fenólicos a lo largo de la distribución de la especie en relación a diferentes variables ambientales (capítulo VII). En conjunto, los resultados de esta tesis permiten aumentar nuestra comprensión de las causas ecológicas que desencadenan la producción de estos compuestos.

IV. Plant polyphenolics: biosynthesis and its role in plant defense

Plant polyphenolics: biosynthesis and its role in plant defense

ABSTRACT

Polyphenolic compounds are the most widespread secondary metabolites present in the plant kingdom. The outstanding diversity of forms, functions and metabolic activities of these compounds derive from many chemicals transformations which begin with two routes, namely the shikimate pathway and the malonic acid pathway. Subsequent transformations take place where many enzymes participate to produce such polyphenolic diversity. In this review, a general scheme of the biosynthesis, the mode of action of selected compounds against different groups of herbivores and pathogens, and the mechanisms of induction of polyphenolics following damage is presented. In addition, the role of modern analytical techniques to quantify and identify these compounds will be discussed, as well as new venues for future research.

Keywords: Herbivory, induction, pathogens, phenylpropanoids, hidrolizable and condensed tannins.

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What are polyphenolic compounds?

Taking into consideration diverse criteria, several definitions for polyphenols and tannins have been proposed over time. These terms have been changing as the understanding on their structure, synthesis and biochemical properties has increased. Originally, these compounds were generally called tannins due to the action they had in the process of tanning leather; none of these compounds were called polyphenols before the 1950s. It was Theodore White who initially defined tannins as compounds able to join to collagen and tan leather. Subsequently Anthony Swain, E-C Bate Smith and Jeffrey Harborne proposed a definition for polyphenols according to their water solubility, molecular weight (500-3000Da), capability to react with iron III salts, permanganate and precipitate several molecules, such as alkaloids, gelatin (denatured form of collagen) and other proteins; this definition itself does not considered the strict binding capability to collagen, which makes possible the tanning of leather. Later, Edwin Haslam adopted this definition and extended it highlighting the molecular structure and he considered the terms "polyphenol" and "tannin" as synonymous. Over the time, each definition started to refine the anterior, and other aspects besides the capability to bind to several molecules where included. However, some definitions appeared and have created confusion at some point, that some simple phenolic compounds, such as resorcinol, are considered polyphenols. The most recent definition of the term "polyphenol" and the definition that will be considered throughout this review is the one proposed by Quideau et al. (2011) which states that “polyphenolic compounds 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”. This definition, thus, considers mainly the number of phenolic rings and the biosynthetic origin of the compounds.

Biosynthesis and structure

The synthesis of polyphenolic compounds begins with two metabolic pathways. One is known as the Shikimate pathway and the other is known as the Polyketide or Malonic acid pathway (Herrmann & Weaver 1999, Taiz & Zeiger 2006). These metabolic routes end up synthetizing three aromatic aminoacids which are subsequently modified through the Phenylpropanoid metabolism and other chemical reactions. Although different polyphenolics, such as tannins or lignin, originate from those pathways, there are important differences in the way in which they are subsequently metabolized (Gross 2008). Therefore, polyphenolics such as lignin, flavonoids and tannins have different biochemical and ecological properties, even when they share common chemical precursors (Waterman & Mole 1994, Haslam 2007).

The metabolic starting point: The Shikimic Acid pathway

In 1885 the Shikimic acid was isolated from the fruit of the japanese plant *Illicium religiosum* commonly called “shikimi-no-ki” (Hebert 1981). Scientific work form Eykmann, Fisher, Freudenberg, among others, allowed its description. But it was not until 1950 that shikimic acid was recognized as a key intermediate in the important biochemical pathway now known

as the “Shikimic acid pathway”. Since then, from an ecological and economic point of view, the Shikimate acid pathway has been extensively studied. This metabolic route represents around 20 % of the global carbon fixed by plants (Herrmann 1995). As this pathway is absent in animals, its study also gave rise to commercially important compounds such as glyphosate, a wide-spectrum herbicide and crop dessicant that blocks a metabolic step in this pathway killing plants, and to the development of antifungal, antibacterial and antiparasite drugs (Steinrucken & Amrhein 1980, Herrmann & Weaver 1999, Bender 2012).

The Shikimic acid pathway participates in the biosynthesis of most plant phenolics, while in bacteria and fungi this role is fulfilled mainly by the Malonic acid pathway. The Shikimic acid pathway, which is restricted to the plastid stroma, leads the conversion of carbohydrate derived compounds (intermediates from glycolysis) into Chorismate, which is the direct substrate that leads the synthesis of the three aromatic aminoacids: L-phenylalanine, L-tyrosine, and L-tryptophan (Figure 1). Because animals lack this pathway they cannot synthetize these essential aminoacids (Weaver & Herrmann 1997, Herrmann & Weaver 1999, Taiz & Zeiger 2006). Although there are specific enzymatic differences in the Shikimate pathway in bacteria, fungi and plants, it usually consists of seven steps. Each step is catalyzed by a particular enzyme. An exception is the fungus *Neurospora*, in which the second step through the sixth are catalyzed by a single enzyme: the multifunctional-enzyme complex arom-enzyme (Ahmed & Giles 1969, Lumsden & Coggins 1977).

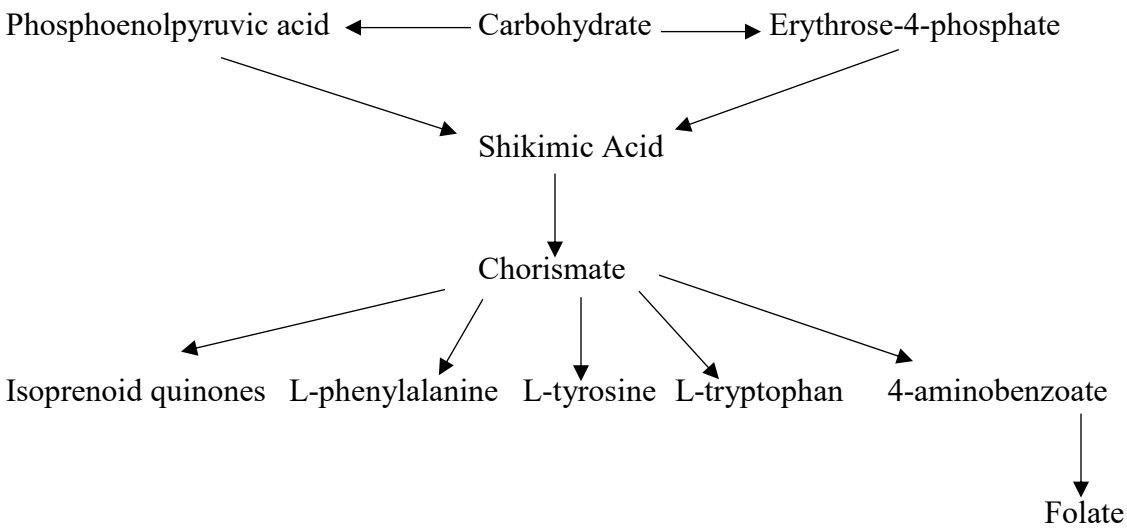

Figure 1. Brief scheme of the Shikimic Acid pathway. Taken and modified from Haslam 1974.

The seven metabolic steps of the Shikimic acid pathway with their respective enzymes were initially characterized in bacterial mutants with the aid of radioactive isotopes. As mentioned above, these seven reactions produce the transformation of Phosphoenolpyruvic acid and Erythrose-4-phosphate (intermediates from glycolysis) to Chorismate. The enzymes involved in each step are listed below, as well as their cofactors (in parenthesis):

1. DAHP synthetase condenses Phosphoenolpyruvic acid and Erythrose-4-phosphate, in order to yield 3-Deoxy-D-arabinoheptulosonate-7-phosphate or DAHP.

2. The enzyme 3-Dehydroquinate synthetase (NAD^{+}, Co^{+2}) catalyzes the reaction from DAHP to 3-dehydroquinate.

3. Then the enzyme 3-Dehydroquinate dehydratase transforms 3-dehydroquinate into 3-dehydroshikimate.

4. The enzyme 3-Dehydroshikimate reductase (NADPH) catalyzes the step from 3-dehydroshikimate to shikimate.

5. Then, Shikimate kinase (ATP) catalyzes the ATP phosphorylation of Shikimate to yield Shikimate-3-phospate.

6. The enzyme 5-Enolpyruvylshikimate-3-phosphate synthetase (PEP) metabolizes shikimate-3-phosphate into 5-enolpyruvylshikimate-3-phosphate which is considered a rare type reaction. Is in this step where the herbicide glyphosate blocks the pathway when blocking this enzyme (its successful function generated revenues for around 480 million usd in 1984, Floss 1986).

7. In the last metabolic step, Chorismate synthetase turns 5-enolpyruvyl-shikimate-3-phosphate into Chorismate, the immediate precursor of the three aromatic aminoacids (Figure 2).

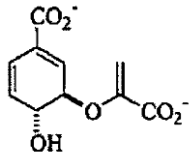

Figure 2. Chorismate, the precursor of the three aromatic aminoacids: L-phenylalanine, L-tyrosine, and L-tryptophan.

After these seven steps occurred, the main stem of the Shikimic acid pathway ends. Then the acid "Chorismate" undergoes subsequent modifications where different branches split out to yield the three aromatic aminoacids and other important compounds (such as Isoprenoid quinones, 4-Aminobenzoate and Folate). For the synthesis of L-phenylalanine and L-tyrosine two enzymatic steps are involved:

1. The enzyme Chorismate mutase transforms Chorismate into Prephenate through a called "Claisen rearrangement" (a powerful carbon-carbon bond forming chemical reaction), then the next two routes can lead either the synthesis of (a) L-phenylalanine or (b) L-tyrosine:

a. For the synthesis of L-Phenylalanine, Prephenate is converted to phenylpyruvate by the enzyme Prephenate dehydratase which is then aminated.

b. For the synthesis of L-tyrosine, Prephenate is converted to *p*-hydroxyphenylpyruvate by the enzyme Prephenate dehydrogenase which is then also aminated.

The biosynthetic branch for L-Tryptophan is longer and it involves the action of five enzymes (Anthranilate synthase, Phosphoribosylanthranilate synthase, Phosphoribosylanthranilate isomerase, Indole-3-glycerol phosphate synthase, Tryptophan synthase α and Tryptophan synthase β). These enzymes yield two key intermediates, Anthranilate and Indole, the latter undergoes amination to synthetize L-Tryptophan. Besides using this pathway for aminoacid and then protein synthesis, plants also use it for the biosynthesis of the hormone auxin, phytoalexins, glucosinolates, and both indole- and anthranilate-derived alkaloids.

Once the aromatic aminoacids are synthesized, they experience further metabolic changes. For L-Phenylalanine a subsequent pathway called the "General Phenylpropanoid Metabolism" leads its transformation to the production of a large number of polyphenolic compounds, including tannins.

Phenylpropanoid metabolism

Once L-Phenylalanine is synthethized, it can be transformed into several hydroxycinnamic acids and their derivatives. There are basically three enzymes involved in these metabolic steps which are collectively known as Group I enzymes: L- phenylalanine ammonia lyase (PAL), the cinnamate-4-hydroxylase, and 4-coumarato-CoA ligase. Later, or even intermediate modifications such as hydroxylations and methylations occur, leading to the formation of the wide variety of acids (Tsai et al. 2006, Vogt, 2010). These acids are known as simple phenylpropanoids.

The PAL enzyme has been widely studied and mainly because it is in the frontier between primary and secondary metabolism. There are multiple copies of the PAL gene, for example, there are four copies in *Arabidopsis*, five in *Populus sp*., and nine copies in rice (Hamberger et al. 2007). Each of these copies respond differently to various stressors, and to the developmental stages of plant tissues (Bhuiyan et al. 2009, Lillo et al. 2008). Therefore, a differential activation of PAL genes leads either to the synthesis of lignin, the production of condensed tannins or the synthesis of flavonoids (Hamberger et al., 2007). One of the mechanisms that inhibit the enzyme PAL is the presence of cinnamic acids, which constitutes a negative feedback due that cinnamic acids are formed by the same enzyme. These acids, in certain concentrations, can strongly inhibit other enzymes involved in the synthesis of other phenylpropanoids (Mavandad et al. 1990, Barz & Mackenbrock 1994). Coumarins derive from the first stages of the phenolic pathway that produce flavonoids. Furanocoumarins, (like psoralen in the Apiaceae family) are phototoxic compounds that, in the presence of light, form intermediate toxic compounds capable to crosslink DNA (Schardl & Chen 2010).

Lignin

One of the factors that allowed the colonization of land by plants was the development of a vascular system able to transport water and nutrients from the soil to the leaves and

finally to the atmosphere in a semi-closed circuit. This water transporting circuit is characterized by its rigidity and structural stiffness, which in addition allowed vascular plants to increase in height. Among the wide number of polyphenolic compounds that have been related with plant defense, lignin, the main component of the tracheids and xylem vessel elements (critical to enhance the mechanical resistance of the vascular system in plants), is also considered as a primary defensive compound due to the increase of rigidity it confers to plant tissues.

Lignin, the second most abundant plant substance, besides giving the opportunity to plants to thrive in a desiccant environment, also aids plants to cope with herbivores and microorganisms. During insect and pathogen attack, hard tissues block, at first instance, their entrance to susceptible plant tissues and deters feeding by herbivores. If it does not stop the attacker, a callus is formed to avoid a further infection, two facts that have enhanced the view of lignin as a defensive chemical compound against insects and pathogens. On one hand, the mechanical restriction to the movement of attackers constitutes one of the most efficient passive barriers against herbivores and pathogens, including specialist and generalist plant herbivores. And on the other, the induction of a callus after an injury or infection has changed the view that lignin acts only as a passive defense, and now is also considered as an active defense (Whetten & Sederoff 1995). If pathogens can cross the plant's cell wall, lignin is synthesized and deposited surrounding the affected and the adjacent cells next to the infection site. The reinforcement of the cell wall thickness diminishes the dispersal of pathogens through vegetal tissue. This type of physiological response is known as a normosensitive response, as they promote the *de novo* synthesis of defense related molecules (Prell & Day 2001).

Lignin is present in the plant cell walls and in the xylem which gives strength and mechanical support to the trunk during secondary growth. This propriety has taken chemist to investigate further in this molecule. Many chemists have been intensively investigated the chemical structure and composition of this material, which is the strongest material on earth for its relative density. Owing to its complexity and difficulty to isolate it, it has been only recently that the synthesis and structure of this compound started to be understood. (Schoonhoven et al. 2005). Lignin is a complex and branched polymer with an extremely high molecular weight that is formed from phenylpropanoid groups. Although the structure of lignin is not precisely known, it is recongnized that is formed from three phenylpropanoid alcohols called: coniferyl, *p*-coumaryl, and sinapyl alcohols, collectively known as monolignols, which derive from many cinnamic acid derivatives. Polymerization is catalyzed by oxidative enzymes (oxidases) and the three monolignols are differentially targeted to the middle lamella, secondary cell wall and fiber forming cell walls. Also, its proportion changes from species to species which adds more complexity (Davin & Lewis 2005).

Flavonoids

Flavonoids are characterized by their 15-carbon skeleton arranged in two aromatic rings connected by a three-carbon bridge. According to the degree of oxidation of the three-carbon bridge they are classified into: chalcones, flavanones, flavones, isoflavones and flavonols,

and are collectively known as yellow and anthocyanin pigments. Flavonoids may undergo several enzymatic modifications, for example: hydroxylation, methylation, glycosylation, sulfonication, acylation and/or prenylation reactions. All these enzymatic reactions result in a great flavonoid diversity. There are around 5000 identified flavonoids. The aromatic ring B and its adjacent 3 carbon side-chain are derived from L-phenylalanine via the Shikimate pathway, whereas ring A is formed by the condensation of three acetate units via the Polyketide pathway that is proposed for the biosynthesis of phloroglucinol and resorcinol derivatives. The immediate precursors of flavonoids are: the hydroxycinnamoyl-CoA and malonyl-CoA, whose condensation is catalyzed by the key enzyme chalcone synthase. Flavanols differ from other flavonoids in that they do not appear as glycosides, there are in a glyconated form or they form esters with Gallic acid. Flavonoids seem to interact with cell membranes. For example, epicatechin is an amphipathic compound, but catechin more lipophilic. Flavanols monomers and dimers diffuse across the membrane and into the cell. Flavonoids, such as quercetin, are also found immersed in plant cuticles, specifically in the inner side of the cuticle.

Flavonoids are best known for their contribution to flower color and role in the attraction of pollinators, however these compounds exert many other functions. They participate in plant growth and development; they regulate hormonal transport and are key components during pollen germination. Flavonoids also act as UV protectants scavenging free radicals to avoid cell damage, these compounds are immersed in the cuticle matrix.

In plant–insect interactions, they have different roles, some act as feeding stimulants, feeding deterrents and oviposition stimulants. Particularly, isoflavonoids (distinctive flavonoids from the Leguminosae family), act as phytoalexins, that is, they have antimicrobial and antifungal function that limit the spread of pathogens in the plant. Also, some flavonoids have antiviral activity inhibiting viral RNA synthesis.

Condensed tannins

Once L-phenylalanine is modified through the general phenylpropanoid metabolism (MGP) chalcones are synthetized, a kind of flavonoid mentioned above that give rise to other flavonoids, as well as the condensed tannins (also known as proanthocyanidins because of their ability to transform into anthocyanins by treatment with strong acids). Once chalcones are formed by action of the enzymes chalcone synthase (CHS) and acetyl CoA carboxylase, undergoe subsequent changes to yield leucoanthocyanidins by the action of the enzyme dihydroflavonol reductase (DFR). In this part of the biosynthesis, the leucoanthocyanidins units can be converted into flavan-3-ols called catechin (Figure 4) or epicatechin (Dixon et al. 2005).

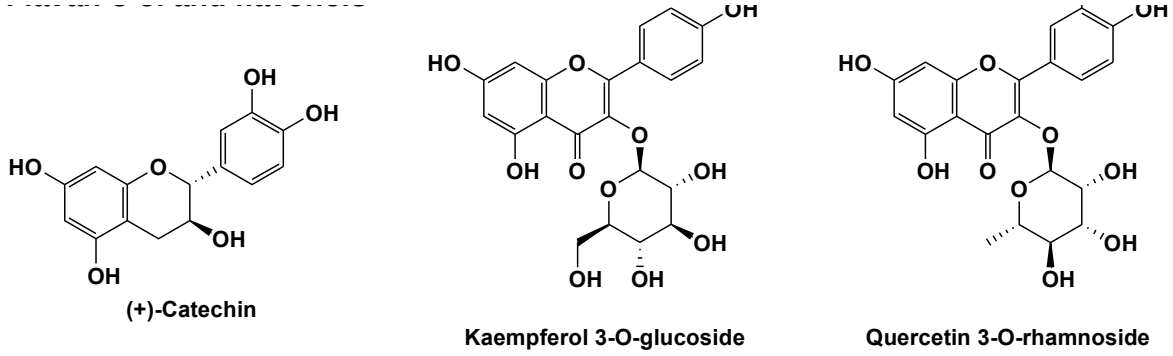

Figure 3. Flavonoid catechin.

Leucoanthocyanidins are reduced thus, by the action of leucoanthocyanidin reductase enzyme (LAR) to synthetize a catechin unit (Verries et al 2008). But if leucoanthocyanidins anthocyanidins are formed by the enzyme anthocyanidin synthase enzyme (ANS) and then reduced by the enzyme anthocyanidin reductase (ANR), epicatechin units are obtained (Paolocci et al. 2005). This set of enzymes are known as Group II enzymes. After catechin and epicatechin units are synthesized, they are transported to the vacuole via a conveyor (Paolocci et al. 2005). Polymerization occurs subsequently to form condensed tannins from the building blocks catechin or epicatechin bonded in the C4-C8 positions (Verries et al 2008). A condensed tannin has two to 20 units flavan-3-ol, however it is unknown exactly how that polymerization occurs.

Hydrolyzable tannins

Hydrolyzable tannins are polymers that are formed from Gallic acid and simple sugars, such as D-glucose. They can be more easily hydrolyzed than condensed tannins. Gallotannins and ellagitannins are two types of hydrolysable tannins. For chemical characterization, it has been considered the presence of two different types of residues: Meta-digalloyl in case the residue gallotannins and 3,4,5,3 ', 4', 5'hexahidroxydiphenoyl (HHDP) for ellagitannins, both derived from 1,2,3,4,6-Pentagaloyl-β-D-glucose (Haslam 2007, Gross 2008). Subsequently Gallic acid monomers are attached to the glucose molecule by the action of the glucosyl transferase enzyme. According to the number of galloyl-glucose units, gallotannins are divided into simple, one to five units, and in complex with six or more units galoylglucose (Clifford & Scalbert 2000, Gross 2008). The term "complex" is also used to describe special tannins are composed of a gallotannin or ellagitannin with more catechin unit. An example of such tannins is Acutisimin (Khanbabaee & van Ree 2001).

Genetic regulation of this type of tannin is less known than the condensed tannins. However, it appears that changes in the expression of enzymes of the Shikimic acid pathway alter their production. Some reasons why these tannins are less studied is because their analysis is more complicated and because they are less stable than condensed tannins (Mueller-Harvey 2001). In addition to their study, the plant material must be transported and stored with more caution (Waterman and Mole 1994).

Analytical tools

Since the pioneering work of Emil Fischer, chemists have obtained great progress to understand the complexity of these compounds. They have isolated several of the enzymes involved in the synthesis of tannins and to identify some of the regulatory genes. However, the pathway leading to the synthesis of condensed tannins or proanthocyanidins poses a challenge to research, because currently, we have not yet managed to clarify all reactions and enzymes involved in the polymerization of tannins, in the identification of the gene or genes involved in the transport of flavan-3-ol units (catechin and epicatechin) to the vacuole, and in the location of cellular site for various enzymes such as LAR and ANR. Similarly, the routes that originate hydrolysable tannins, although shorter, still require further investigation. With the support of genetics, genomics and metabolomics engineering, these questions will probably be answered in a not distant future.

One of the most promising complementary approaches to the new generation chemical techniques is to quantify the oxidative capacity of tannins. Through this analysis, it can be known the potential anti-oxidant or pro-oxidant capacity of polyphenolic compounds in different chemical environments, which might allow inferences over their defensive properties or even on the effect of these compounds on the digestive system of mammals and invertebrates (Salminen et al. 2011). Other types of studies to accompany the quantification of the concentration of polyphenols, such as bioassays, will help to determine the role of the studied chemical on the fitness of the herbivores. These types of manipulative studies will allow to test if polyphenols have indeed a defensive role. In addition, more study on the mechanisms that promote the production of polyphenols and how are these affected by the variation in temporal and spatial scales in the abiotic and biotic factors that may regulate its production is required. In conjunction, the synergistic study of these compounds will provide us a clearer understanding of the mechanisms that promote the evolution of plant defenses and will allow us to explore new materials and substances to employ in the industry.

Biochemical approaches

In the case of tannins, to determine whether they possess a defensive role in plant-herbivore and plant-pathogen interactions, it is indispensable the use of several and simultaneous methodological approaches and the employment of correct analytical techniques. Most ecological and physiological work on tannins have rely on colorimetric quantification for total phenols, condensed tannins and hydrolyzable tannins, which are widely used worldwide, but so far they have been proved unable to establish the type of relation between the degree of herbivory, the richness of herbivores within plants and the levels or types of polyphenols (Forkner et al. 2004, Adams et al. 2009). For example, Appel et al. (2001) quantified the amount of tannins in 16 woody species that differ in their concentration of tannins due to the species or to the seasonality in the collection time. These authors employed the Folin-Denis colorimetric techniques, which do not allow for the resolution required to establish variation across species and treatments. In response to these concerns, some authors propose the use of other analytical techniques, such as liquid chromatography coupled to mass spectrometry, which are powerful tools that provides highly detailed information on the

nature of these compounds (Rautio et al. 2007, Allwood & Goodacre 2009). As the level of details reveled by modern analytical techniques increase, the probability of disentangle the function of secondary compounds involved in plant-herbivore and pathogen interactions is higher (Roininen et al. 1999). By this type of approach accompanied with other complementary techniques might help to understand the role of polyphenols in plant defense.

Chromatographic methods

The progress of chemical ecology and the development of state-of-the-art analytical equipment, such as mass spectrometry, and high performance liquid and gas chromatography have greatly increased our knowledge on the structure and function of secondary metabolites. This has taken us several steps forward in our understanding of the chemical compounds that are implicated in plant-insect and plant-pathogen interactions.

Different approaches should be considered to disentangle de role of polyphenols and tannins. Analytic chemistry and molecular data have provided a venue for new and exciting research on the subject. For example, in recent years much has been known about the synthesis of polyphenols (tannins) and the mechanisms that regulate it.

Polyphenolics related to plant defense and resistance against insects and pathogens

Plants are constantly attacked by a great array of organisms, from microscopic pathogens to mega-herbivores weighting over 4 tons, such as elephants. Despite this constant attack, most of the plants that we see today are thriving in their local ecosystems. Hairston et al. (1960) propose that the "world is green" mainly because there is a strict control of the overconsumption of plants by the first order heterotroph organisms, the superior order of predators, and by the competition among organisms. This hypothesis ignored the fact that plants have evolved diverse mechanisms of defense, which is perhaps the main restriction for the depletion of green resources worldwide (Murdoch 1966). Further work on the coevolution between plants and herbivores allowed to establish the importance of an almost unlimited set of chemical compounds that function as defenses against herbivores. Currently a wealth of studies has shown that mechanisms of defense involving secondary compounds are widespread across the plant kingdom (Schoonhoven et al. 2005).

The first mention in the scientific literature of the term "secondary compound" comes from Kossel (1891) and Czapeck (1921). These authors considered that these compounds were only by-products or waste products irrelevant for the survival of plants, due that they were not related to primary metabolism and thus, were not involved in the main biochemical reactions able to sustain plant life (e. g. photosynthesis or cellular respiration). Therefore, secondary compounds were considered either waste products or temporal steps towards an end-function (Bourgaud et al. 2001). Given their low concentration of some of those compounds in plant tissues, and the deficient analytical techniques of that time, it was natural that no further research studies were performed to explore the function and structure of the secondary metabolites. It was not until 50 years ago that scientists worldwide start to discover the plethora of secondary compounds that shape the complex network of interactions (either competitive or mutualistic) involving almost every single neighbor plant and their predators.

Even today, only a fraction of the wide variety of secondary metabolites that plants synthetize have been described on their chemical structure, and even less research has explored their function in the plant-insect and plant-pathogen interactions. For some metabolites, such as terpenes, there is no doubt of their negative effect over herbivore's fitness, however, there are some families of compounds where there is no consensus on their function. This is the case of polyphenolics, a family of compounds that have captured the attention of biologists and chemists.

One of the factors that has led to the confusion of the defensive role of polyphenolics, as the case of tannins, is the differential effect produced in herbivores. The effect of tannins depends on their chemical structure and the characteristics of the herbivore that consumes them. It has been seen that the same tannin can produce different effects in different herbivores, or that tannins with similar structures produce different effects on the same species of herbivore (Ayres et al. 1997). This phenomenon has been observed in lepidopteran insects such as beetles and, in higher organisms such as squirrels. The tannins in acorns substantially affect red squirrels but not gray squirrels. Apparently, gray squirrels have developed behavioral, digestive and enzymatic mechanisms to prevent the harmful effects of these compounds (Haslam 2002). Some studies conducted on large spatial scales have tried to determine whether a relationship exists between the level of plant damage caused by insects and concentration of phenols and tannins (Moles et al. 2011a, Moles et al. 2011b). However, there is not a clear trend in all studies. Not always the most damaged plants are those with a lower concentration of polyphenols (Adams et al. 2008). One of the explanations for the variation in the concentration of phenols observed, which is not related to herbivory, is the incidence of light (Close & McArthur 2002). However, this commonly used explanation must be taken with caution since the incidence of light mainly promotes the production of flavonoids and not tannins. Tannins and flavonoids have different properties. Flavonoids act as antioxidants capable of sequestering free radicals and protect plants from photodamage whereas, hydrolysable tannins, contrary to flavonoids act as pro-oxidant agents capable of producing free radicals and reactive forms oxygen under alkaline pH conditions. Therefore, the quantification of total phenols is not always an appropriate indicator to draw conclusions about the defensive role of polyphenolics.

Induction: a plant damage response

There is evidence that the expression of the PAL gene changes in response to mechanical damage, pathogen infection and high levels of UV-B (Mellway et al. 2009). Expression of genes involved in the synthesis of tannins: The enzyme chalcone synthase (CHS) together with phenylalanine ammonia lyase (PAL) are considered the most important enzymes in the synthesis of tannins. This enzyme plays an essential role to produce various phenolic compounds, including tannins. The activity of these enzymes appears to be strongly coordinated as the promoters (DNA regions that facilitate transcription of a particular gene, usually located near the gene that is being regulated) of both genes have common sequences (Dixon & Paiva 1995). One of the genes that regulate the expression of PAL is the MYB134 CHS gene, which codes for R2R3 MYB transcription factor (a protein which facilitates the binding of RNA polymerase). In *Populus spp.* this gene is over-expressed in response to

mechanical damage, to infection by pathogens and to high levels of incidence of UV-B light, which leads to an increase in the production of condensed tannins (Osier & Lindroth 2006, Miranda et al. 2007). The genes responsible to regulate and ultimately to increase the expression of the genes encoding PAL and CHS also seem to increase the transcription of the genes coding for the enzymes of Group II such as DFR, ANR and LAR (Mellway et al. 2009).

In commercially important species other MYB genes have been identified, which apparently are also involved in the regulation of tannins. In grapevines (*Vitis spp*), it was found that VvMybPA1, VvMYB5a and VvMYB5b genes play an important role during the coloration and ripening of grapes to activate the CHS, DFR gene and ANS (Bogs et al. 2007, Deluc et al. 2008), thus modifying the composition and concentration of anthocyanins and flavonols. MYB genes also appear to have a high degree of specificity to this metabolic pathway. In addition to the transcription factors encoded by the MYB genes, other genes have been identified other important factors in the regulation of tannins, such as the ones that codify for the BPF-1 protein, which is expressed in the presence of pathogens in parsley and Myc factors and WD40 (Costa e Silva et al. 1993, Koes et al. 2005). These last two factors are not as specific to the metabolic pathway of tannins, as they also regulate other processes in plants such as trichome differentiation and production of seed mucilage (Lewis & Walker et al. 1999).

Other factors that affect the levels of tannins, although the genetic or physiological basis is not fully resolved, are the concentration of soil nutrients (mainly nitrogen, N). Plants grown under low amounts of N tend to accumulate more tannin (Ruohomäki et al. 1996, Hemming & Lindroth 1999). This type of response has been studied in several species (particularly in *Populus spp.*), and under different conditions of soil quality. Usually, there is an increase of tannins in nutrient-poor soils, however, the response varies depending on the species and genotype (Hunter & Schultz 1995, Osier & Lindroth 2001, Harding et al. 2002, Harding et al. 2009). The physiological significance of the relationship between soil nutrients and synthesis of tannins is not easily explained because the balance theory between carbon and nutrient budgets are not easy to apply in several cases, due to the difficulty to establish precise experimental protocols (Hamilton et al. 2001).

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V. Specific polyphenols and tannins are associated with defense against insect herbivores in the tropical oak *Quercus oleoides*

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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

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, 330°C. 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

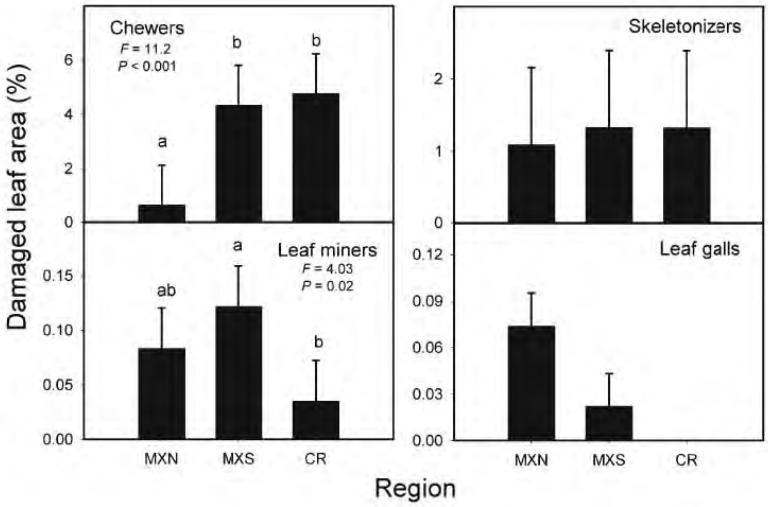

Fig 1 Damaged leaf area (%) produced by four herbivore guilds in three regions encompassing the distribution of *Quercus oleoides*. MXN=Northern Mexico, MXS=Southern Mexico, CR=Costa Rica

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

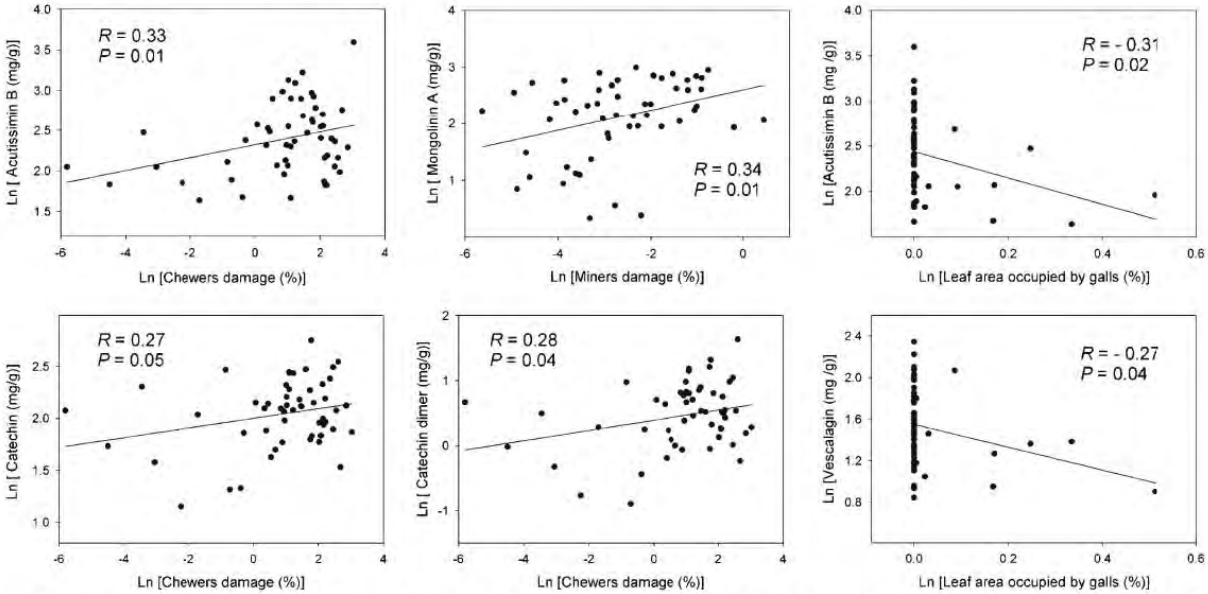

Fig 2 Pearson correlations between the concentration of specific polyphenols and tannins (mg/g) and foliar damage (%) in 54 individuals from 9 populations of the tropical oak *Quercus oleoides*

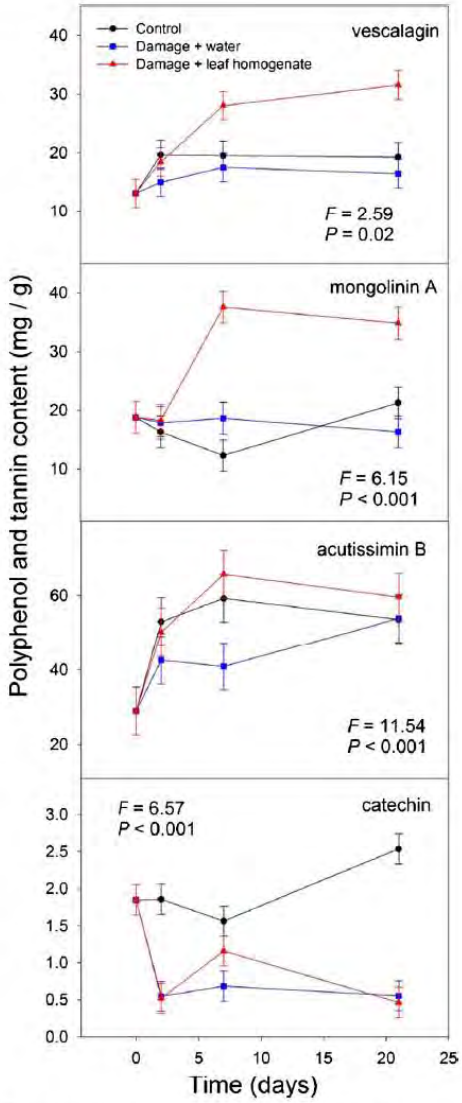

Fig 3 Polyphenol and tannin content in 1–yr-old *Quercus oleoides* saplings in response to mechanical damage (Damage+water) and simulated herbivore attack (Damage+leaf homogenate)

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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Material suplementario del artículo Moctezuma et al. 2014

FIGURE S1 Typical LC-FLD chromatogram of *Quercus oleoides* leaf extract. Peaks were identified by mass-to-charge ratio as well as fragmentation spectra. (1) hexahydroxydiphenoyl-glucose, (2) di-hexahydroxydiphenoyl-glucose, (3) vescalagin, (4) stenophyllanin, (5) vescavalonic acid, (6) acutissimin B, (7) mongolinin A, (8) cocciferin D3, and (9) cocciferin D2.

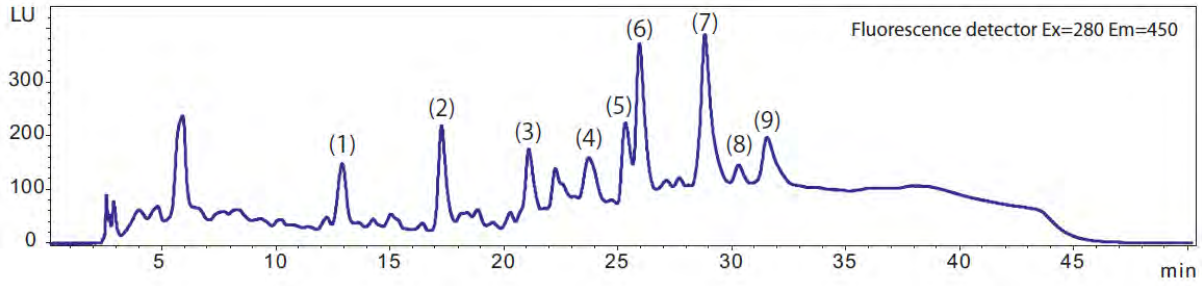
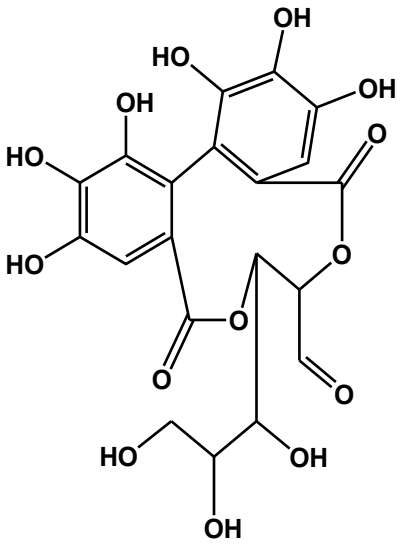
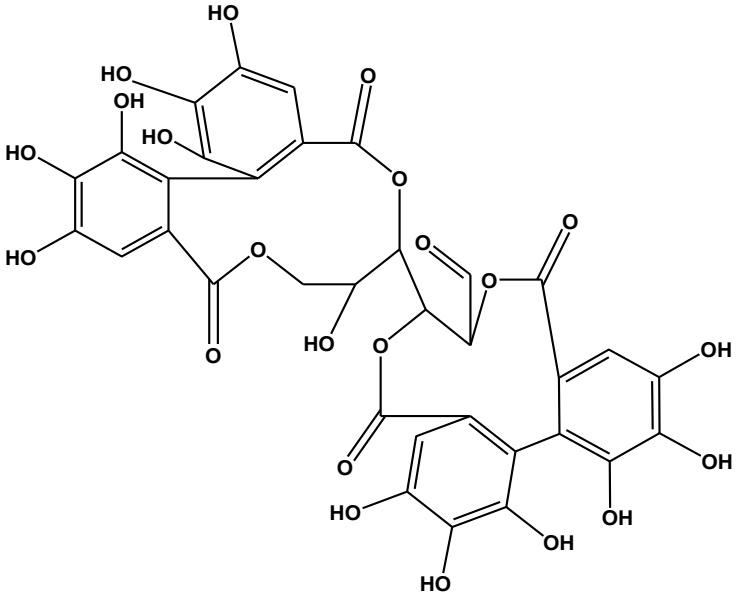


FIGURE S2. Structural formulas of the polyphenols and tannins found in *Quercus oleoides*.

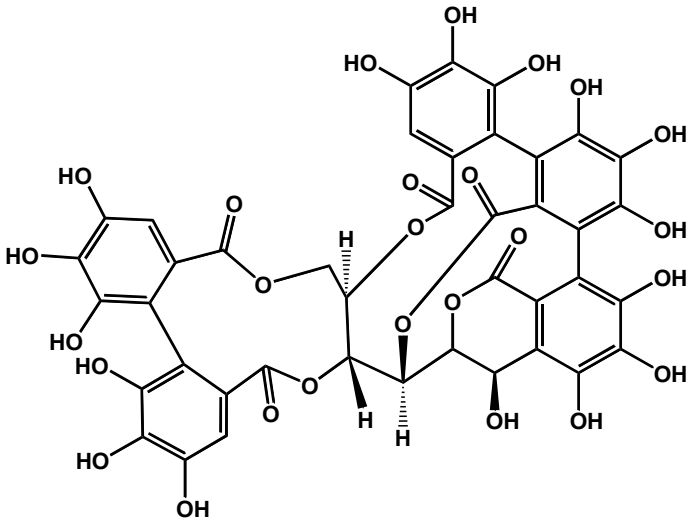
Hydrolyzable tannins



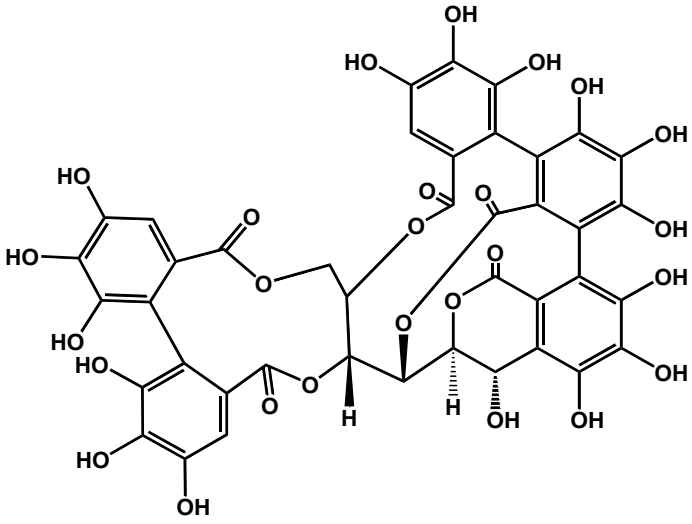
Hexahydroxydiphenoyl glucose



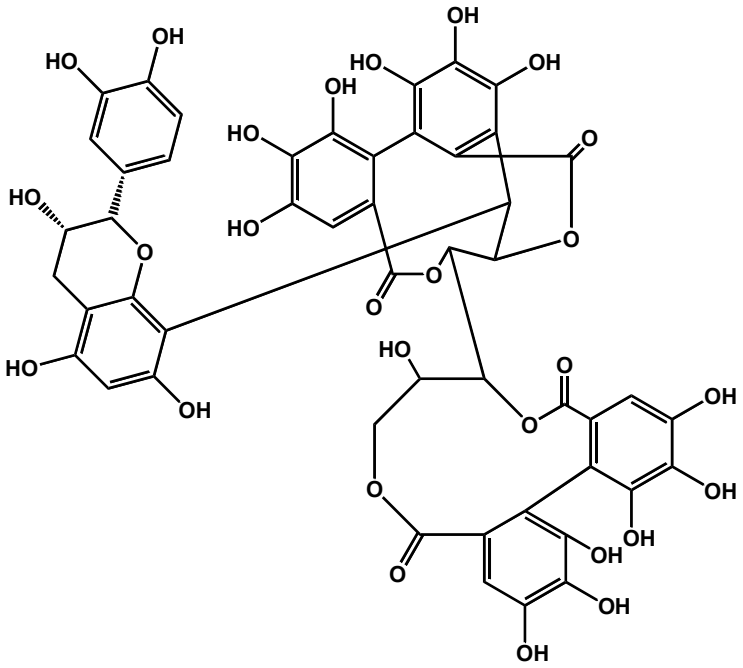
Di-hexahydroxydiphenoyl glucose



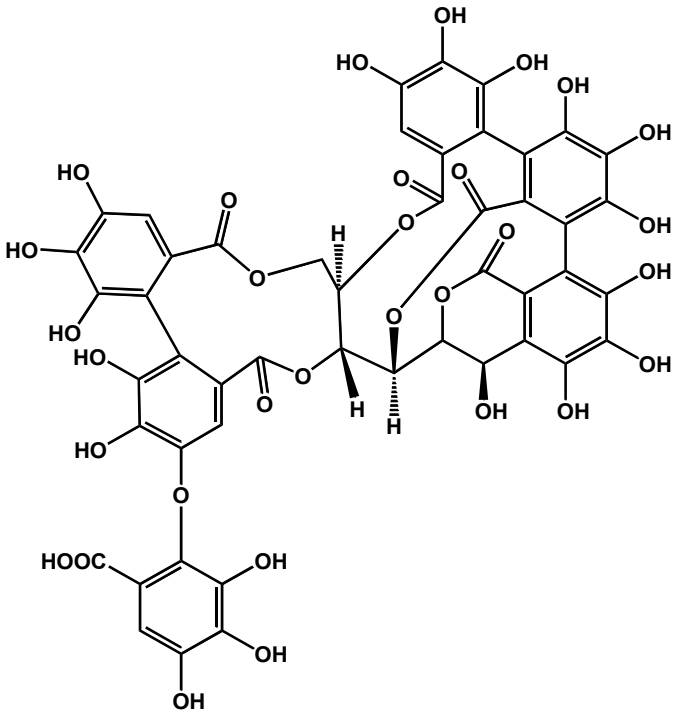
Vescalagin



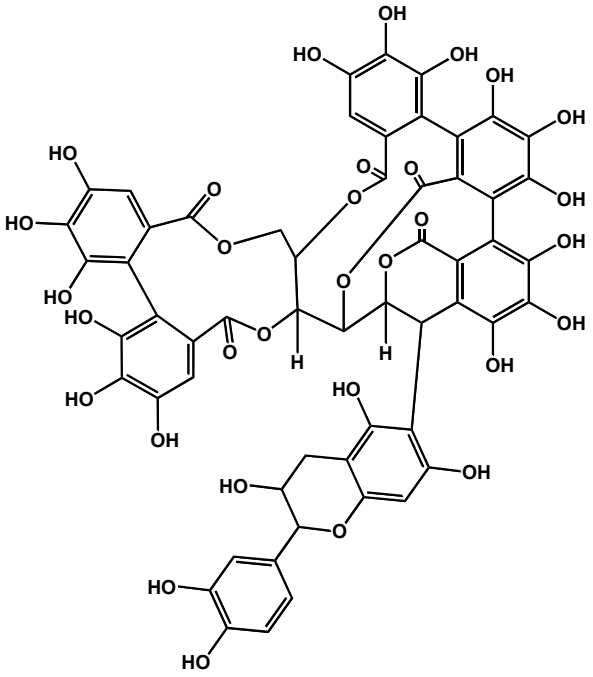
Castalagin



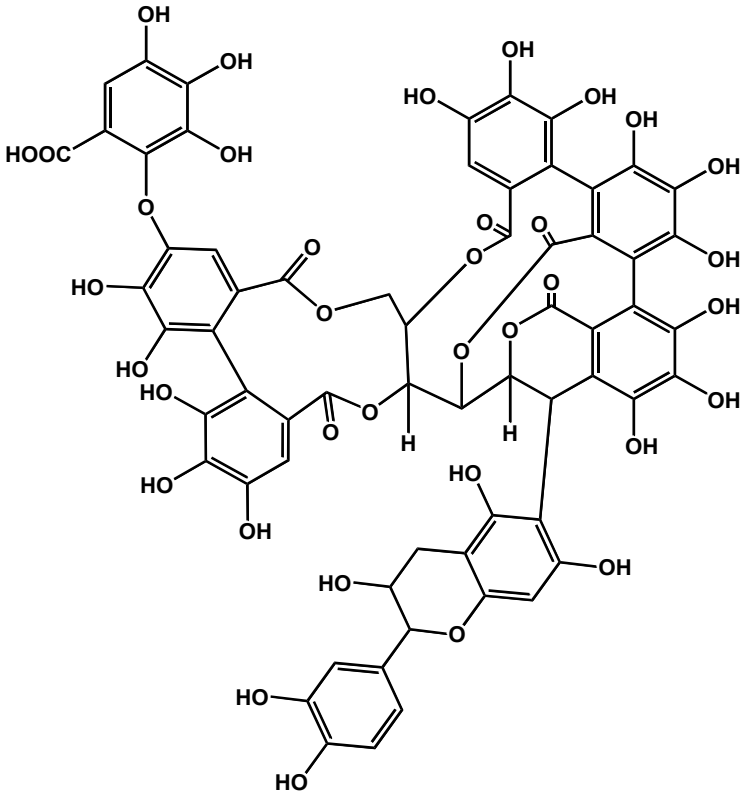
Stenophyllanin



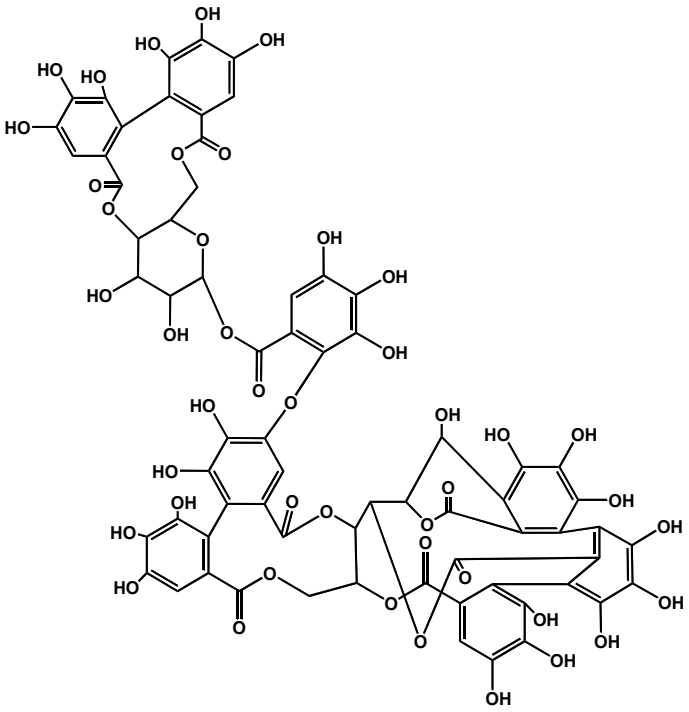
Vescavalonic acid



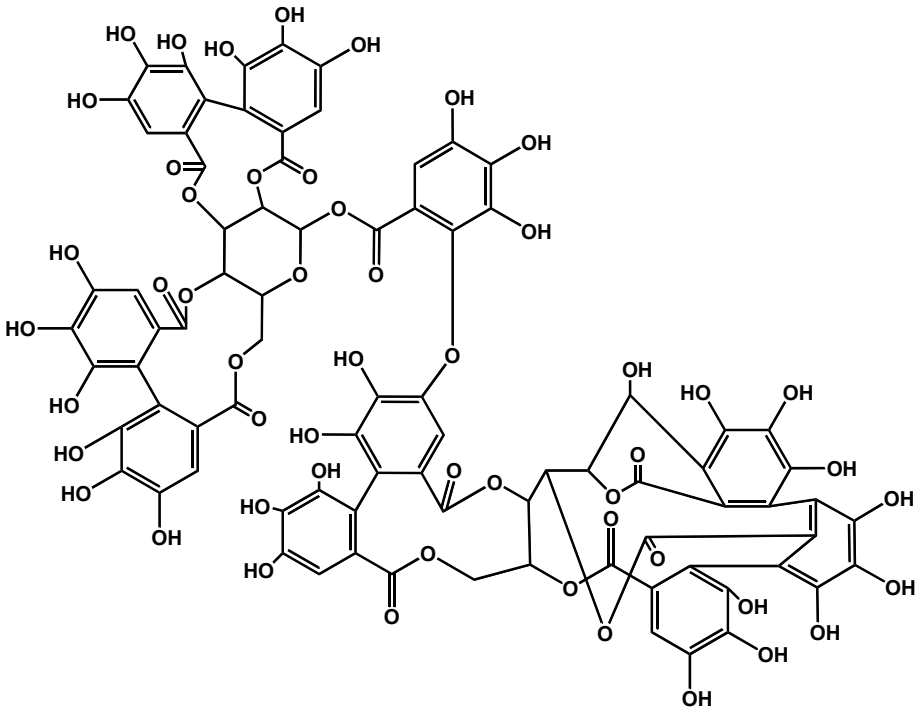
Acutissimin B



Mongolinin A

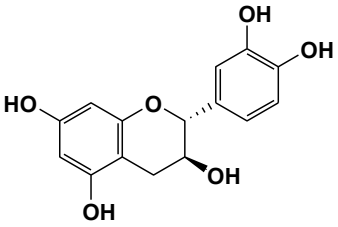


Cocciferin D3

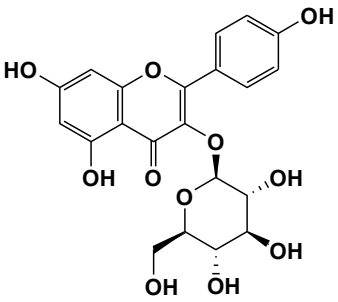


Cocciferin D2

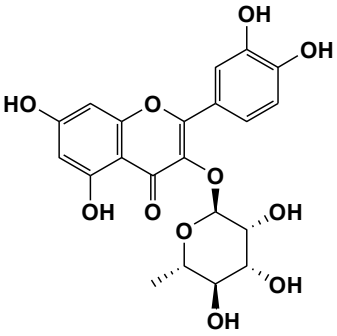
Flavan-3-ol and flavonols



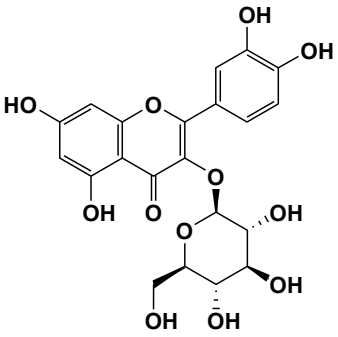
(+)-Catechin



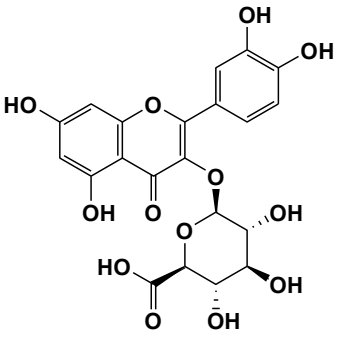
Kaempferol 3-O-glucoside



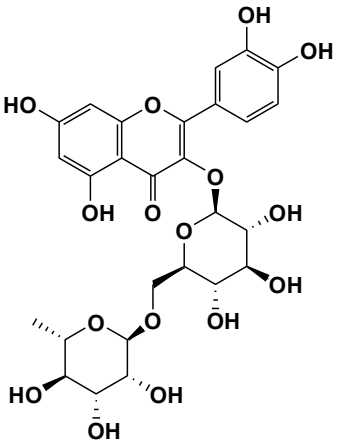
Quercetin 3-O-rhamnoside



Quercetin 3-O-glucoside



Quercetin 3-O-glucoronide



Quercetin 3-O-rutinoside

Table S1. Study locations of *Quercus oleoides*

Region / Population	Abb.	N	W	Altitude (m)
Mexico Norte	*MXN*			
Tampico North	TAMT	22° 17’ 47’’	97° 52’ 39’’	39
Tampico South	TAMV	22° 11’ 36’’	97° 49’ 46’’	53
Poza del Llano	POZ	21° 29’ 50’’	97° 49’ 12’’	12
Mexico Sur	*MXS*			
Acayucan	ACA	18° 00’ 16’’	94° 55’ 34’’	108
Sayula	SAY	17° 54’ 26’’	95° 01’ 08’’	123
Las Choapas	CHO	17° 59’ 38’’	94° 07’ 21’’	41
Costa Rica	*CR*			
Santa Elena	SAN	10° 55’ 11’	85° 36’ 40’’	283
Santa Rosa	ROS	10° 52’ 17’’	85° 35’ 49’’	278
Guachipelin	GUA	10° 41’ 56’’	85° 29’ 02’’	115

Table S2. Mean leaf herbivore damage produced by four guilds of herbivorous insects per region and population. G/L = gall / leaf ratio, G = leaf area occupied by galls, Ch = chewers, Sk = skeletonizers, Mi = leaf miners). For population abbreviations refer to table S1.

Herbivore guild		Regions / populations										
		CR				MXS				MXN		
		SAN	ROS	GUA		SAY	ACA	CHO		POZ	TAMT	TAMV
G / L		0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00
% G		0.00	0.00	0.00		0.07	0.00	0.00		0.11	0.08	0.06
% Ch		2.43	8.63	8.51		8.23	9.91	3.37		4.24	0.14	1.63
% Sk		0.18	0.81	0.32		0.53	0.44	0.22		0.19	0.02	0.09
% Mi		0.06	0.03	0.05		0.38	0.33	0.10		0.23	0.04	0.20

Table S3 Pearson correlations between leaf damage and tannin content. Herbivore damage is presented as percentage of leaf consumed (G/L = gall / leaf ratio, G = leaf area occupied by galls, Ch = chewers, Sk = skeletonizers, Mi = leaf miners). Significant correlations in bold, n = 54. For compound abbreviations, refer to Table 1.

	G / L	% G	% Ch	% Sk	% Mi
Hglu	-0.03	-0.02	0.01	0.08	0.07
DHglu	-0.07	-0.19	0.00	-0.24	-0.09
Ve	-0.22	**-0.27**	0.07	-0.04	-0.14
St	-0.10	-0.08	0.10	0.16	-0.10
VA	0.19	0.17	-0.23	-0.10	-0.07
AB	**-0.30**	**-0.31**	**0.33**	0.11	-0.11
MA	0.16	0.13	-0.03	0.12	**0.34**
CD2	0.12	0.11	0.06	0.19	0.20
CD3	0.09	0.05	0.02	0.01	0.22
Cat	-0.09	-0.16	**0.27**	0.15	0.18
Kglu	0.09	0.11	-0.19	0.01	0.17
Qrh	0.20	0.20	-0.24	0.07	-0.19
Qglu	-0.01	0.03	-0.08	-0.16	0.09
Qgln	-0.02	0.00	-0.10	0.11	0.00
Cdi	-0.22	-0.25	**0.28**	0.22	0.06
Qru	0.02	0.02	-0.07	-0.09	-0.10
Un	0.00	-0.01	0.11	-0.14	0.09

VI The pathogen cell wall component chitosan induces the synthesis of the hydrolyzable tannins Cocciferin D2 and Cocciferin D3

The pathogen cell wall component chitosan induces the synthesis of the hydrolyzable tannins Cocciferin D2 and Cocciferin D3 in the oak *Quercus oleoides*

Abstract

Few studies have characterized the response of individual plant secondary compounds that might be involved in the defensive reactions of plants against fungal attacks. Here, we evaluated the potential induction of polyphenols to simulated fungal attack, by producing artificial leaf damage and applying a solution of chitosan, a fungal cell wall component that triggers immunity reactions against pathogens on plants on one-year old saplings of the tropical oak *Quercus oleoides*. We found a set of 18 polyphenolic compounds in leaves, however, only two compounds, namely Cocciferin D2 and Cocciferin D3 increased in concentration after damage and addition of chitosan. Our methodology allowed for the detection of compounds involved in the response to fungal attack in trees, which might aid in the prospection for fungal biocontrol agents.

Keywords: Induction of foliar damage, plant secondary metabolites, plant defense against pathogens, *Quercus oleoides.*

Introduction

Although a widely accepted view of the ecological world assumes that plants have a chemical arsenal of secondary compounds to deter damage from herbivores and pathogens, few studies have screened for the specific compounds involved in the defense against fungal pathogens, which are involved in the most catastrophic outbreaks affecting plants. Across the world fungal and bacterial pathogens are involved in outbreaks affecting the production of some of the most important cultivars, thus acting as an every-day threat to the welfare of millions of people worldwide. For example, the outbreaks of the filamentous ascomycete fungi *Magnaporthe orzya*, the causal factor of the rice blast disease, might be devastating, and producers normally assume as regular losses of 10 to 30% of their annual productivity to this fungus. Another fungi, *Botrytis cinerea*, is responsible for agronomic losses over 1 billion Euros per year (Dean et al. 2012). Thus, it is critical to survey for possible biocontrol agents and to understand the mechanisms of action of plant defense against fungi.

In general, as in any immune system, the interaction between fungi and plants have been shaped by a co-evolutionary "arm race", and thus a multitude of variations of defensive systems and pathogenic modes of infection have been found across the plant and microbe realms. In general, pathogens might be differentiated by their feeding guild, whereas biotrophic and hemibiotrophic pathogens feed on living or nearly living tissues, and necrotrophic fungi feeding on dead tissues. Thus, the severity of action and the mechanisms of contagion and disease are different across guilds (Davidsson et al. 2013). As pathogenic agents might strongly compromise plant survival and fitness, plants have taken advantage of detection of microbial motifs that are highly preserved across bacteria and fungi, particularly on pathogen-associated molecular patterns, PAMPS (Malinovsky et al. 2014) to enhance defensive responses aiming to isolate or eliminate the infectious agents. In addition, plants also rely on the detection of damage associated to fungal or bacterial disease, such as

fragments of plant cell wall, or other damage-associated molecular patterns (DAMPS, Boller and Felix 2009) to improve the detection and early response against pathogens. However, it can be hypothesized that plants would acquire a selective advantage if improving the cellular and physiological response to PAMPS in relation to DAMPS to stimulate more effective defensive responses to plant disease, and it has been found that both mechanisms induce the plant immune system to prevent further infection (Jones and Dangl 2004).

Chitosan, a homopolymer of β-(1→4)-linked N-acetylglucosamine (GlcNAc) units is one of the most widely studied PAMPS, given its ubiquity in fungi (Hadwiger 2013). This ubiquity has enhanced its prospective use as an elicitor of plant defenses against pathogen attack, even at the commercial scale (Shibuya and Minami 2001). These defensive responses attributed to chitin or chitosan induction of plant defenses include the biosynthesis of terpenoid phytoalexins, reactive oxygen species, jasmonic acid and callose (Hadwiger et al. 1980; Kohle et al. 1985; Conrath et al. 1989; Ren and West 1992; Yamada et al. 1993; Nojiri et al. 1996). However, few experiments have tested which are the substances directly related to microorganisms damage, and their pace of production, in long lived plants that rely on other compounds that can be induced given tissue damage in plants, such as tannins and other polyphenols.

Tannins, particularly those from oak (*Quercus* spp.) seem to have promising antimicrobial effects. It has been shown that tannins from oaks (*Q. stellata*, Q. *havardi* and *Q. marilandica*) have stronger antimicrobial activity against human-borne pathogens, such as *Salmonella*, *Klebsiella*, *Streptococcus* and *Escherichia* (Min et al. 2008), and the set of tannins present in *Q. incana* reduces bacterial activity of the microbiome on bovine rumen (Makkar et al. 1988). In this note, a fine-grain chemical characterization of the set of polyphenols present in leaves of the tropical oak, *Quercus oleoides*, a tropical and subtropical distributed oak from Mexico to Costa Rica, and a set of experiments aiming to detect the compounds that increase in concentration following damage are presented, conveying in a methodology able to detect possible biocompounds useful for plant defense.

Material and Methods

Study Species and Sampling. Quercus oleoides (Fagaceae, subgenus Quercus, section Quercus; Schltdl. et Cham.) is distributed from the northern part of Mexico (state of Tamaulipas) to Costa Rica, mainly in coastal zones, between 0 and 500 m asl. The distribution of *Q. oleoides* encompasses a wide range of mean annual temperatures (23 - 28°C) and precipitation (700 - 3200 mm) (Valencia 2004). Three populations were selected in Costa Rica to collect acorns: Santa Elena, Santa Rosa and Guachipelin (Table 1).

Table 1. Populations of Costa Rica where acorns were obtained.

Costa Rica	*CR*			
Santa Elena	SAN	10° 55’ 11’	85° 36’ 40’’	283
Santa Rosa	ROS	10° 52’ 17’’	85° 35’ 49’’	278
Guachipelin	GUA	10° 41’ 56’’	85° 29’ 02’’	115

To avoid confounding effects due to variation in elevation and possible hybridization with other oaks, only populations occurring between 0 and 300 m asl were selected. Only trees that were scattered on the edges of forests were sampled. At each site, the geographical coordinates and elevation of the sampled individual 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.

Simulation of pathogen damage. To test if the biosynthesis of polyphenolics can be induced by pathogen cell wall components, we established a controlled experiment using acorns collected in December 2010 from the three populations of Costa Rica mentioned above. Acorns were stored at 4°C in plastic bags and were randomly planted in April 2011 under greenhouse conditions using 50 percent peat moss, 25 percent vermiculite, and 25 percent agrolite as substrate. Seedlings were grown for one year, until height and leaf number oscillated around 35 cm and 10 leaves respectively. To avoid variation in tannin content due to leaf age (Close et al. 2005), only the two youngest apical pairs of fully expanded leaves (excluding buds and young small red leaves) were used for the experiment.

Three treatments were established to study the induction of polyphenolics by pathogen cell wall components: 1) control, where no damage was produced to the leaves; 2) mechanical damage, where leaves were damaged with a metallic brush and treated with of a solution of acetic acid 1%, and 3) pathogen simulated damage where leaves were wounded with a metallic brush and treated with a solution of quitosan 0.25%. Quitosan is a potent elicitor that induces the synthesis of the Phenylalanine ammonia-lyase enzyme (PAL enzyme) which is an important enzyme for the synthesis of phenolics. Before preparing the solution, quitosan was ground into powder with a ball mill (Mixer Mill MM400 Retsch Co., Düsseldorf, Germany) and then it was dissolved in a solution of acetic acid 1%, pH was adjusted to 5.5 with NaOH. Quitosan solution was sprayed to damaged leaves avoiding spraying close leaves. The same solution without quitosan was used for pathogen control treatment. Mechanical damage was created on 10 percent of the leaf area by puncturing. The brush also damaged the cuticle and this was evident after several days following damage (the final damaged area was around 30 %). The initial concentration of polyphenols was quantified before damage (day zero) and 2, 7 and 21 days after damage. Leaves were transported under liquid nitrogen, ground into powder, and lyophilized before chemical

analysis. In all cases (i.e. factors = treatment × time), five independent plants were used as replicates.

Polyphenol identification and quantification. Leaves were placed in liquid nitrogen immediately after cutting, transported to the laboratory, and stored at -70 ºC. After finishing collecting the samples (21 days), the leaves were ground under nitrogen, lyophilized (Alpha 1-4 LD Plus, Martin Christ, Niedersachsen, Germany) and stored at -20°C in vacuum-sealed bags.

Polyphenols (hydrolyzable tannins, flavan-3-ols and flavonols) were extracted using 20 mg of leaf powder dissolved in 1.6 ml of acetone 70 % during overnight (12 hours) at 4° C using constant agitation. Samples were centrifuged 10 minutes at 13 rpm and the supernatants were placed into individual HPLC vials and acetone was evaporated under nitrogen flow. A second extraction was performed by adding 1.2 ml of fresh solvent (acetone 70%) to the pellet, samples were placed at 4° C under constant agitation for four hours. Samples were centrifuged again for 10 min at 13rpm and supernatants (1.2 ml) placed in the same HPLC vial and dried again under nitrogen blow. Finally, the combined dried extracts were re-suspended in 600 µl of a solution of methanol and acetonitrile 1:2 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). Size 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 percent B in A; 35-40 min, 40 percent B; 40-45 min, 40-0 percent B; and 45.1-50, min 0 percent B. The eluent was monitored by fluorescence detection with excitation at 280 nm and emission at 450 nm. The total mobile phase flow 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 was 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 2). Liquid chromatography on an Agilent 1200 HPLC system (Agilent 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 percent A; 1-7 min, 0-65 percent B in A; 7-8 min 65-100 percent B in A; 8-9 min 100 percent B and 9-10 min 100 percent 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, 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 proanthocyanidin 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 proanthocyanidin 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 -40V; DP -390V); for kaempferol glucoside, m/z 446.9 → 285 (CE -40V; DP -390V); for quercetin rhamnoside, m/z 446.9 → 301 (CE -40V; DP -390V); for quercetin glucuronide, m/z 476.9 →301 (CE -40V; DP -390V); for quercetin rutinoside, m/z 608.9 →301 (CE -40V; DP -390V); and finally m/z 678.9 → 517 (CE -40V; DP -390V) 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, 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.

Statistical Analyses. Pathogen simulated damage 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).

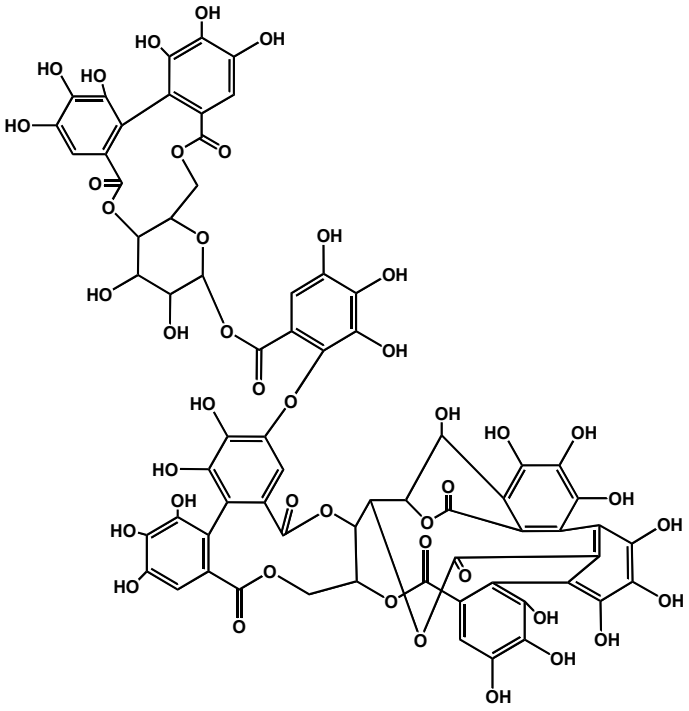
Results

A set of 18 compounds where found in leaves of *Q. oleoides* (Table 2). Although compounds widely varied in their concentration and properties, from this set, only two compounds showed evidence of induction following mechanical damage and application of chitosan, namely Cocciferin D-2 and Cocciferin D-3 (Figure 1).

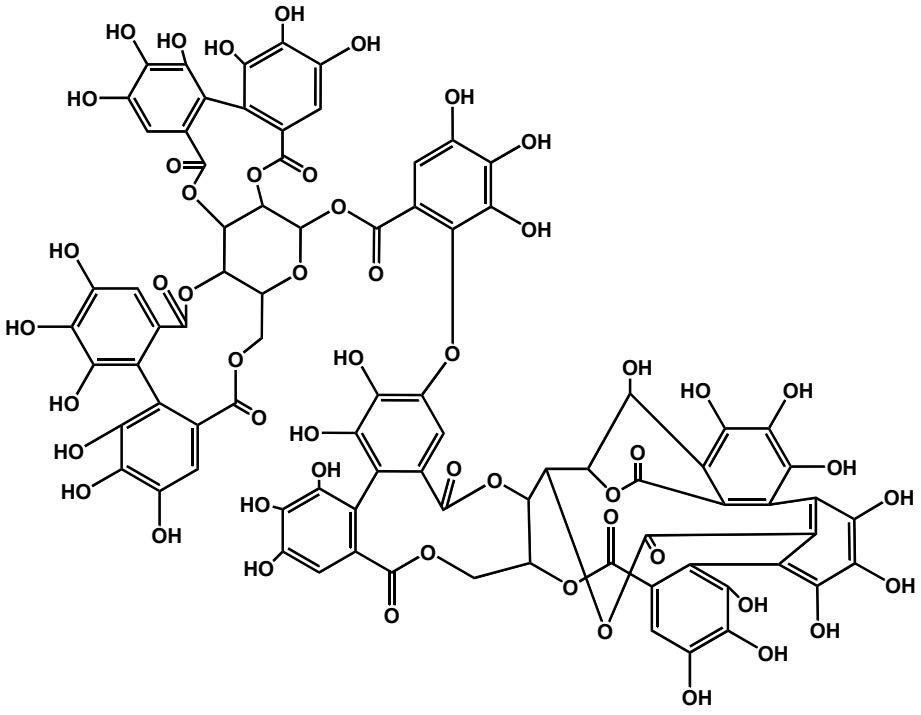
Table 2 Phenolic compounds identified in *Q. oleoides* leaf extracts.

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	CD2	---	783	30.2	1264, 1059, 932
Cocciferin D2	CD3	---	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; MS2, fragment masses used in identification



Cocciferin D3



Cocciferin D2

Figure 1. Molecular structure of the hydrolyzable tannins Cocciferin D3 and Cocciferin D2.

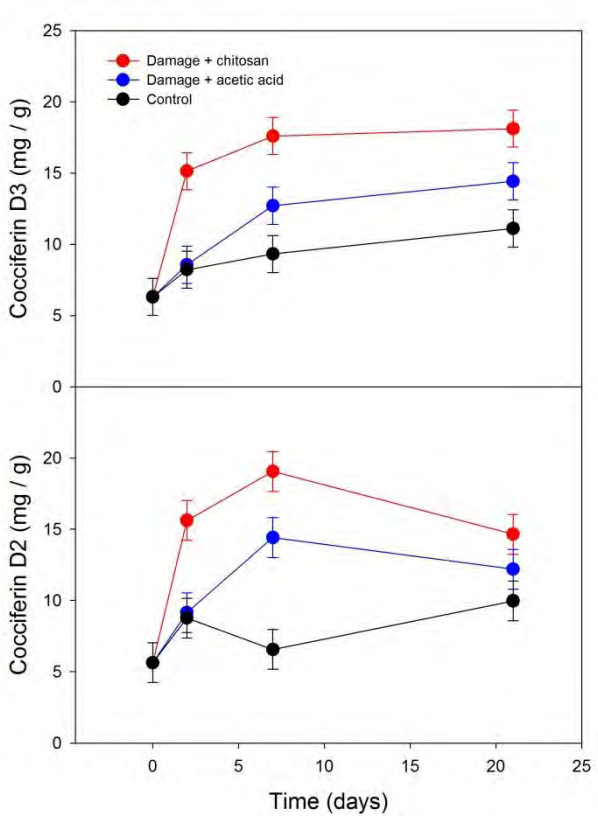


Figure 2. Differential production of polyphenols Cocciferin D2 and D3, in response to artificial damage and addition of a solution of chitosan in 1 year old saplings of the tropical oak *Quercus oleoides*.

Discussion

This note reports a temporal study that determines which compounds might be related to plant defense against pathogens. By inducing the production of polyphenols after simulating the entrance of pathogens by mechanical damage and the use of chitosan, we could determine that at least two polyphenols are reacting to the damage and presence of chitosan. This compound might be a recognition motif that plants may detect and respond to. Cocciferin is a dimeric ellagitannin that was first described in *Quercus suber* and *Quercus coccifera* from Algeria when searching for compounds that may have an antimicrobial function (Ito et al. 2002). This compound is formed by a dimer of Castavaloninic acid and Pedunculagin (Salminen et al. 2004), and to our knowledge this is the first report describing its induction by chitosan, a component of the cell wall of fungi.

We found that the concentration of both induced compounds changed in the three treatments, including our control along time, thus suggesting that these compounds are subject to ontogenetical effects. Given that leaves in this type of oaks tend to be long-lived, it is expectable that plants should aim to improve the protection of their foliar investment in mature leaves, by increasing the concentration of protective agents within tissue. This has been previously shown in *Quercus robur*, a winter deciduous species where the concentration of tannins follow a seasonal pattern to increase during late summer, which is associated with

the emergence of the winter moth, *Operophtera brumata* (Feeny 1970). In this sense, it would be expected that the same patterns should operate in evergreen species, i.e. to increase the concentration of defensive compounds in fully expanded and mature leaves, that are responsible for most photosynthetic revenues (Kikuzawa and Lechowicz, 2011). However, this steady increase in the concentration of inductible compounds may vary across time if damage is present. In this regard, our experiment also shows that there are differences in the production of defensive compounds, which vary if the damage is purely mechanical vs. mechanical + chemical. This indicates that the plants may invest in higher amounts of concentration of defensive metabolites according to the situation (also known as hypersensitive reactions, Chen 2008), where the plant might increase the amount of defensive compounds from a basal increase dependent of ontogeny, but if subject to damage, it may choose to invest towards a preventive infection (purely mechanical damage) or may invest in an otherwise inevitable infection, thus increasing their concentration of secondary metabolites at significantly higher levels than what would be expected if no motifs of fungal pathogens be present. Interestingly, the patterns of temporal variation in Cocciferin D3 shows that following the climax of infection, the plant may reduce their concentration of this metabolite. This may be due to the plant having resolved the threat of infection (no probable infection may be expected, given that the chitosan employed was obtained from crustacean exoskeletons and it had a chemical analytical degree), either by preventing the spread of the infection or by confining to a programmed cell death the sites of infection (Chen 2008). In either case, what is remarkable is the ability of plants to dynamically respond to foliar damage and probable infection by modulating the concentration of defensive compounds. Other reports (McDowell 2011, McDowell et al. 2011) have proposed that plant death is enhanced when hydraulic stress, carbon starvation and pathogen attack interact in plants across time and space. Whether the concentration of inductible compounds is dependent to the amount of stored photosynthates is a question that merits further research given the risk of trees and perennial crops to succumb to pathogen attack after drougths.

Previous reports have detected oak compound that possess antimicrobial or anti-fungal properties by bioassays designated to enhance the efficiency of widely used medical compounds, such as ketoconazole, and have found that proanthocyanidins have good anti-fungal properties (Karioti et al. 2011). The medicinal value of oak compounds has been recently described, particularly given the high phenolic content and anti-oxidant properties of oak leaf infusions (Rocha-Guzman et al. 2009; Sanchez-Burgos et al. 2013), but relatively few has been reported on the potential induction of these compounds by surrogated agents simulating infection or herbivore attack (Moctezuma et al. 2014). By comparing the response of saplings subject to foliar damage by insects vs foliar damage by pathogens, what is remarkable to point out is that the type of compound that is induced is different, whereas in insect damage a different set of phenolic compounds is induced (catechin, acutissimin, vescalagin and mongolinin, Moctezuma et al. 2014). Given the *in vivo* plant reaction towards increasing the production of Cocciferin D-2 and D-3, further research on the potential anti-fungal proprieties of these compounds would be necessary. This type of analysis may be complementary to the search for biomedical useful compounds using bioassays, and additionally may allow establishing the mechanisms of plant defense against fungal attacks.

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VII. Variation in polyphenol content across the distribution range of the tropical oak *Quercus oleoides*

Variation in polyphenol content across the distribution range of the tropical oak *Quercus oleoides*

Abstract- Latitude is one of the main drivers of phenotypic variation. Leaf traits such as leaf toughness and the content of chemical compounds are related to differences in temperature, light and insect pressure. In the present study we assessed the latitudinal variation in polyphenol content and its relation to climatic variables such as temperature and light incidence. Across three regions and nine populations that encompassed the geographic distribution of the tropical oak *Quercus oleoides* (ranging from northern Mexico to Costa Rica). We quantified leaf area and leaf mass per unit area (LMA, a surrogate for leaf mechanical resistance), as well as the composition and concentrations of hydrolyzable and condensed tannins using normal and reversed-phase high-performance liquid chromatography (HPLC), respectively. Leaf mass per unit area and the content of eight compounds significantly varied across regions, with the populations from Southern Mexico being significantly different in their chemical profile. Data on polyphenol content, temperature and precipitation were summarized by principal component (PC) analyses, and the two first PC for each set of variables were correlated using Pearson correlations. The first PC summarizing data on herbivory, and the second PC of tannin content were positively associated with the first temperature PC, with higher herbivory and tannin content in the lower latitudes, thus suggesting that increasing temperatures may enhance the strength of plant-insect interactions in our study species.

Key Words- Chemical variation, condensed tannins, Costa Rica, flavan-3-ols, flavonols, herbivory, HPLC, hydrolyzable tannins, Mexico, oak chemistry.

Resumen- Uno de los principales factores que modifican la variación fenotípica es la latitud. En este trabajo se evaluó la variación latitudinal en la composición de taninos y su relación con variables climáticas en el encino tropical *Quercus oleoides*, a lo largo de tres regiones y nueve poblaciones que engloban la distribución geográfica de la especie (desde el noreste de México hasta Costa Rica). Cuantificamos el área foliar por unidad de masa (LMA, como subrogado de la resistencia mecánica foliar), así como la composición y concentración de taninos hidrolizados y condensados mediante el uso de cromatografía líquida de alto desempeño (HPLC) en fases normal y reversa, respectivamente. El LMA, el área foliar dañada por masticadores y minadores, y el contenido de ocho compuestos varió significativamente a través de las tres regiones, siendo el Sur de México diferente en su perfil químico. La información de herbivoría, contenido de taninos, temperatura y precipitación fueron sintetizados usando análisis de componentes principales (PC), y los primeros dos componentes de cada variable fueron correlacionados usando correlaciones de Pearson. El primer PC de herbivoría y el segundo PC de contenido de taninos estuvieron asociados con el primer PC de temperatura, donde las latitudes menores tienen mayores temperaturas y mayores niveles de herbivoría y contenido de taninos, sugiriendo que los cambios climáticos

pueden modificar la fortaleza de las interacciones planta- insecto en nuestra especie de estudio.

Palabras clave – Flavan-3-oles, flavonoles, herbivoría, HPLC, Taninos condensados, Taninos hidrolizables, variación latitudinal.

One of the most critical traits in plant defense is the production of secondary metabolites, which has been classically recognized as a factor determining the performance of plants and herbivores (Shoonhoven et al 2007). Their role in plant-insect interactions began to acquire attention several years ago, and by now thousands of these compounds have been isolated and their structure determined (Seigler 1998). However, despite the wide attention that they have received, our understanding of the manner in which these compounds vary spatially throughout latitudinal gradients is still scarce. For example, the total content of phenolic compounds has been found to increase from south to north in an Australian subtropical forest (Hallam and Read 2006). However, this trend was not reflected in other presumed defensive strategies, such as an increase in leaflet toughness or glucoside concentration (Hallam and Read 2006). For example, in Acacia falcata there were not latitudinal gradients in herbivory (ranging from 3 to 5%), although leaf toughness was lower and specific leaf area higher in the lowest latitudes (Andrew and Hughes 2005), and there was no correlation of leaf mining with rainfall or latitude in different vegetation types (mallee, heath, woodland and rainforest) in Australia (Sinclair and Hughes 2008). Even more, inverse trends in herbivory have been reported by J. M. Adams and Y. Zhang (2009) as a possible consequence of "outbreaks", where the intensity of herbivory increases and are reflected by herbivory rates over 10 % in the north part of the gradient, and a series of recent meta-analyses found no relation between latitude and herbivory or traits that confer plant resistance (Moles et al. 2011, 2013). Other studies have found little evidence to indicate pervasive latitudinal gradients in the production of secondary compounds (Adams et al 2008, Moles et al. 2011, Moles et al. 2012).

The exploration of the biogeographic patterns of plant-insect interactions, in particular foliar damage due to insect attack and their associated array of plant chemical arsenals is an important open question, particularly as it has been proposed that current global temperature increments will give rise to new outbreaks, potentially increasing herbivory rates (Coley 1998). Therefore, we studied the geographic variation of chemical profiles of polyphenols of *Quercus oleoides*, a tropical oak distributed both in subtropical and tropical regions, and their association with latitude and climate. This species was chosen because there is an state-of –the-art tannin chemical characterization of its polyphenols, and because is a species that allowed us to control the effects of latitude and climate, ranging from Tamaulipas State, in Northern Mexico, to Guanacaste, Costa Rica. This wide latitudinal distribution includes a wide diversity of environments and encompasses the two main genetic groups of this species (Cavender-Bares et al. 2011). We hypothesized that there would be a differential effect of latitude and climate over the concentration of polyphenols which will allow predicting how climatic variations would influence communitarian and ecosystemic processes.

Methods and Materials

Study Species and Sampling- Quercus oleoides (Fagaceae, subgenus Quercus, section Quercus; Schltdl. et Cham.) is distributed from the Mexican state of Tamaulipas to Costa Rica, mainly in coastal zones, between 0 and 500 m. 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 the northern Gulf of Mexico, three in the southern Gulf of 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 and the geographical coordinates and elevation of the sampled individual were recorded at each site. There was a minimum distance between populations of 10 km and a minimum distance between trees of 10 m. The sampling was conducted at the end of the rainy season, from November to December 2009. To standardize the sampling, selected trees were 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 was obtained from Moctezuma et al. (in prep.) for four herbivore guilds on six individual trees per population. From each individual, approximately 30 leaves on sun-exposed and south-facing branch at a height of 4 m were randomly selected, transported from the field in coolers and scanned at a resolution of 300 dpi on the same day using an Epson Perfection V700 Photo scanner. Leaf damage by each type of herbivore was calculated using WinFOLIAPro software (2009 Regent Instruments Inc.). Damage was classified in four herbivore guilds: chewers, skeletonizers, miners and gall-forming insects (Cranshaw 2004, Adams and Zhang 2009).

Quantification of Leaf Area and Leaf Mass per Area (LMA)- From the same set of 30 leaves that were used to measure the amount of foliar damage, we calculated the leaf area and leaf mass per unit area (LMA). Leaf area was calculated with WinFOLIAPro software. After collection, leaves were oven-dried at 70 °C for a minimum of three days, and the LMA was calculated as the dry mass in g / leaf area in cm2 (Cornelissen et al. 2003).

Extraction and analyses of phenolic compounds- A detailed account of the chemical methods to extract, purify and analyze phenolic compounds can be found in Moctezuma et al. (in prep.). Briefly, the concentration of phenolic compounds was quantified in ten randomly selected fully expanded leaves from the same branch that was used to assess foliar damage. Leaves were placed in liquid nitrogen after cutting and were lyophilized (Alpha 1-4 LD Plus, Martin Christ), pulverized (Mixer Mill MM400 Retsch Co., Germany), and stored at -20 °C in vacuum-sealed bags until extraction. Phenolics were extracted following Moctezuma et al. (in prep.) using acetone and methanol. Hydrolyzable tannins were analyzed by high performance liquid chromatography-fluorescence detection and tandem mass spectrometry (LC-FLD and LC-ESI-MS), 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). Flavan-3-ols and flavonols were analyzed by reversed liquid chromatography-tandem mass spectrometry (LC-ESI-MS-MS), using an Esquire 6000

ESI ion-trap mass spectrometer after separation by liquid chromatography on an Agilent 1200 HPLC system (Agilent Technologies; Santa Clara, CA, USA), using a 50 X 4.6 mm XDB C18 column with a particle size of 1.8 μm (Agilent). From these analyses a dataset of 17 compounds was found, from which 16 unique phenolic compounds were successfully identified (Moctezuma et al. in prep.). This same set of compounds was present in all the populations and regions. From this set of compounds, nine were hydrolyzable tannins / ellagitannins and eight were flavan-3-ols and flavonols (including a total of 23 structural isomers), one of which could not be identified (Table 1, Moctezuma et al. in prep.).

Climatic database - A set of eleven temperature-related variables and eight precipitation related variables were obtained from WorldClim climate database (http://www.worldclim.org/bioclim, Hijmans et al., 2005). These variables were extracted from each population using Diva GIS (http://www.diva-gis.org/), at a resolution of 1 × 1 km2. Current climatic conditions were extracted from WorldClim global grids in the generic format, and this was converted to bmp format to be read in Diva GIS. After this procedure, climatic data was extracted manually using the 19 layers of climate.

Statistical Analyses- Prior to the analyses, data was ln-transformed to improve normality and homogeneity of variance, with the exception of gall damage per area and weight, which were (ln-transformed + 1). The herbivory, leaf area, and LMA, and the concentration of hydrolyzable tannins, flavonols and flavan-3-ols were compared across populations and regions by means of one-way nested ANOVAs (d.f. = 2, 8), where populations where nested within regions. These analyses were followed by comparisons of the means between the three regions (Costa Rica, Southern Mexico and Northern Mexico) using Tukey's honestly significant difference post-hoc tests. The patterns of covariation in tannin content and the amount of leaf damage per each herbivore guild were summarized using principal component analysis (PCA) on the correlations, where the scores of the first and second principal components of the PCA were extracted to compare their variation across regions. In addition, eleven temperature-related variables and eight precipitation-related bioclimatic variables were also summarized (WorldClim climate database, Hijmans et al., 2005) by PCA, and the first two principal components of the tannin, herbivory and climatic dataset were correlated using Pearson correlations. To detect clustering patterns at the regional scale, a hierarchical clustering algorithm was employed per population, using the Ward's method to calculate the standardized distance between clusters, using JMP 8.0, and the results were subsequently displayed using a dendrogram.

Results

Across populations and regions, there were significant differences in leaf area, LMA (Figure 1), and herbivory from chewers, leaf miners and galls (Moctezuma et al. in press). The concentration of four hydrolyzable tannins and five flavonols/flavan-3-ols significantly differed across regions (Figure 3, Table 2). The first principal component that summarized the covariation among the 17 compounds significantly varies across regions, with southern Mexico significantly being different from Costa Rica and northern Mexico ($F = 7.93$, $P <$

0.001, Figure 3, Figure S2). Populations were also clustered by their tannin profiles using UPGMA with Ward's method for calculating cluster distances and, concordant with the PCA, only Southern Mexico populations were clustered together (Figure 4).

To understand the relation between climatic variation and the patterns of herbivory and foliar defense, we summarized the variation in herbivory previously reported in Table S2 from Moctezuma et al. 2014 which describes the percentage of herbivory of ten trees from nine populations across the range of distribution of our study species regarding four types of herbivore guilds, vs the foliar tannin content and environmental variables by using PCAs, and we extract the first two PC to explore the relationships between summarized variables. We found that the first PC of herbivory and the second PC of tannin content were significantly associated with the first PC summarizing eleven temperature variables. This association implies that the lowest levels of herbivory and overall tannin content are present in sites with lowest temperature values (Figure 5). No significant correlations were found between herbivory and tannins PCs with precipitation PCs.

Discussion

As shown by our results, latitude and temperature seems to be negatively associated with the levels of foliar damage and the concentration of polyphenols in our study species. This implies that lower latitudes have higher amounts of certain foliar damage and certain secondary metabolites. These issues are currently subject to controversy, as the previously prevalent notion that lower latitudes harbor higher intensity in herbivory or secondary metabolite production has been subject to scrutiny, both using study cases and meta-analyses (Schenke et al. 2009, Adams and Zhang 2009, Moles et al. 2011). As our results suggests, not all the compounds are subject to geographical clines. It is expectable that herbivory pressure to be niche dependent (Thompson 2005), where latitude will determine variation in the amount of damage due to different types of herbivores, and as a response to this and to other environmental pressures imposed by latitude and temperature, to trigger the production of secondary metabolites.

Variation in tannin concentration, foliar damage and functional traits along the latitudinal gradient. For eight out of 17 compounds and for chewers, the herbivore guild that was responsible for most foliar damage (Moctezuma et al 2014), there is concordance with previous reports that reported higher levels of damage and higher concentration of secondary metabolites at lower latitudes. In contrast, in nine out of 17 compounds and in three herbivore guilds, we were unable to find latitudinal trends. Even more, we were unable to find gall producing insects in Costa Rica, a remarkable fact that could be a consequence of a strong biogeographic barrier that also modifies oak genetics (Cavender-Bares et al. 2011). The fact that Costa Rica, the southern-most region of our study, had no gall damage and significantly less leaf mining is in marked contrast to reports that convey higher herbivory pressure towards the tropics. It is surprising although, that the chemical profile was only significantly different in southern Mexico, as reflected both by the PCA and by the similitude analysis. This area is characterized by a high prevalence of rainfall throughout the year, in contrast with northern Mexico and Costa Rica, which are seasonally dry tropical and subtropical

forests. Even more, we consider that the importance of tannins in plant defense is not equal among the different compounds, and some compounds should be taking a more important role in plant defense than others (Moctezuma et al. in prep.). Thus, although other factors may be determinant in the production of the bulk of tannins, they are secondarily influenced by climate, as depicted by the fact that the second PC of tannins was significantly associated with temperature. Given the variation across populations in terms of herbivory and tannin concentration, we might expect that foliar resistance and defense is varying across sites following a mosaic pattern (Thompson 2003), where some local herbivores modulate the variation of the chemical phenotype towards a single type of chemical signature within the possible ontogenetic repertoire of the species. This repertoire seems to be more ample in tropical sites, which may be possible given the longer term persistence of insects and leaves, thus predisposing to a wider array of possible interactions between plants and insects (Moles et al. 2011a)

We also find that leaf area and LMA varies according to latitude, where Costa Rica had a lower area and higher LMA than the other two regions. The *Q. oleoides* populations from Costa Rica are located within the dry tropical forest, and they are among the few evergreen species within those forests. Being evergreen in a dry tropical forest should impose restrictions in the water use of this species, a fact that should be reflected in the leaf construction and architecture. In fact, *Q. oleoides* is considered among the most drought tolerant species in the area, being able to preserve its leaves even during extreme drought (Brodribb and Holbrook 2005). How the interaction between drought tolerance and resistance against herbivore attack operates remains an interesting open question. Future surveys should integrate more precise analytical chemical techniques to evaluate the pervasiveness of these patterns and solve the controversial basis of the latitude-dependent gradient of herbivory and plant defenses.

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Table 1. Chemical variation in *Q. oleiodes* among regions and populations. Nested ANOVA effects for 3 regions (Costa Rica, South Mexico, North Mexico), each region including 3 populations**.**

Polyphenols		**Region**			**Populations**		
	Abr.	**SS**	***F***	***P***	**SS**	***F***	***P***
Hydrolyzable tannins							
Hexahydroxydiphenoyl-glucose		2.21	1.54	0.22	**11.03**	**2.57**	**0.03**
Di-hexahydroxydiphenoyl-glucose		1.38	0.35	0.7	3.34	0.28	0.94
Vescalagin		**30.41**	**4.29**	**0.019**	15.75	0.74	0.61
Stenophyllanin		**54.09**	**8.15**	**< 0.001**	17.91	0.90	0.50
Vescavalonic acid		**16.60**	**5.22**	**0.009**	17.87	1.87	0.11
Acutissimin B		**407.5**	**8.24**	**0.009**	**339.6**	**2.28**	**0.05**
Mongolinin A		69.76	1.31	0.28	190.8	1.19	0.32
Cocciferin D2		0.07	0.05	0.95	6.09	1.61	0.16
Cocciferin D3		13.86	2.8	0.07	20.49	1.38	0.24
Flavan-3-ols and flavonols							
Catechin		**106.4**	**12.75**	**< 0.001**	33.92	1.35	0.25
Kaempferol glucoside		1.18	2.8	0.07	1.23	0.97	0.46
Quercetin rhamnoside		**2.79**	**3.84**	**0.03**	**6.23**	**2.85**	**0.02**
Quercetin glucoside		**98.97**	**3.52**	**0.04**	103.7	1.23	0.31
Quercetin glucuronide		6.77	1.08	0.35	15.71	0.83	0.55
Catechin dimer		**7.87**	**6.06**	**0.005**	4.51	1.16	0.35
Quercetin rutinoside		0.12	1.0	0.38	0.29	0.83	0.55
Unknown		**0.35**	**8.51**	**< 0.001**	**1.08**	**8.51**	**< 0.001**

FIGURE LEGENDS

Figure 1. Leaf area and leaf mass per area (LMA) in *Q. oleoides* in three regions. CR = Costa Rica, MXS = Southern Mexico, MXN = Northern Mexico.

Figure 2. Hydrolyzable tannin, flavon-3-ol and flavonoid content in *Q. oleoides* in three regions. The first and second Principal components summarizing the variation in chemical profiles were compared by one way ANOVA. CR = Costa Rica, MXS = Southern Mexico, MXN = Northern Mexico.

Figure 3. Principal component analysis (PCA) synthesizing the concentration of 18 flavan-3-ols and flavonols and nine hydrolyzable tannins of 54 individuals of three regions encompassing the distribution of *Q. oleoides*. Triangles = Costa Rica (CR), Empty circles = Southern Mexico (MXS), Filled circles = Northern Mexico (MXN). The PCA- scores of MXS were significantly different from those of MXN and CR ($F = 7.93$, $P = 0.001$).

Figure 4. Hierarchical grouping of nine populations of the tropical oak *Q. oleoides,* made on the basis of their tannin chemical composition. Clusters were obtained using the un-weighted pair group method (UPGMA), using Ward's minimum variance method for calculating standardized distance between clusters. For population abbreviations, refer to Table S1.

Figure 5. Relationships between the first principal components summarizing four types of herbivory, polyphenol content (second principal axis) and eleven bioclimatic traits related with temperature of *Q. oleoides* in three regions. CR = Costa Rica, MXS = Southern Mexico, MXN = Northern Mexico.

Fig. 1.

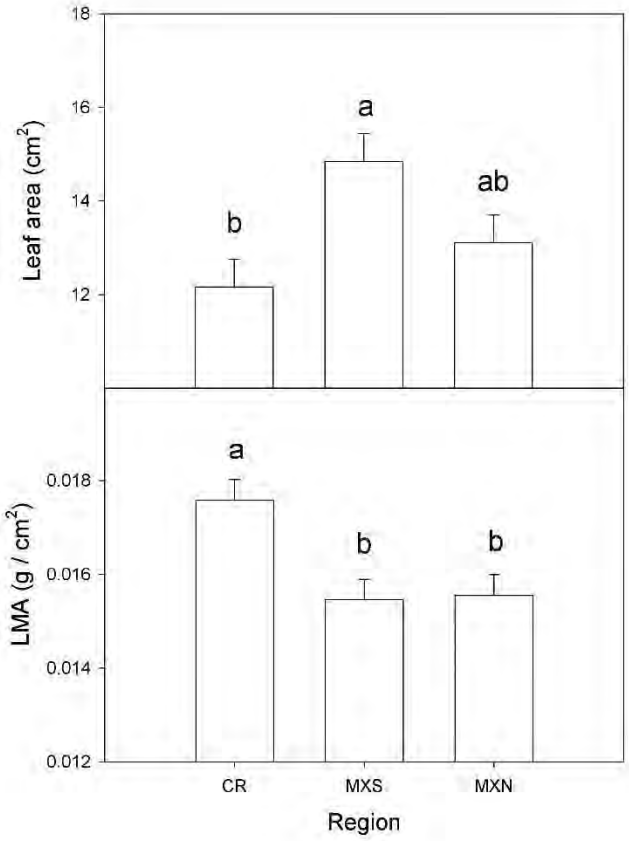
18
16
14
12
Leaf area (cm²)
a
ab
b
0.018
0.016
0.014
0.012
LMA (g / cm²)
a
b
b
CR
MXS
MXN
Region

Figure 2.

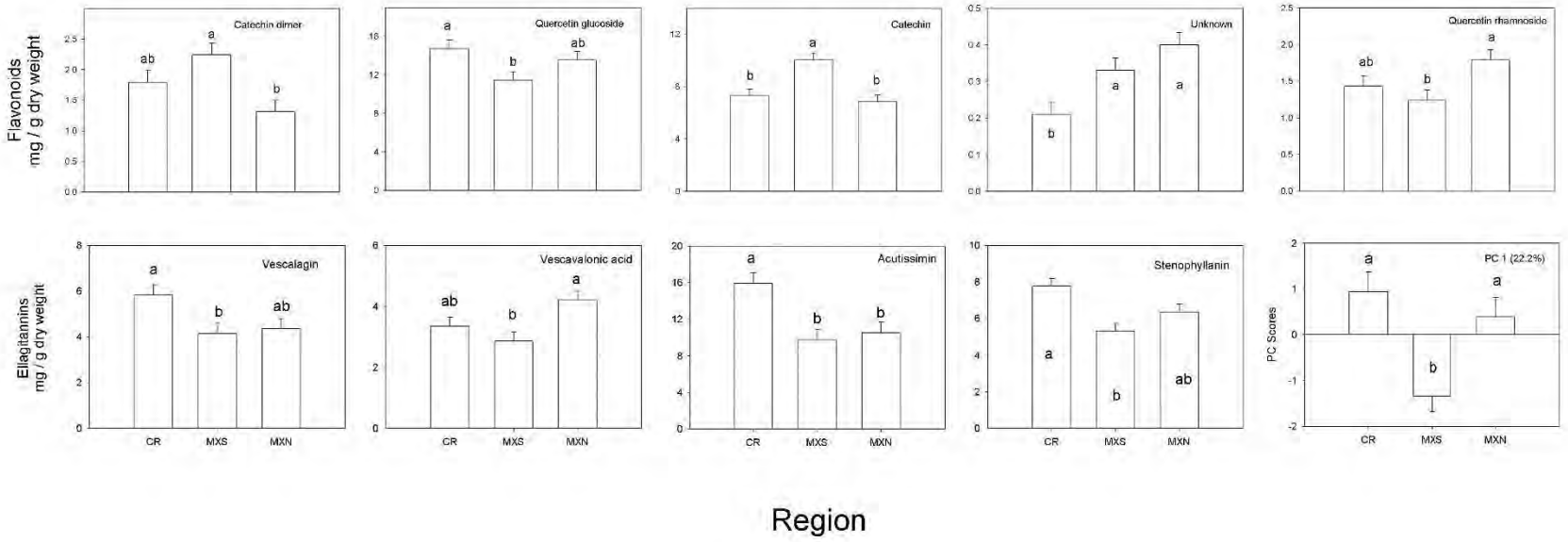
Flavonoids
mg / g dry weight
Catechin dimer
Quercetin glucoside
Catechin
Unknown
Quercetin rhamnoside
Ellagitannins
mg / g dry weight
Vescalagin
Vescavalonic acid
Acutissimin
Stenophyllanin
PC 1 (22.2%)
PC Scores
CR
MXS
MXN
Region

Figure 3.

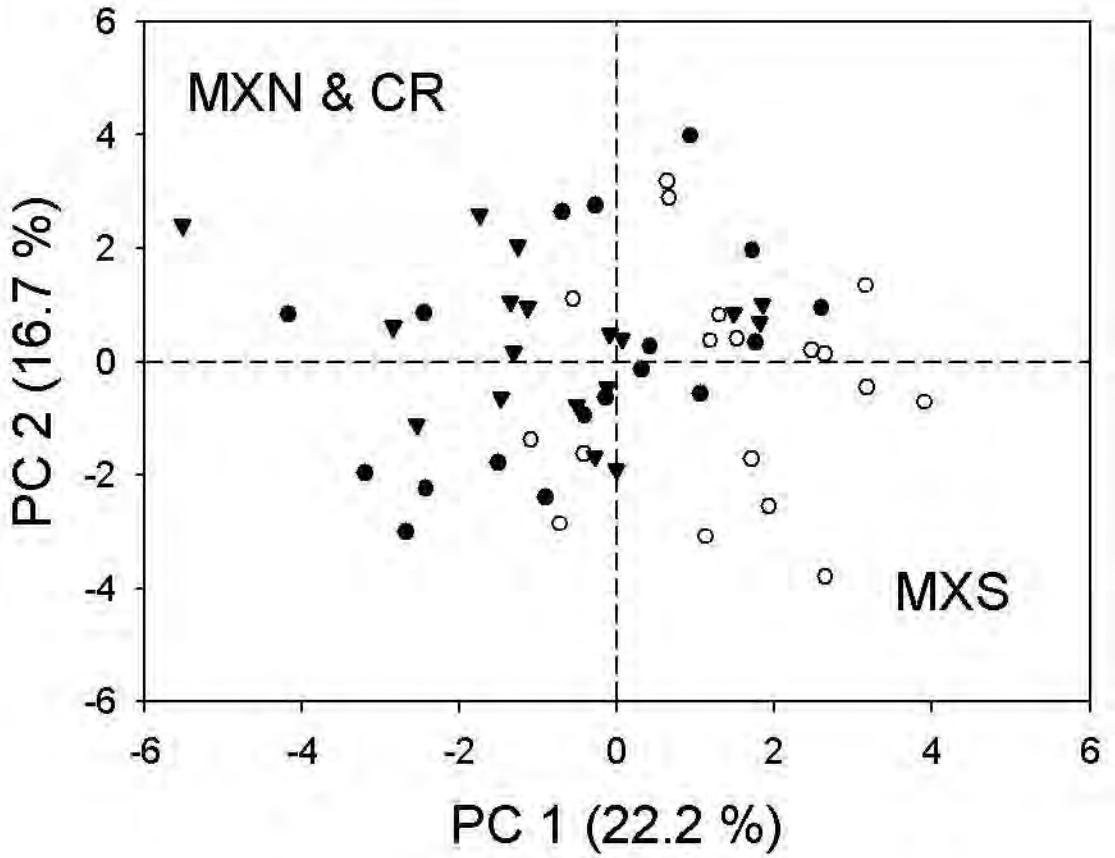

Fig. 4.

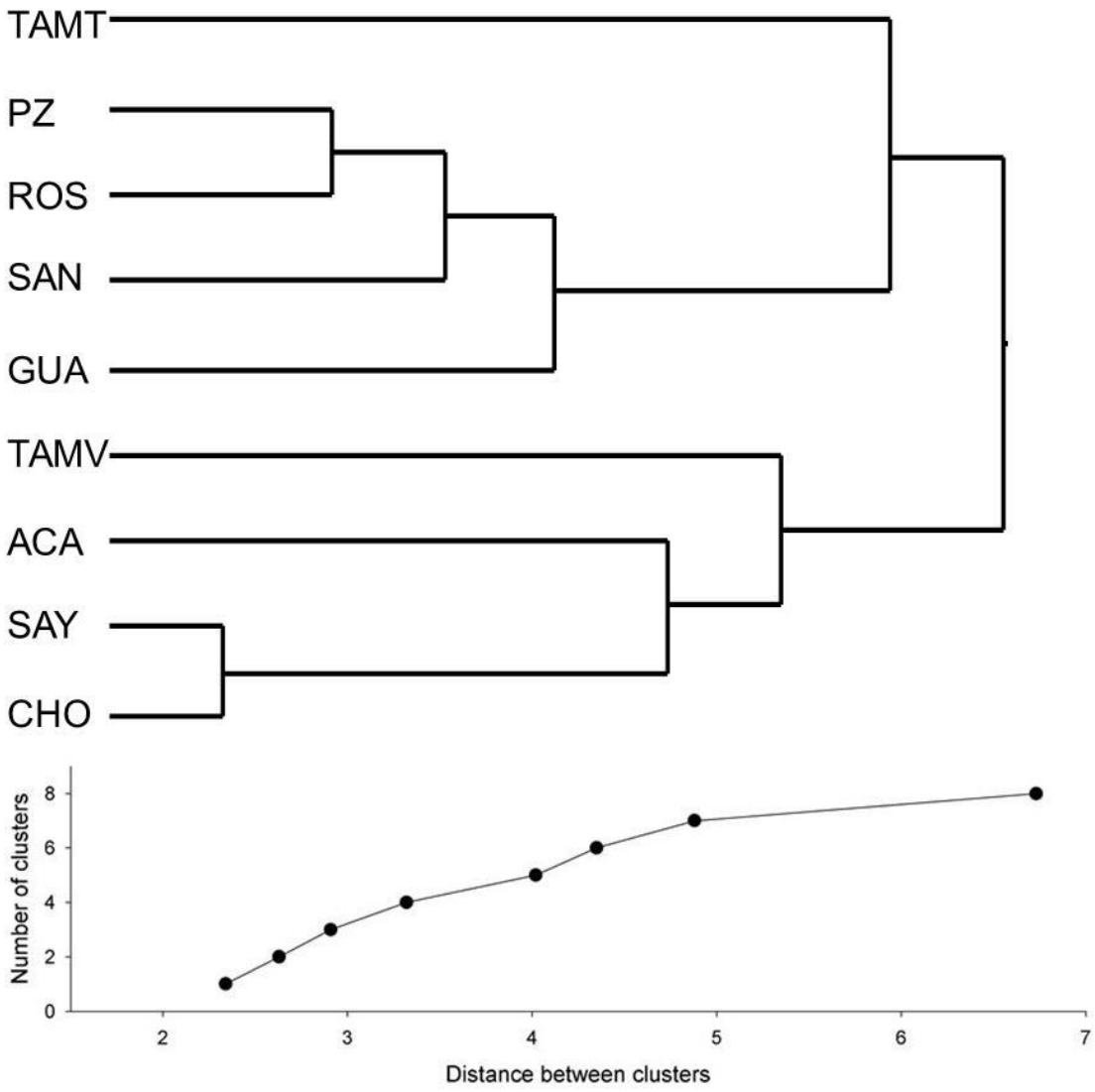
TAMT
PZ
ROS
SAN
GUA
TAMV
ACA
SAY
CHO
Number of clusters
0
2
4
6
8
2
3
4
5
6
7
Distance between clusters

Fig 5.

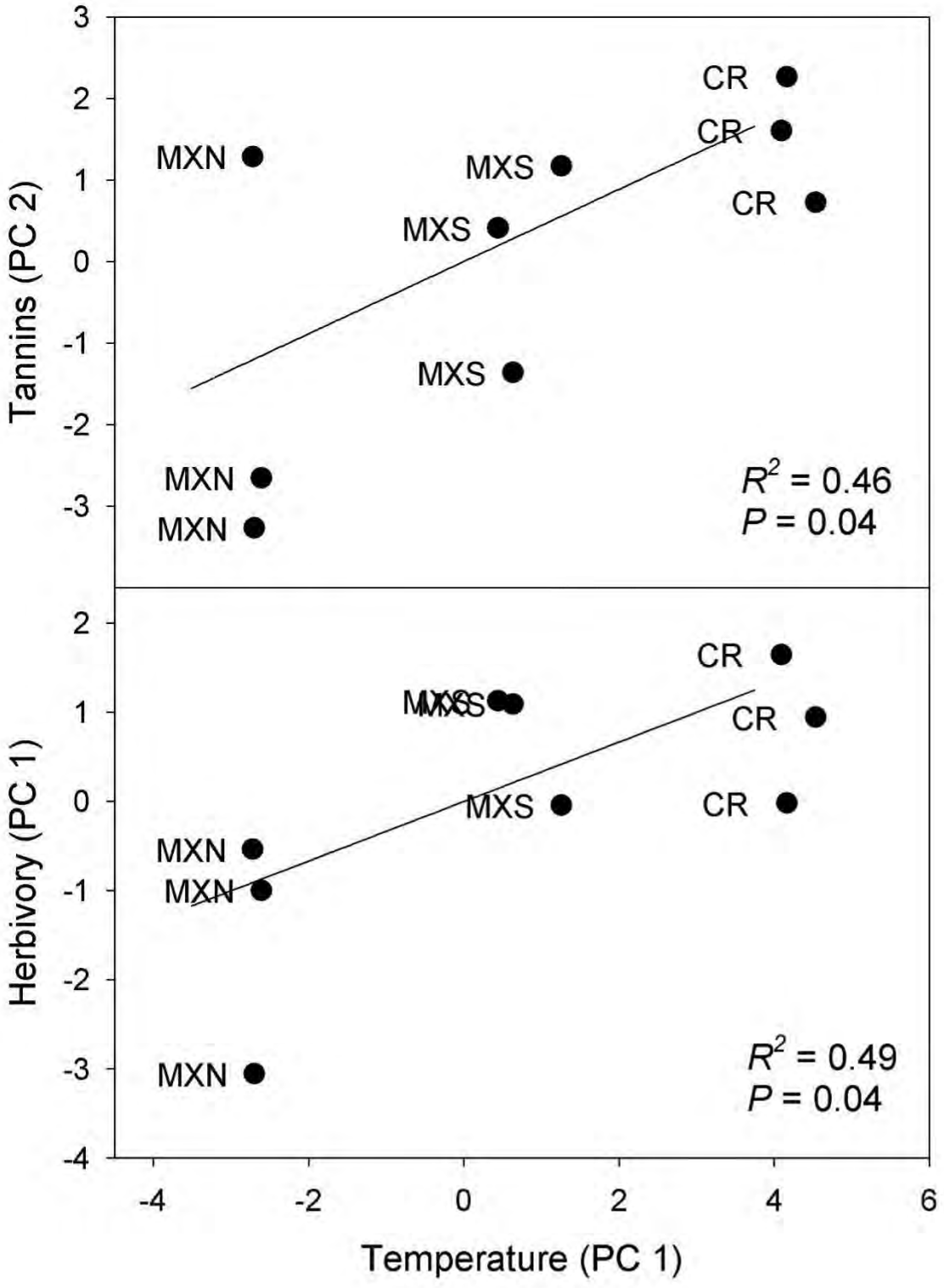
Tannins (PC 2)
Herbivory (PC 1)
Temperature (PC 1)
CR
CR
CR
MXS
MXS
MXS
MXN
MXN
MXN
R^2 = 0.46
P = 0.04
R^2 = 0.49
P = 0.04

Table S1 Studied locations of *Quercus oleoides*

Region / Population	Abb.	N	W	Altitude (m)
Mexico Norte	*MXN*			
Tampico North	TAMT	22° 17’ 47’’	97° 52’ 39’’	39
Tampico South	TAMV	22° 11’ 36’’	97° 49’ 46’’	53
Poza del Llano	POZ	21° 29’ 50’’	97° 49’ 12’’	12
Mexico Sur	*MXS*			
Acayucan	ACA	18° 00’ 16’’	94° 55’ 34’’	108
Sayula	SAY	17° 54’ 26’’	95° 01’ 08’’	123
Las Choapas	CHO	17° 55’ 49’’	94° 09’ 05’’	41
Costa Rica	*CR*			
Santa Elena	SAN	10° 55’ 11’	85° 36’ 40’’	283
Santa Rosa	ROS	10° 52’ 17’’	85° 35’ 49’’	278
Guachipelin	GUA	10° 41’ 56’’	85° 29’ 02’’	115

Table S2 Mean content (mg/g dry weigth) of hydrolyzable and condensed tannins per region and population. For compound abbreviations, refer to table 1, for population abbreviations refer to table S1

Compound	Region / Population								
	CR			MXS			MXN		
	SAN	ROS	GUA	SAY	ACA	CHO	POZ	TAMT	TAMV
Hglu	2.63	3.03	3.81	2.78	2.38	3.23	2.77	3.26	2.03
DHglu	2.84	2.48	2.47	2.99	3.31	2.50	2.77	3.23	2.81
Ve	6.16	4.82	6.55	4.36	4.27	3.82	5.07	3.82	4.24
St	7.14	7.24	8.93	5.25	5.48	5.26	7.00	6.42	5.65
VA	3.08	2.96	4.02	2.79	2.91	2.87	3.19	5.32	4.10
AB	15.60	19.12	13.07	9.57	9.76	9.94	15.25	6.65	9.67
MA	8.91	8.78	8.92	12.94	8.09	13.80	9.27	7.61	12.59
CD2	1.24	1.98	1.57	1.70	1.35	1.95	1.99	1.07	1.98
CD3	1.78	1.34	1.74	3.25	2.43	2.55	2.66	1.41	3.82
Cat	7.53	7.73	6.70	10.22	9.54	10.39	8.51	5.50	6.61
Kglu	1.11	0.72	1.00	1.17	0.80	0.83	1.35	1.09	1.32
Qrh	1.05	1.45	1.80	1.08	1.51	1.14	1.38	2.44	1.55
Qglu	16.72	12.89	14.59	13.32	9.69	11.36	13.69	14.76	12.19
Qgln	4.88	4.57	4.49	3.49	3.15	5.05	4.43	3.83	3.45
Cdi	1.89	2.02	1.47	2.43	1.83	2.46	1.76	0.97	1.18
Qru	0.62	0.44	0.53	0.40	0.54	0.33	0.60	0.49	0.48
Un	0.19	0.24	0.20	0.38	0.24	0.39	0.26	0.21	0.73

Table S3 Mean leaf area and mean leaf mass per unit area per region and population. For population abbreviations refer to table S1

Trait	Regions / populations								
	CR			MXS			MXN		
	SAN	ROS	GUA	SAY	ACA	CHO	POZ	TAMT	TAMV
LA	10.55	12.78	13.15	14.06	14.53	15.95	10.20	14.87	14.24
LMA	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.01	0.02

VIII. DISCUSIÓN GENERAL Y CONCLUSIONES

En esta tesis se evaluó el efecto del daño foliar producido por diferentes tipos de agresores sobre la producción de varios compuestos fenólicos en el encino tropical *Quercus oleoides*. En términos generales, se encontró que no todos los compuestos cumplen la misma función, dado que algunos compuestos incrementan su producción en respuesta al daño producido por agentes químicos relacionados con patógenos (quitosán), otros en respuesta al daño producido por herbivoría y otros más por diferentes efectos, tales como la ontogenia o el desarrollo de mayor masa foliar por unidad de área. Se encontró además que hay gran variación tanto del daño como de los perfiles químicos a través del ámbito de distribución de la especie. En conjunto, esta investigación permite relacionar la alta diversidad estructural y química de los compuestos fenólicos con diferentes funciones en *Quercus oleoides*.

Para determinar si los taninos presentan un rol defensivo en las interacciones planta-herbívoro y planta-patógeno, en esta tesis fue indispensable el uso de técnicas analíticas adecuadas. Esto se realizó porque las cuantificaciones colorimétricas de fenoles, taninos condensados y taninos hidrolizables totales, la cuales han sido ampliamente utilizadas, hasta el momento no han logrado esclarecer el tipo de relación que hay entre los polifenoles y los niveles de herbivoría y la riqueza de herbívoros en las plantas (Forkner et al. 2004, Adams et al. 2009). Por ejemplo, Appel et al. (2001) midieron la cantidad de taninos en 16 especies leñosas que sabían tenían contenidos diferentes de taninos debido a la especie o a la temporada colectadas. Las técnicas de Folin (colorimétricas) no permitieron ver las variaciones esperadas. Algunos autores hacen hincapié en el uso de técnicas analíticas como la cromatografía líquida y la espectrometría de masas, ya que son herramientas poderosas que proporcionan información altamente detallada sobre la naturaleza de estos compuestos (Rautio et al. 2007, Allwood y Goodacre 2009).

Aunque en esta tesis no se realizaron experimentos de inducción en individuos adultos, se encontró que la mayoría de las plantas adultas estudiadas en campo tuvieron el mismo conjunto de compuestos fenólicos que las plantas jóvenes a las cuales se les realizaron los experimentos de inducción. Los mismos picos de cromatografía que se pudieron resolver exitosamente se encontraron tanto en plantas jóvenes como en los individuos adultos. Esto parece indicar que la diversidad de compuestos fenólicos en esta especie es un carácter conservado entre poblaciones y a lo largo de la ontogenia de la especie, ya que en todas las poblaciones tanto de adultos como de juveniles se identificaron los mismos tipos de compuestos.

Un aspecto que se trató de estandarizar lo más posible en esta tesis fue el estado fenológico de los individuos adultos seleccionados, que ocurrió durante la fase de producción de bellotas, que en esta especie corresponde al invierno en el subtrópico (Tamaulipas) y a la temporada de secas en la región tropical (Veracruz y Costa Rica). Sin embargo, por no haber información previa, no conocemos sobre la diversidad de herbívoros asociados a esta especie de encino y menos aún de su variación fenológica. Es posible que algunas poblaciones hayan estado sujetas al ataque de diferentes grupos de herbívoros durante la estación seca-invernal y que esto haya ocasionado en parte la tasa de consumo foliar diferencial debida a los cuatro gremios de herbívoros estudiados. Sin embargo, esta especie es perenifolia y no tira todas sus hojas durante la temporada seca, incluso en sitios de bosque tropical caducifolio, como en Guanacaste, Costa Rica. Por poseer hojas no

caducifolias, es probable que las hojas estudiadas en esta tesis reflejen la historia foliar por períodos largos de tiempo, por lo tanto, pueden reducir el efecto de la variación fenológica de los herbívoros por estación. Por otra parte, también es esperable que la composición de herbívoros sea variable a través de las poblaciones, pues cada población presentó variación en precipitación y temperatura que puede influir en la riqueza de insectos asociados a cada población, y más aún, por tratarse de áreas geográficas diferentes (Costa Rica pertenece al área biogeográfica Neotropical y las poblaciones de Tamaulipas estarían insertadas en el Neártico). Estos factores podrían explicar la variación en los patrones de daño foliar a través de las distintas poblaciones y regiones.

La variación geográfica también puede haber influido en el éxito de germinación de las semillas, el cual fue muy variable entre poblaciones. Del mismo modo que se pretendió estandarizar lo más posible la selección de sitios e individuos, el tratamiento de las semillas fue estándar, donde cada vez que se colectaron las bellotas se aplicó un tratamiento preventivo con insecticidas y con fungicidas. Desafortunadamente, cuando se encontraron las bellotas, particularmente del norte de Veracruz y Tamaulipas, estas presentaban altas tasas de ataque por coleópteros curculiónidos. Esto contribuyó de manera importante a reducir el éxito de la germinación de las semillas colectadas en esta región, con lo cual se tuvo que depender de semillas de otras regiones para realizar los experimentos de inducción. Por último, un aspecto desafortunado es que las condiciones sociales de algunas regiones estudiadas limitaron el número posible de visitas para colectar semillas de las diferentes poblaciones, puesto que se planearon visitas anuales a las diferentes poblaciones para realizar colectas repetidas, pero las condiciones de seguridad no fueron óptimas para poder realizar dicho diseño. Sin embargo, aún con estas dificultades, existen maneras alternativas de poder realizar estudios futuros que incrementen nuestro conocimiento de la naturaleza de interacción entre herbívoros y encinos en México.

Para escalar esta investigación se podrían utilizar otras aproximaciones metodológicas complementarias. Por ejemplo, las cuantificaciones de polifenoles podrían ir acompañadas de bioensayos con herbívoros nativos. Estos tipo de estudios son recomendables porque permiten realizar experimentos con especies de herbívoros adaptados a consumir la planta de interés, lo que daría resultados más precisos. Esto se intentó abordar durante la fase inicial de esta tesis, es decir, se intentó criar herbívoros nativos para posteriormente relacionar el consumo de hojas de plántulas con la producción de varios compuestos fenólicos (experimento de inducción). Se colectaron larvas de lepidópteros que se encontraron consumiendo hojas de *Q. oleoides* en el campo, sin embargo, por el bajo número de individuos encontrados, y por ser de diferentes especies, no fue posible implementar un diseño experimental ni mucho menos una cría experimental de herbívoros nativos. Para tratar de solventar esta situación, se intentó realizar un bioensayo utilizando como herbívoro el gusano cogollero, *Spodoptera frugiperda,* una plaga que ataca varios cultivos de importancia económica. Sin embargo, este tipo de modelos fue muy difícil de estandarizar, dado que el éxito del consumo de hojas de encino fue muy bajo y variable por no ser un herbívoro adaptado a la familia Fagaceae. Esto, incluso cuando se estandarizó con larvas del mismo estadio (*instar)* de etapa intermedia (estadio 4).

Para obtener un modelo de herbivoría más realista, se requiere de estudios futuros que determinen cuál es el herbívoro de mayor abundancia en ésta u otra especie que pudiera servir

como organismos modelo para entender las interacciones de las restantes 160 especies de encinos de México. Por ejemplo, en Europa se conoce bien que *Operophtera brumata* ("winter moth") y *Lymantria dispar* ("gypsy moth") son herbívoros abundantes que consumen encinos en ese continente. Estas especies de herbívoros no se pueden emplear en América Latina, por ofrecer un riesgo de invasión biológica muy alto, tal como ha sucedido en Estados Unidos y Canadá. Más aún, todavía no se conocen herbívoros con esas características en México y que sean susceptibles de ser reproducidos bajo condiciones de laboratorio. Con herbívoros nativos a México se podrían establecer experimentos de herbívoria más precisos en nuestro país. Estos bioensayos podrían relacionarse también con otras características de las hojas como contenido de proteínas, aminoácidos y resistencia mecánica de las hojas. De este modo, se tendría una visión más completa de las disyuntivas que enfrentan los herbívoros durante la selección de su alimento e indicar qué especies de encino serían más vulnerables al ataque de plagas.

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