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Implicaciones fenológicas, anatómicas y químicas entre *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) y los psílidos gallícolas *Calophya rubra* Blanchard y *Calophya mammifex* Burckhardt & Basset (Psylloidea: Calophyidae)

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RESUMEN GENERAL

Las agallas vegetales son estructuras especializadas inducidas por organismos parásitos, generalmente insectos, capaces de alterar el desarrollo normal de los tejidos vegetales. *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) es un arbusto nativo de Chile, considerado un superhospedero de agallas de insectos, sobre el cual al menos tres especies de psílidos gallícolas (Hemiptera: Psylloidea) inducen agallas en sus hojas y tallos. Aunque se considera que el desarrollo de las agallas se produce bajo la influencia del genotipo del hospedero, del inductor y del medioambiente, el papel de estos factores en la determinación de la morfología de las agallas aún no se ha esclarecido. Los superhospederos de insectos gallícolas constituyen un sistema modelo ideal para estudiar los factores que determinan los patrones de desarrollo de las agallas. Por ello, a través de herramientas anatómicas y químicas se estudiaron los factores que determinan la estructura final de las agallas inducidas por *Calophy*a spp. en las hojas y tallos de *S. polygama*, bajo la influencia del clima mediterráneo del sur de Chile. *Calophya mammifex* Burckhardt & Basset induce agallas globoides en hojas y *C. rubra* Blanchard induce agallas cónicas en tallos. El clima desfavorable del sur de Chile determina un ciclo de vida univoltino y un período de diapausa en ambas especies de insectos. Independientemente de la especie inductora, las agallas son mayormente parenquimáticas, con tres compartimentos bien diferenciados, abundante sistema vascular y rediferenciación de tricomas que se superponen en la apertura de la agalla. Además, en ambos morfotipos se detectó la presencia de dos compartimentos de tejidos especializados, un tejido similar al nutritivo y un tejido de almacenamiento común, ambos ricos en metabolitos primarios. De igual manera, se observó la compartimentalización de los polifenoles, separados del compartimento medio donde se alimentan los inductores. Estas características han sido asociadas al hábito alimenticio de los inductores succionadores del floema. Sin embargo, cada morfotipo de agalla mostró peculiaridades: presencia de drusas y haces vasculares en las agallas de hoja, y la formación de unidades vasculares y parénquima más hiperplásico e hipertrófico en las agallas de tallo. El contenido de fenoles fue diferente entre ambos morfotipos de agallas y con los órganos hospederos, lo que evidencia la influencia de los órganos hospederos en la estructura y composición química de las agallas. Las características fenológicas, anatómicas, y químicas de las agallas de calófidos se desarrollan bajo la acción sinérgica de los insectos inductores, los órganos hospederos y el clima. Esto determina que las agallas de calófidos sean estructuras complejas que garantizan la nutrición y protección de *C. mammifex* y *C. rubra* en las condiciones climáticas desfavorales del clima Mediterráneo chileno.

GENERAL ABSTRACT

Galls are specialized plant structures induced by a parasite organism, usually an insect, which alters the normal developmental patterns of plant tissues. *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) is a shrub native to Chile, considered a superhost of galling herbivores, on which at least three psyllids species have been observed parasitizing their leaves and stems. Even though gall development occurs under the influence of host-plant genotype, environment, and inducer genotype, the role of these factors in determining gall morphology is not clearly understood. A superhost for galling herbivores constitutes an ideal model system to study gall developmental patterns. The factors that determine the final gall structure induced by *Calophya* spp. on leaves and stems of *S. polygama*, under the Mediterranean climatic conditions of southern Chile, were evaluated through anatomical and chemical tools. *Calophya mammifex* Burckhardt & Basset induces globoid leaf galls and *C. rubra* Blanchard induces conical stem galls on *S. polygama*. The Mediterranean climate determines a univoltine life cycle and a diapause period for *C. mammifex* and for *C. rubra*. Regardless of the inducing species, the galls are mostly parenchymal, with three distinct compartments, abundant vascular system and trichomes redifferentiation that overlap at the gall opening. In addition, in both morphotypes, the presence of two specialized tissue compartments, a nutritive-like tissue and a common storage tissue, rich in primary metabolites, was detected. Also, polyphenols compartmentalization was observed, those who are separated from the median compartment, where the inductors are fed. These features have been associated with the feeding habit of sucking phloem of the inducers. However, each gall morphotype showed peculiarities: leaf galls with druses and vascular bundles, and the formation of vascular units and more hyperplastic and hypertrophic parenchyma in the stem galls. The polyphenol contents in both galls differed between them and with the host organs, which evidences the influence of the host organs on the structure and chemical composition of the galls. Anatomical, histochemical and phenological features of calophyid galls are developed under the synergic action of insects, host plant potentialities, and climate conditions, determining complex structures towards nutrition and protection of the *C. mammifex* and *C. rubra* under the unfavorable conditions of the Mediterranean climate.

INTRODUCCIÓN GENERAL

Morfogénesis de las agallas inducidas por insectos

Las agallas son estructuras vegetales especializadas inducidas por organismos parásitos, generalmente insectos, capaces de alterar los patrones de desarrollo normal de los tejidos hospederos (Kinsey 1920; Felt 1940). Los insectos gallícolas inducen alteraciones anatómicas y metabólicas en respuesta a las secreciones inyectadas por la larva durante la alimentación o por las hembras durante la oviposición (Shorthouse & Rohfritsch 1992; Higton & Mabberly 1994; Stone & Schönrogge 2003; Dias et al. 2013a).

El desarrollo de las agallas se produce en cuatro etapas: inducción, crecimiento y desarrollo, maduración y senescencia (Rohfritsch 1992; Dreger-Jauffret & Shorthouse 1992; Arduin et al. 2005). Posterior a la inducción se inicia la formación de la corteza de la agalla alrededor del sitio de inducción a través de procesos de hiperplasia tisular e hipertrofia celular (Mani 1964; Rohfritsch 1992; Oliveira et al. 2006). Tales procesos se incrementan con la actividad continua de alimentación del insecto (Rohfritsch 1992). Durante la fase de crecimiento y desarrollo se forman los tejidos típicos de la agalla (Rohfritsch 1992; Kraus 2009; Oliveira & Isaias 2010b). Las agallas más compleja manifiestan diferenciación de capas de tejidos vegetales especializados para la protección y nutrición del inductor (Stone & Schönrogge 2003; Kraus 2009). Los estudios anatómicos de estas agallas muestran zonas de tejidos especializados bien definidas estructuralmente. En la zona más interna que limita la cámara larval, se forman células nutritivas (tejido nutritivo), seguida de una zona de protección, frecuentemente con células lignificadas, y en algunos casos tejidos de reserva (Arduin & Kraus 1995; Rohfritsch 1999; Dorchin et al. 2002; Kraus et al. 2003; Oliveira & Isaias 2010a, b).

La fase de maduración es la principal fase trófica del insecto (Isaias et al. 2014a). La actividad de alimentación se produce activamente del tejido nutritivo y/o de los contenidos traslocados desde los tejidos de reserva localizados en la corteza externa (Bronner 1992; Kraus 2009). La fase de senescencia se produce con la salida del imago, lo que provoca importantes cambios en los tejidos de la agalla como resultado del cese de alimentación del insecto (Rohfritsch 1992).

Durante el desarrollo de las agallas, algunas capas de tejidos acumulan sustancias nutritivas (Bronner 1992) y/o de defensas (Isaias et al. 2014b). Las sustancias nutritivas generalmente se almacenan en tejidos nutritivos o de reservas, desde donde son traslocados hasta el sitio de alimentación del insecto gallícola (Price et al. 1986, 1987; Bronner 1992). Las sustancias de

defensa o tóxicas, generalmente compuestos fenólicos, con frecuencia se acumulan en las capas externas de las agallas ((Nyman & Julkunen-Titto 2000; Oliveira et al. 2006; Formiga et al. 2011; Isaias et al. 2014b).

La estructura y metabolismo de las agallas dependen del taxón y hábito alimenticio del gallícola (Rohfritsch 1992), y generalmente difieren del órgano hospedero. De acuerdo a ello, se asume que la morgonénesis y metabolismo de la agalla está bajo el control del inductor, por lo que se considera a las agallas un fenotipo extendido del insecto gallícola (Abrahamson et al. 1991). Los análisis histo-citométricos relacionados con la morfogénesis de los órganos hospederos hasta la senescencia de las agallas permiten dilucidar cuantitativamente los patrones histológicos involucrados en el desarrollo final de las agallas (Isaias et al. 2014b). De igual modo, la acumulación y compartimentalización de los metabolitos primarios y secundarios en las agallas pueden ser detectados histoquímicamente, lo que constituye una valiosa herramienta para interpretar el posible rol nutritivo y/o defensivo de las mismas (Isaias et al. 2014b).

Aunque la morfogénesis de las agallas se ha relacionado con el hábito alimenticio del gallícola (Rohfritsch 1992), esto no explica por sí mismo la gran diversidad de forma y estructura de agallas que se encuentran en la naturaleza, incluso entre insectos con el mismo hábito alimenticio (Oliveira et al. 2014). A pesar de que las agallas se considera un fenotipo extendido del insecto gallícola (Abrahamson et al. 1991), están formada por células y tejidos de la planta. De esta manera el desarrollo de las agallas puede estar limitado por las potencialidades morfogenéticas del tejido vegetal hospedero (Isaias et al. 2014b).

En dependencia del grado de reactividad del tejido infestado se desarrollarán diferentes morfotipos de agallas con distintos grados de similitud a su órgano hospedero (Formiga et al. 2015). Por tanto, la morfología final de la agalla dependerá de la plasticidad del tejido de la planta hospedera, pues este tejido impondrá mayor o menor limitaciones morfogenéticas al desarrollo de la agalla (Isaias et al. 2014b). De esta manera, las agallas más simples deberán ser más parecidas a su órgano hospedero (Floate 2010). En este sentido, las agallas inducidas en órganos de menor plasticidad fenotípica, como los tallos comparados con hojas (Mauseth 1988), se desarrollan bajo mayor restricción mofogenética, y deberán ser más parecidas entre sí que las que se desarrollan en órganos con mayor plasticidad fenotípica como las hojas (Isaias et al. 2013; Ferreira & Isaias 2013).

Al ser infestado un mismo hospedero vegetal por distintas especies gallícolas, cada especie de insecto induce reorganizaciones distintas en los tejidos vegetales y produce una agalla típica

para cada especie de insecto (Meyer 1987; Shorthouse & Rohfritsch 1992; Isaias et al. 2014b). Este fenómeno se produce en los superhospederos de agallas que muestran un gran potencial morfogenético para responder a estímulos provenientes de diferentes especies de insectos gallícolas (Formiga et al. 2015), por lo que cada tejido hospedero impone sus propias limitaciones morfogéneticas al desarrollo de las agallas (Oliveira et al. 2014).

Peculiaridades anatómo-metabólicas de las agallas de insectos succionadores

Las agallas, como otro órgano normal de la planta, tienen su propia morfología e histología (Meyer & Maresquelle 1983), aunque con amplia variedad de formas (Isaias et al. 2014a) determinada por la susceptibilidad de la planta hospedero y las diferencias fisiológicas en el comportamiento de los inductores (Raman 2007b); en particular, el hábito alimenticio (Crespi & Worobey 1998) y la estructura de las piezas bucales del inductor (Raman 2011). Por ejemplo, características como la manera en que el inductor se alimenta y su posición dentro de la cámara, pueden ser determinantes para la forma final de la agalla (Rohfritsch 1992). De hecho, agallas inducidas por diferentes taxones de artrópodos suelen presentar distintas formas, grados de modificación de tejidos, complejidad, y respuestas celulares diferentes (Isaias et al. 2014a). Por lo tanto, la planta responde de forma específica y especializada al modo de alimentación del inductor (Mani 1964; Rohfritsch 1992), lo que altera el patrón anatómico del órgano vegetal a diferentes niveles y genera agallas diferentes (Oliveira et al. 2008).

En el caso de inductores hemípteros (Hemiptera), que se alimentan del contenido de las células del parénquima o directamente del floema (Dreger-Jauffret & Shorthouse 1992; Oliveira & Isaias 2010b), inducen una mayor variedad de formas de agallas, aunque con menor especialización de sus tejidos. La corteza de estas agallas está compuesta principalmente por parénquima hiperplásico e hipertrófico, con haces vasculares intercalados y tejidos esclerificados en la madurez (Meyer 1987), por lo que poseen pobre organización y son estructuralmente similares al tejido del órgano que las originó (Floate 2010).

Las agallas de psílidos (Hemiptera: Psylloidea), supuestamente, no acumulan sustancias de reserva, por lo que no desarrollan tejido nutritivo diferenciado, ni inducen formación de gradientes histoquímico y citológico (Bronner 1992; Dias et al. 2013a; Oliveira et al. 2014). Sin embargo, en estas agallas se han descrito tejidos que rodean los haces vasculares con células que almacenan almidón, denominados tejidos de almacenamiento común (*common storage tissue* CST) (Álvarez et al. 2009; Oliveira & Isaias 2010b; Isaias et al. 2011; Muñoz-Viveros et

al. 2014). Además, en el Neotrópico se han reportado casos específicos de agallas de psílidos con tejidos de células que almacenan almidón y azúcares reductores, y que tienen un núcleo activo y citoplasma denso (Carneiro et al. 2014; Carneiro & Isaias 2015; Richardson et al. 2016). A estos tejidos se les han denominado tejidos parecidos a nutritivos (*nutritive like tissues* NLT) (*sensu* Richardson et al. 2016). En base a estos resultados, según Ferreira et al. (2017) dentro de las agallas inducidas por insectos succionadores de floema se pueden encontrar: (i) agallas más simple que no desarrollan tejidos de almacenamientos o solo desarrollan CST; (ii) agallas más complejas que inducen formación de NLT alrededor de la cámara ninfal y de los haces vasculares y CST en la corteza externa de la agalla.

Papel de los metabolitos secundarios en las agallas

La composición química de las plantas hospederas está estrechamente relacionada con la elección del insecto inductor (Abrahamson et al. 2003). Específicamente, para los psílidos se ha señalado que la composición única de metabolitos secundarios (MS) del hospedero, pudiera ser la base de la fidelidad en la elección del hospedero (Hodkinson 2009). Pequeñas disimilitudes en los perfiles químicos de los hospederos podrían marcar la diferencia a la hora de escoger un hospedero y un órgano de la planta (Raman 2007a).

En este sentido, los compuestos volátiles en las agallas, como los terpenos, se han relacionado con funciones de señalización para la localización del sitio de oviposición (Tooker et al. 2005; Germinara et al. 2011; Isaias et al. 2014a), y la localización de la hembra para el acto de cópula (Tooker et al. 2002; Tooker & Hanks 2004). Además, estos compuestos probablemente están involucrados en la defensa de la agalla contra patógenos y depredadores (Gerchman & Inbar 2011; Martinez 2010; Rostás et al. 2013).

Los cambios inducidos por los insectos gallícolas en la composición de MS de las agallas pueden ser histoquímicamente demostrado (Isaias & Oliveira 2014). Los test histoquímicos son pruebas *in situ* que permiten la detección y localización de los MS en las capas de tejidos especializados de las agallas. Las células de las capas más externas de la corteza de la agalla generalmente acumulan metabolitos secundarios, como alcaloides, flavonoides, compuestos fenólicos y taninos (Nyman & Julkunen-Titto 2000; Oliveira et al. 2006; Formiga et al. 2011; Isaias et al. 2014b), lo que puede indicar el papel defensivo de estos compuestos. Especialmente, los compuestos fenólicos muestran una marcada variación cualitativa y cuantitativa en respuesta a diferentes etapas fisiológicas y de desarrollo de los órganos vegetales (Kause et al. 1999); así

como a factores ambientales, como la intensidad de la luz y la disponibilidad de nutrientes (ver Herms & Mattson 1992 y sus referencias). De acuerdo a estos elementos, las agallas podrían seguir los patrones de acumulación temporal de compuestos fenólicos de los órganos hospederos en respuesta a diferentes factores. A pesar de que se conoce de la presencia de fenoles en los tejidos de las agallas, se cuenta con pocos datos para evaluar la importancia relativa de la variabilidad temporo-espacial de los compuestos fenólicos en las agallas a consecuencia de las diferentes etapas de desarrollo de la agalla y de diferentes estaciones fenológicas de crecimiento.

Implicaciones fenológicas en la relación planta hospedero-insecto gallícola

Las características morfológicas, químicas, fisiológicas y fenológicas de las plantas están moduladas por los cambios estacionales en las condiciones medioambientales (Floate et al. 1996). La fenología de la planta hospedera puede influir de manera crítica en la comunidad de insectos gallícolas asociados (Yukawa 2000; Yukawa & Akimoto 2006), por lo que los ciclos de vida de ambos organismos deben estar sincronizados para garantizar la especificidad de la relación.

El período de crecimiento vegetativo es el tiempo preferencial para la inducción de agallas, ya que los tejidos jóvenes son más reactivos al estímulo del inductor (Rohfritsch & Anthony 1992), porque son ricos en nutrientes y contienen cantidades más bajas de compuestos fenólicos y defensivos (Feeny 1976; Rhoades & Cates 1976). La producción de tejidos nuevos es una respuesta fisiológica de las plantas a los ritmos estacionales de factores abióticos como el clima (Van Schaick et al. 1993), especialmente a la disponibilidad de agua (Sarmiento & Monasterio 1983). Debido a este requerimiento, en las zonas templadas hay una fuerte tendencia a que los insectos gallícolas posean un ciclo de vida univoltino (Weis et al. 1988; Hodkinson 2009), ya que la producción de tejidos nuevos está restringida a una estación específica del año (Rohfritsch & Anthony 1992). Sin embargo, la disponibilidad de recursos alimenticios y las condiciones ambientales en el Neotrópico favorecen ciclos de vidas multivoltinos (Weis et al. 1988; Lima 2008; Dias et al. 2013a, b), como en el caso de psílidos gallícolas asociados a dos especies siempre verdes de Brasil: *Richeria grandis* Vahl (Lima 2008) y *Schinus engleri* Barkley (Dias et al. 2013b).

En climas templados, con alta estacionalidad como el centro-sur de Chile, los veranos secos y calurosos alternados con inviernos frios y húmedos (Giorgi & Lionello 2008), pudiera ser un factor limitante para el desarrollo de ciclos de vidas multivoltinos, incluso cuando las plantas

hospederas son siempre verdes. En estas condiciones climáticas la mejor estrategia para los insectos gallícolas pudieran ser el desarrollo de ciclos de vida univoltino (Hodkinson et al. 1999; Hodkinson 2009), y la formación de características anatómicas, morfológicas y químicas en las agallas que las protejan contra el clima desfavorable. Sin embargo, hasta la fecha no se cuenta con estudios de la influencia del clima Mediterráneo de Chile en el ciclo de vida y caracaterísticas anatómicas y químicas de las agallas.

Super-hospederos de agallas: sistema *Schimus polygama* - *Calophya* spp.

Schinus polygama (Cav.) Cabrera, conocido comúnmente como huigán, es un arbusto aromático y medicinal, previamente considerado nativo de Argentina, Brasil, Bolivia, Uruguay y Chile (Rodríguez 2011). Sin embargo, la taxonomía y sistemática del género *Schinus* en América del Sur es confusa y aún está bajo revisión. De acuerdo a comunicación personal del Dr. Daniel Burckhardt[1] y para los fines de esta tesis, se considera a *S. polygama* distribuida solamente en Chile y la zona fronteriza con Argentina, mientras que la especies distribuida en Brasil se corresponde a *S. engleri*. Además, se ha considerado asumir como nombre de la especie *Schinus polygama* como se indica en *Plant List* (http://www.theplantlist.org/), y no *S. polygamus* como previamente se ha considerado en el primer capítulo de esta tesis.

En hojas, ramas, flores y yemas foliares de *S. polygama* en Chile se ha descrito el desarrollo de agallas de insectos de los órdenes Hemiptera (Psylloidea) y Lepidoptera (Cecidosidae) (Sáiz & Núñez 1997; Burckhardt & Basset 2000), por lo que pudiera ser considerado un super-hospedero de agallas. Dentro de la superfamilia Psylloidea se han descrito dos géneros de psílidos gallícolas: *Tainarys* con dos especies y *Calophya* con tres especies (Burckhardt & Basset 2000). *Calophya* spp. induce diferentes tipos de agallas: *C. rubra* Blanchard produce agallas de ramas tipo envolvente; *C. scrobicola* Burckhardt & Basset agallas de punteaduras y *C. mammifex* Burckhardt & Basset agallas tipo bolsillo, estas dos últimas en hojas (Burckhardt & Basset 2000; Sáiz & Núñez 2000). Las especies del género *Tainarys* inducen agallas de enrollamiento de hoja (Burckhardt & Basset 2000).

Cuando un mismo hospedero vegetal es infestado por distintas especies gallícolas, cada una de ellas induce reorganizaciones distintas en los tejidos vegetales, y produce una agalla típica para cada especie (morfotipo) (Meyer 1987; Shorthouse & Rohfritsch 1992; Isaias et al. 2014b). No

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obstante, cuando los linajes de insectos están estrechamente relacionados tienden a influir de forma similar en los tejidos de la planta hospedero (Rohfritsch 1992), debido a las semenjanzas en sus hábitos de alimentación. Entonces, es de esperar que las agallas de calófidos inducidas en los tallos y hojas de *S. polygama* sean muy similares entre sí. Sin embargo, la interacción sinérgica entre el genotipo de la planta hospedero, el genotipo del inductor y el medioambiente pueden determinar un fenotipo específico de agalla para cada especie de insecto (Weis et al. 1988). Los susperhospederos de insectos gallícolas han sido el foco de estudios anatómicos y ecológicos en la Región Neotropical (Moura et al. 2008, 2009; Oliveira et al. 2008; Oliveira & Isaias 2009, 2010b; Carneiro et al. 2013, 2014; Carneiro & Isaias 2015). Sin embargo, en el clima Mediterráneo de América del Sur (Chile), estos estudios han sido muy escasos y sus resultados contradictorios.

De acuerdo a las intricadas relaciones que se establecen entre el insecto gallícola y la planta hospedera, se presume que el desarrollo de las agallas siga patrones químicos y estructurales determinados por los organismos involucrados. A pesar de ello, los factores ambientales pueden influenciar el ciclo de vida de la planta hospedera y de los insectos inductores, lo que genera peculiaridades fenológicas, estructurales y químicas en las agallas. El sistema *S. polygama*-*Calophya* spp. constituye un modelo ideal para explorar esta hipótesis. Estudios fenológicos, anatómicos, histoquímicos, cromatográficos y espectroscópicos pueden contribuir a esclarecer la interacción entre el genotipo de los dos organismos involucrados y la acción del clima Mediterráneo del sur de Chile. De esta manera, se podrán responder las siguientes preguntas de investigación: ¿el clima mediterráneo del centro-sur de Chile influye en las peculiaridades fenológicas, histoquímicas y anatómicas de las agallas de calófidos inducidas en *S. polygama*?; ¿cómo los insectos gallícolas manipulan las características morfogenéticas e histoquímicas de las hojas y tallos de *S. polygama* durante el desarrollo de las agallas de calófidos?; ¿la morfogénesis de las agallas de calófidos está determina por las restricciones impuestas por los órganos hospederos o por los hábitos de alimentación de los inductores de las agallas?; ¿las características estructurales y los perfiles de metabolitos de las agallas de calófidos indican inversión en estrategias de defensa y/o nutritiva?; ¿la compartimentación de los metabolitos secundarios en las agallas de calófidos sigue un patrón genérico o específico?; ¿los niveles de compuestos fenólicos en las agallas está influenciado por los patrones de sus órganos hospederos?; ¿existen convergencias en la distribución espaciotemporal de los compuestos fenólicos en las agallas de calófidos?

El estudio de las alteraciones de los tallos y hojas de *S. polygama* durante el establecimiento de calófidos inductores, y la formación de los diferentes morfotipos de agalla pueden revelar las relaciones que se establecen entre los genotipos involucrados bajo la influencia del clima mediterráneo. El presente trabajo constituye la primera aproximación al estudio de la influencia del clima mediterráneo sobre las peculiaridades fenológicas, anatómicas, y químicas de agallas de insectos relacionados filogenéticamente, *Calophya* spp., que se desarrollan bajo las mismas potencialidades morfogenéticas del superhospedero *S. polygama*. En este sentido, para dar respuestas a las preguntas de investigación enunciadas, y lograr mayor comprensión del sistema *S. polygama* - *Calophya* spp., la tesis se estructura en cuatro capítulos:

I. Leaf and stem galls of *Schinus polygamus* (Cav.) Cabr (Anacardiaceae): anatomical and chemical implications
II. Anatomical and phenological implications of the relationship between *Schinus polygama* (Cav.) (Cabrera) (Anacardiaceae) and the galling insect *Calophya rubra* (Blanchard) (Hemiptera: Psylloidea)
III. Factors influencing the morphogenesis of galls induced by *Calophya mammifex* (Calophyidae) on leaves of *Schinus polygama* (Anacardiaceae)
IV. Spatiotemporal variation in phenolic levels in galls of calophyids on *Schinus polygama* (Cav.) Cabrera (Anacardiaceae)

CAPÍTULO I

Leaf and stem galls of *Schinus polygamus* (Cav.) Cabr (Anacardiaceae): Anatomical and chemical implications

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Abstract. Galling insects commonly induce anatomical and metabolic changes in their host plant tissues, which is true for *Schinus polygamus* (Cav.) Cabr. (Anacardiaceae) in Chile. Currently, anatomical and chemical changes induced by galling insects in stems and leaves of *S. polygamus* were analyzed. Methanolic extracts of non-galled and galled tissues were analyzed by gas chromatographyemass spectrometry (GC-MS). Differences in the secondary metabolite profiles, and their relation with plant responses to gall development were evidenced. Transverse sections of non-galled host organs and galls were done and observed under light and scanning electron microscopies. One stem gall (conical) and one leaf gall (globoid) morphotypes were identified. The globoid and conical galls have dense trichomes, large nymphal chambers, and develop mainly by tissue hyperplasia. The chemical profiles of stems, leaves and galls are distinct, except for the concomitant detection of pyrogallol in galls. The highest abundance of terpenes and phenols in gall tissues were identified, and two triterpenes were firstly reported for the non-galled tissues of *S. polygamus*. Host plant tissues are highly responsive to the Psyllidae stimuli toward the over development of a phenolic-rich parenchyma, which ends up favouring the *Calophya* sp. establishment and gall development.

Keywords: Calophya; galling insects; host plant; nymphal chamber; psyllids, secondary metabolites

1. Introduction

Schinus polygamus (Cav.) Cabr (Anacardiaceae), commonly known as hardee peppertree, is an aromatic and medicinal shrub native to Argentina, Bolivia, Uruguay, Brazil and Chile (Rodríguez, 2011). In some South American countries, such as Chile and Brazil, *S. polygamus* presents numerous phytosanitary problems caused by herbivorous and pathogenic insects (Damasceno et al., 2010). This species also have been reported as a host of a diversity of galling insects (Sáiz and Núñez, 1997) capable of inducing galls on leaves, branches, and flowers (Sáiz and Núñez, 1997; Burckhardt and Basset, 2000). Accordingly, *S. polygamus* can be defined as a super-host of galling insects (Dias et al., 2013a), which may be Diptera (Cecidomyiidae), Hemiptera (Psylloidea), and Lepidoptera (Cecidosidae) (Sáiz and Núñez, 1997; Burckhardt and Basset, 2000).

Galls are specialized plant structures induced by a parasite organism, usually an insect, which alters the normal developmental patterns of plant tissues (Felt, 1940). The galling organisms induce anatomical and metabolic alterations, probably, in response to secretions injected by the larvae during the feeding activity or by the female during oviposition (Dias et al., 2013a). The galling insects are capable of inducing morphogenetic changes in their host plants towards obtaining food or/ and shelter (Rohfritsch, 1992). Cell hypertrophy, tissue hyperplasia, inhibition of some developmental programs and cytological changes occur during the development of galls (Ferreira and Isaias, 2013). Moreover, gall-inducing herbivores have the ability to manipulate plant tissues growth and development for their own benefit, and can also manipulate their chemical composition (Moura et al., 2008; Dias et al., 2013b). This chemical manipulation redirects plant cell responses towards the determination of tissues with higher nutritional quality, the inner nutritive tissues, and tissues rich in defensive compounds, the outer tissue layers, which may help in protecting the galling insects against their natural enemies (Stone and Schonrogge, 2003; Formiga et al., 2009).

Galls usually contain a large amount of nutrients and low concentrations of defensive compounds (Hartley, 1999; Isaias et al., 2014). Nonetheless, some variations in this general pattern may occur, and galls may contain higher concentrations of defensive compounds in comparison to the non-galled tissues of their host plants (Hartley, 1999). The chemical profile of *S. polygamus* tissues revealed leaf volatile compounds synthesized in response to the attack of herbivores (Valladares et al., 2002; Damasceno

et al., 2010). The composition of such volatiles varies between non-galled leaves and galls (Damasceno et al., 2010), mostly in relation to mono and sesquiterpenes. However, there is a lack of information about non-volatile secondary metabolites (SM), as well as their functions in galled tissues of *S. polygamus*. Also, studies on the structural profile due to galling stimuli on *Schinus* species are restricted to *Calophya duvauae* Scott-*S. polygamus* system (Dias et al., 2013a), and a phytochemical profile of this species in galled and non-galled conditions has yet to be determined. Herein, we compare the structural and chemical potential of two distinct host plant organs, the stems and leaves of a single species, *S. polygamus*, to respond to two different galling herbivores stimuli, and focus on the following questions: (i) Are there structural traits of the host organs potentiated towards the development and survival of the galls? (ii) Do the chemical profiles indicate in- vestment in chemical defensive strategies in galls? (iii) Are the galling insects associated to stems and leaves capable of inducing convergent responses on their host organs?

2. Materials and methods

2.1. *Study area and processing of plant material*

Branches of *S. polygamus* were sampled at the town of Chillán Viejo, kilometer 4, Ñuble Province, Biobío Region (36°39' 32" S 72°16' 43" W), Chile. Plant species identification was confirmed by specialists from the Department of Botany of the University of Concepcion (CONC). A voucher specimen was deposited in CONC under the accession number 180330.

The branches were visually inspected, with a magnifying glass to detect the presence of galls. Gall morphotypes were classified and described according to Isaias et al. (2013).

2.2. *Structural analyses*

For studies in scanning electron microscopy (SEM), samples of leaf and stem galls were fixed in 2.5% glutaraldehyde in sodium phosphate buffer, pH 7.2, at 4 °C, for 24 h. The samples were washed twice in 0.1 M phosphate-buffered saline (PBS) for 10 min, post-fixed in 1% osmium tetroxide in 0.1 M PBS, at 4 °C, for 2 h, and washed with the same buffer twice for 10 min. Fragments were dehydrated in 30e100% ethanol series and were dehydrated a second time in liquid CO_2 by a critical point dryer (Balzers® Union

FL-9496, Holland) (O'Brien and Mccully, 1981). The sections were mounted on aluminum stubs with carbon film, and metalized in gold (approximately 400 Å) in a Sputter Coater (Edwards® S 150, U.S.) for 3 min at 30 mA. Observations were done under a scanning electron microscopy (JEOL® JSM - 6380 LV, Japan).

For the studies in light microscopy (LM), leaf and stem galls were fixed in 4% Karnovsky in 0.1 mM phosphate buffer for 24 h (O'Brien and Mccully, 1981; modified to pH 7.2) and stored in 70% ethanol. For the preparation of permanent slides, fragments were dehydrated in a 50-100 *n*-butyl series, and embedded in Paraplast® (Kraus and Arduin, 1997). Serial sections (12-18 mm) were obtained in a rotary microtome and stained in 0.5% safranin-astra blue (9:1) (Kraus and Arduin, 1997). The slides were observed and photographed under a light microscope (Leica® ICC50 HP).

2.3. *Methanolic extraction and identification of chemical compounds*

Non-galled stems and leaves, and stem and leaf galls were isolated and macerated with 100% methanol for 7 days, at room temperature. The non-galled stem bark was removed prior to methanol extraction. Methanol extracts were filtered through Whatman® No 1 filter paper, and undergo the identification process of secondary metabolites (SM).

The extracts were concentrated at a reduced pressure with a rotary evaporator until completely dry. Subsequently, the crude extract was sequentially extracted with *n*-hexane, ethyl acetate, and distilled water. The hexanic and ethyl acetate extracts were concentrated again at a reduced pressure in a rotary evaporator, diluted in ethyl acetate, and monitored by thin- layer chromatography (TLC). For the identification of chemical compounds in each fraction, these extracts were subjected to gas chromatography coupled with mass spectrometry (GC-MS) in the Agilent® 7890A equipment, with the Agilent® 5975C mass detector, using a capillary column of fused silica type HP5-MS, 30 m, 0.25 mm internal diameter, and 0.25 mm thick, under the following characteristics: Temperature: 250 °C; Detector (mass): 280 °C; Oven: initial 100 °C for 5 min, increasing to 8 °C/min up to 250 °C, and maintained for 15 min. The adjustment of the detector as a scanner varied from 50 to 500 amu. Flow of carrier gas (electronic grade helium) at 1 mL/min. The characterization was carried out by means of comparison with the NIST® database.

3. Results

3.1. *Features of stem and leaf galls*

Leaves and branches of *S. polygamus* are infested by gall-inducing insects (Fig. 1A), which can be recognized by two gall morphotypes, a stem and a leaf gall. Stem galls are conical, dark brown, and have a bunch of trichomes towards the apical portion (Fig. 1B). Sometimes several galls may coalesce, but each gall has one larval chamber. Leaf galls are globoid, red, with the aperture located at the tip of an abaxial projection surrounded by abundant trichomes (Fig. 1C). There is a depression on the adaxial surface of leaf lamina, which is green (Fig. 1D). The globoid galls are isolated, and may vary from a single to many galls on the same leaf lamina.

The nymphal chambers, both of stem conical and of leaf globoid galls, are large, surrounded by concentric layers of cells (hyperplasia) (Fig. 2A-D). The shape of the nymphal chamber is different in the two gall morphotypes, round-shaped in leaf galls (Fig. 2A-B); and elongated in stem galls in a funnel shape that narrows towards the opening area of the gall and is wider towards the gall inner portion (Fig. 2C-D). Three tissue layers are observed in stem galls (Fig. 2D-E), while in leaf gall a homogeneous tissue is found (Fig. 2B). Moreover, the presence of trichomes was revealed in the opening area of stem galls, which is projected inwards (Fig. 2C). In both galls, each chamber hosts a single inducing insect, belonging to the superfamily Psylloidea (family Calophyidae), presumably to the genus *Calophya*.

3.2. *Chemical profile of secondary metabolites in host organs and galls*

The chemical profile of the non-galled host organs and both galls are distinct (Table 1), except for the concomitant detection of pyrogallol both in stem and leaf galls, which represents the unique similarity in new synthesis of molecules due to galling stimuli. The SM of the family of phenols corresponds to 50% and 25% of the total diversity of molecules in leaf and stem galls, respectively. Stem galls maintain the presence of steroids also detected in non-galled stems, but with differences in the chemical structure of such metabolites.

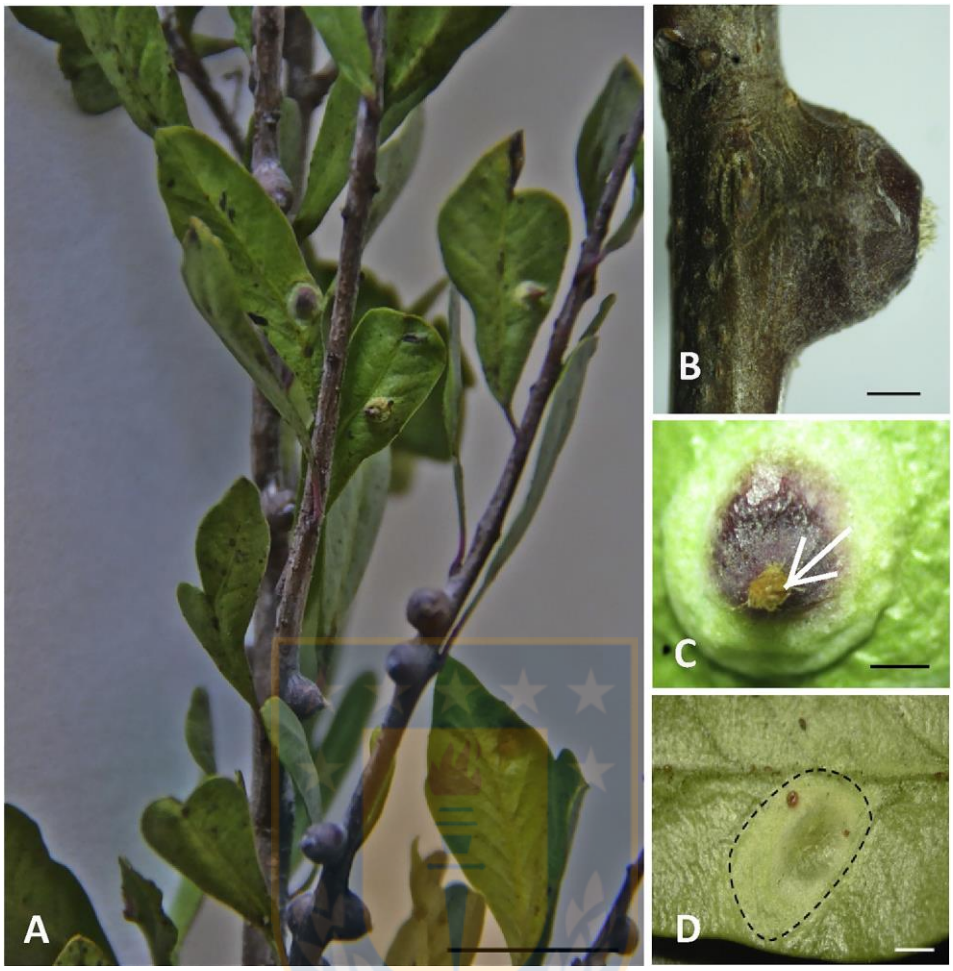

Fig. 1. Galls of Hemiptera (Psylloidea: Calophyidae) on *Schinus polygamus* (Cav.) Cabr. (Anacardiaceae). A: Branches with leaf and stem galls. B: Detail of a conical stem gall. C-D: Details of leaf globoid galls, C: Abaxial surface view evidencing the projection with trichomes on gall aperture (arrow), D: Adaxial view (dotted area). Bars: B, C, D = 1 mm; A = 1 cm. Fuente: Elaboración propia

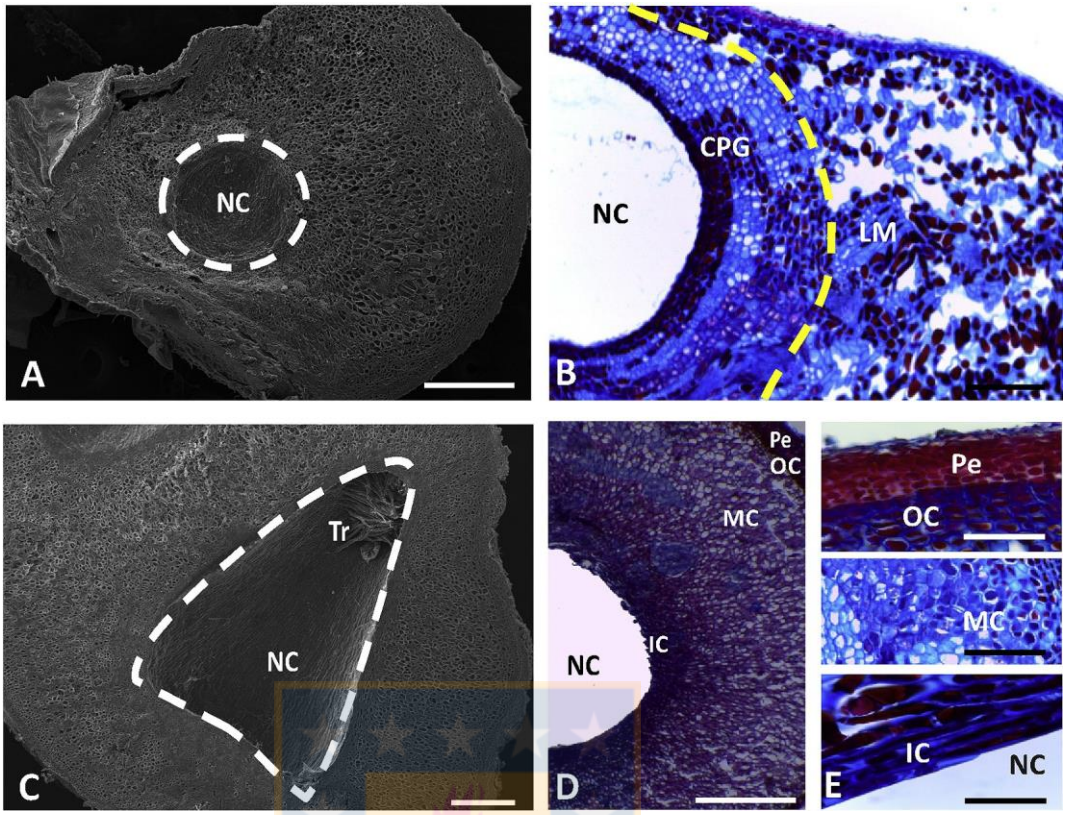

Fig. 2. Leaf and stem galls on *Schinus polygamus* (Cav.) Cabr. (Anacardiaceae). AeB: Leaf gall showing tissue hyperplasia and round nymphal chamber in SEM preparation (A), and LM preparation evidencing the homogeneous parenchyma (B). C-E: Stem gall with tissue hyperplasia and elongated nymphal chamber in SEM preparation (C) where trichomes in gall aperture can be seen, and LM preparation where the three tissue layers are evidenced (D) and details of each three tissue layers (E). Bars: E = 50 mm; B, D = 200 mm and A, C = 500 mm. Abbreviations: NC: nimphal chamber; CPG: cortical parenchyma of gall; LM: leaf mesophyll, Tr: trichomes; IC: internal cortex; MC: middle cortex; OC: outer cortex; Pe: periderm. Fuente: Elaboración propia

Generally, the predominating compounds in *S. polygamus* are terpenes (61.9%), mainly sesquiterpenes. In overall analyses, the non-galled stems and leaves have terpenes but with differences in their chemical structures. The non-galled stems have the greatest diversity of sesquiterpenes, which are not detected in non-galled leaves, and are not conservative in galls, as well. Current analyses detected a-amyrin and ursenal triterpenes for the first time in *S. polygamus*.

The uniqueness of the chemical profile of leaf galls is represented by the detection of nitrogen compounds, aromatic hydrocarbon benzofuran and quinone; while the uniqueness of the chemical profile of stem galls is represented by the detection of fatty acid esters.

4. Discussion

4.1. *Structural traits and gall survival*

Gall morphological and anatomical characteristics are usually related to protective mechanisms against unfavorable environmental conditions, especially desiccation (Stone and Schonrogge, 2003) and natural enemies of the galling insects (Rohfritsch, 1992; Oliveira et al., 2006; Oliveira and Isaias, 2010). For example, trichomes are related to mechanical protection against invading organisms (predators and parasitoids) and to stabilization of temperature and humidity inside the nymphal chamber (Oliveira et al., 2006; Álvarez et al., 2009).

Trichomes in stem and leaf galls of *S. polygamus* along with the hyperplasia of cortical parenchyma can protect the galling insect both against natural enemies and unfavorable environmental conditions. Enhance in trichomes differentiation in leaf galls can represent an overpotentialization of the ordinary morphogenetical pattern of the host plant tissues for trichomes are rarely observed in leaves of *S. polygamus* (Dias et al., 2013a). Such manipulation of host plant morphogenesis has also been induced by *C. duvauae* in leaf galls *S. polygamus* (Dias et al., 2013a), *Euphalerus ostreoides* Crawf-*Lonchocarpus muhelbergianus* Hassl (Oliveira et al., 2006), and *Baccharopelma dracunculifoliae* Burckhardt on *Baccharis dracunculifolia* DC (Arduin et al., 2005), in Brazil. The presence of trichomes was reported, too, for galls induced by *Calophya mammifex* Burckhardt & Basset on *Schinus longifolius* (Lindl.) Speg. and has also been interpreted as a mechanical defence mechanism (Agudelo et al., 2013).

For stem galls, the trichomes differentiate in the dermal system of the nymphal chamber, which originated from the folding of the stem epidermis towards the inner gall surface. Even though the mother cells of a phellogen are present close to the nymphal chamber, suber differentiation does not occur. As a consequence of the spatial replacement, trichoblasts differentiate from cells previously determined to be discharged by suberization. Moreover, the parenchyma cells, which originate the cork cambium beneath the epidermis, turn into adult stem cells (Sugimoto et al., 2011). Their differentiated state is likely to follow internal (developmental) and external signals (stress) that force such cells to redifferentiate to become competent for switching their fates (Lev-Yadun, 2003). Redifferentiation processes are uncovered by multiple phenomena in plants

(Lev-Yadun, 2003), and in galls, plant cells under the influence of gall-inducing insects have their competence reprogrammed, and often assume rapid cell cycles and new cell fates for the neo-ontogenesis of plant galls (*cf*. Carneiro et al., 2014).

Both stem and leaf galls show hyperplasia of cortical parenchyma, a typical process of gall formation (Oliveira and Isaias, 2010; Moura et al., 2009a), which is one of the most common processes in various Hemiptera galls (Isaias et al., 2011; Dias et al., 2013a, b; Formiga et al., 2015). Hyperplasia is induced in young tissues, which have a greater capacity for cell division, and may respond promptly to the stimuli of the gall inducers (Rohfritsch, 1992). Such process results in increased biomass in galls, and can confer a high number of nutritive cells, as well as thickens gall wall, which ends up mechanically protecting the galling insect against unfavorable environmental conditions and natural enemies attack (Stone and Schonrogge, 2003).

4.2. *Chemical defence strategies*

The chemical profile of the non-galled host plant organs is not maintained at gall developmental sites. The phenolics - pyrogallol - were the only secondary metabolite (SM) accumulated both in leaf and stem galls. The triterpene a-amyrin detected in non-galled leaves does not accumulate in leaf galls, otherwise, accumulated metabolites which enhanced their nutritional value (nitrogen compounds), but also their anti-microbial activity, such as the benzofuran (Nascimento et al., 2004) and the quinone. The accumulation of sesquiterpenes, common in non-galled stems, is not detected in stem galls, and may indicate the use of fatty acid precursors also to increase the nutritional value of gall tissues, as proposed by Tooker and de Moraes (2009) for *Gnorimoschema gallaesolidaginis* Riley galls on *Solidago altissima* L.

The chemical accumulation of SM, especially phenolics, has been usually related to their defensive role (Nyman et al., 2000; Agudelo and Ricco, 2012). Eventually the SM have also been associated with the success of the galling insects, as observed for *E. ostreoides-L. muhelbergianus* (Oliveira et al., 2006) and for Cecidomyiidae-*Aspidosperma spruceanum* Müell. Arg. systems (Formiga et al., 2009).

Table 1. Biologically important secondary metabolites identified in extracts of non-galled leaves, non-galled stems, and leaf and stem galls of Schinus polygamus (Cav.) Cabr. (Anacardiaceae).

	Compound	Relative Peak Area (%)	Chemical Family
Non-galled leaves	α-Amyrin	11.7	Triterpene
Leaf galls	1,2,3-Benzenetriol (pyrogallol)	12.33	Phenol (tannins)
	6H-Purin-6-one, 1,7-dihydro-2- (N-methyl guanine or hypoxanthine)	5.23	Nitrogen compound
	Ethanone, 1,1'-(6-hydroxy-2,5-benzofurandiyl)bis (euparone)	8.94	Aromatic hydrocarbon benzofuran
	Anthraquinone, 1-(methoxyphenyl) (1-methyl phenyl -anthraquinone)	10.66	Phenol (Quinone)
Non-galled stems	α-Bergamotene	2.43	Sesquiterpene
	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)- (7-epi-α cadinene)	6.32	Sesquiterpene
	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1α,7β,8aα)]-(eremophylene)	5.75	Sesquiterpene
	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-(Δ cadinene)	7.0	Sesquiterpene
	Tricyclo[4.4.0.02,7]dec-3-ene-3-methanol,1-methyl-8-(1-methylethyl)-(15- copaene)	12.91	Sesquiterpene
	Pregn-4-ene-3,20-dione, 18,21-dihycroxy (4-eno-18,21-dihidroxi-3,20-diona)	7.94	Steroidal
	Urs-12-ene-28-al (ursenal)	8.44	Triterpene
	Copaene	4.71	Sesquiterpene
	Cyclohexene, 1-methyl-4-(5-methylene-4-hexenyl)-, (S)	3.05	Sesquiterpene
	Spathulenol	6.29	Sesquiterpene
	6-isopropenyl-4-8a-dimethyl-1,2,3,5,6,7,8,8a-octohydro-napthalen-2-ol	5.40	Sesquiterpene
	1-piperideneacetonenitrile	3.43	Amine
Stem galls	1,2,3-Benzenetriol (pyrogallol)	25.22	Phenol (tannins)
	Tridecanoic acid, methyl ester (myristic acid methyl ester)	24.62	Fatty acid ester
	9, 12, 15-Octadecatrienoic ac, methyl ester (methyl linoleate)	30.80	Fatty acid ester
	Ergosta-4,6,22-trien-3ª-ol (ergosta trienol)	3.06	Steroidal

The phenolic compounds were the major SM detected in leaf and stem galls of *S. polygamus*. Particularly, pyrogallol (tannins) was recognized both in globoid leaf galls and in the conical stem galls. This compound has antiseptic, antioxidant, fungicide, and insecticide properties (Balasubramanian et al., 2014), so it is also believed to enhance the galls chemical protection against natural enemies.

Another phenolic compounds detected in leaf galls belong to the quinone family. Experimental evidences suggest that sedentary insects may stimulate a defensive mechanism based on the oxidation of phenols to quinones in plant tissues (Miles and Oertli, 1993). However, herbivores that feed on tannin-rich plant material, as the leaf galls in current results, seem to possess some chemical adaptation to remove tannins from their digestive systems, as proposed by Taiz and Zeiger (2006). We infer that psyllids have developed oxidation mechanisms of quinone to non-toxic polymers, and overcome the defensive mechanism of their *S. polygamus* host plants. Also, the deterrent effect of quinones (Nyman and Julkunen-Tiitto, 2000) could improve the chemical microenvironment and its effect against natural enemies of the galling insect, which do not have the above mentioned detoxification mechanism.

The terpenes play controversial ecological and physiological roles in plant-insect relationships (Rand et al., 2014), which are virtually poorly explored (Rostás et al., 2013). The terpenes are probably involved in the defense against pathogens and predators of galling insects (Rostás et al., 2013) or as attractants for gall natural enemies, such as the parasitoids (Tooker and Hanks, 2006).

The α-bergamotene and the spathulenol are sesquiterpenes identified exclusively in non-galled stems. The a-bergamotene was previously identified as traces (<1%) in leaves of *S. polygamus*, and may possibly attract predators of free-living herbivores, and can alternatively protect gall tissues against herbivores (Damasceno et al., 2010). Such an effect was found in the leaves of *Nicotiana attenuate* Steud under the attack of *Manduca quinquemaculata* Haworth, whose oral secretion can stimulate the production of a-bergamotene, which attracts a predator insect (Kessler and Baldwin, 2001). The spathulenol is part of the chemical composition of the essential oil in many plants, and has been detected in leaf extracts of *Melampodium divaricatum* Rich. and *Conyza albida* Willd. ex Sprengel, where it was related to repellency against ants and to antimicrobial activity, respectively (Hubert and Wiemer, 1985; Pacciaroni et al., 2000). Based on experimental evidences

(Hubert and Wiemer, 1985; Pacciaroni et al., 2000; Damasceno et al., 2010), we propose that the spathulenol in the non-galled stems of *S. polygamus* can function as a chemical defense against natural enemies, as well.

Current analyses detected two SM in *S. polygamus* for the first time, the a-amyrin, detected just in non-galled leaves, and the ursenal, detected exclusively in non-galled stems. The a-amyrin has been reported in leaves of medicinal and oleo- resinous plants, including some species of the genus *Schinus* (Lloyd et al., 1977; Frontera and Tomas, 1994). However, the a-amyrin has not been previously detected in leaves and fruits of *S. polygamus* examined in Chile (Erazo et al., 2006), Argentina (González et al., 2004), and Brazil (Damasceno et al., 2010). Likewise, ursenal is another triterpene that has not been reported for this plant genus or species (Murray et al., 2012). Both, a-amyrin and ursenal, are related to important biological functions against various health-related conditions, as inflammation, microbial, fungal, and viral infections and cancer cells (Liu, 1995; Hernández et al., 2012). The presence of these SM in the non-galled tissues of *S. polygamus*, support the folk medicinal uses of this species, such as the antipyretic, anti-inflammatory and analgesic activities of the aerial parts of the plant (Erazo et al., 2006), but seems to be impaired by gall induction.

4.3. *Convergent and divergent adaptive traits in the two types of galls*

The structural analyses of the stem and leaf galls of *S. polygamus* demonstrated the typical anatomical profiles of the Anacardiaceae and *Schinus* species (Álvarez et al., 2009; Agudelo and Ricco, 2012; Agudelo et al., 2013; Dias et al., 2013a). Nevertheless, the structural and chemical profile of both host organs is quite distinct, and some convergent similarities were observed in response to the stimuli of their associated galling herbivores. Trichomes, hyperplasia of cortical parenchyma, and accumulation of phenolics were convergently induced by galling stimuli.

The overdifferentiation of trichomes and the hyperplasia of cortical parenchyma could be associated with the feeding mouth apparatus of the psyllids (piercing stylets) (Burckhardt, 2005). Such morphological features are independent of the galling herbivore taxa, but presumably associated with their feeding habits. Dias et al. (2013a) described a convergence in tissue composition of *C. duvauae* galls on *S. polygamus* and those of *A. lantanae* on *L. camara*, both with piercing stylets. The mode of feeding and the number of galling herbivores per chamber should influence cell responses and consequently the

generation of distinct gall morphotypes (Isaias et al., 2014).

However, not only the feeding habits of the gall-inducing insects are crucial to the establishment of plant cell responses, but the morphogenetic potentialities and constraints of the host plant tissues are also decisive (Isaias et al., 2014). The interactive signaling both from the galling insects and from the host plants seems to cause a gradient of stimuli (Oliveira and Isaias, 2010; Carneiro et al., 2014). This gradient may trigger the differences in the external and internal morphological features of both the stem and leaf galls on *S. polygamus*, despite of the fact that they are induced by psyllids with the same feeding habits and on the same host plant species.

The psyllid induces leaf and stem galls on *S. polygamus* (Sáiz and Núñez, 1997; Burckhardt and Basset, 2000) with different morphotypes (color and shape), nymphal chamber and organization of cortical parenchyma. Phylogenetic analyses of aphids (Stern, 1995), gall wasps (Stone and Cook, 1998), thrips (Crespi et al., 1997), and sawflies (Nyman et al., 2000) report that the galling insects, and not the host plants, determine the location, size, and shape of the galls. In *Lantana camara* L., two leaf galls, induced by different galling agents: *Aceria lantanae* Cook and *Schismatodiplosis lantanae* Rubsaamen induces distinct plant tissues reorganization, and forms typical gall structures (Moura et al., 2008), which reinforces such premise. Nevertheless, even though the influence of the host plant or host plant organs potentialities is commonly neglected, stems are considered to host simpler galls than leaves (Formiga et al., 2015). Despite, in current model of study, stem and leaf galls are anatomically similar, and the differences observed could be determined by the potentialities of the host plant organs, once the mode of feeding of both galling insects is similar. Our results support the hypothesis that the inducing agent as well as the potentialities and constraints of the host plant morphogenesis are responsible for the determination of gall morphotypes.

Also, current results indicate the accumulation of steroids as the only chemical convergent feature maintained from non-galled condition toward stem galls. Plant cells synthesize a complex array of sterol mixtures, which has essential roles at the cellular level (Hartmann, 1998). On the other hand, phenolics accumulation both in leaf and stem galls is the only remarkable chemical convergence, probably linked to the influence of the psillyids on *S. polygamus*.

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CAPITULO II

Anatomical and phenological implications of the relationship between *Schinus polygama* (Cav.) (Cabrera) (Anacardiaceae) and the galling insect *Calophya rubra* (Blanchard) (Hemiptera: Psylloidea)

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ABSTRACT

- The success of galling insects could be determined by synchronization with host plant phenology and climatic conditions, ensuring suitable oviposition sites for gall induction and food resources for their survivorship. The anatomical, histochemical and phenological synchronization strategies between *Calophya rubra* (Blanchard) (Hemiptera: Psylloidea) and its host, the evergreen plant *Schinus polygama* (Cav.) (Cabrera) (Anacardiaceae), in the Mediterranean climate of southern Chile was herein evaluated, and was compared to that of the congeneric *C.* cf. *duvauae* (Scott) from Brazil and closely related host plant *S. engleri* in the subtropical climate.
- The anatomical, histometric, histochemical and vegetative phenology study of the stem and galls was conducted from June 2015 to December 2016.
- Based on the anatomical, histometric and histochemical analysis, the conical stem gall traits imply in gains over the non-galled stem toward the galling insect survivorship, but the maintenance of phellem, secretory ducts and pith are indicative of conservative developmental traits that cannot be manipulated by *C. rubra*. Our results indicate that the climatic conditions of the Mediterranean climate zone limit *C. rubra* immatures' activity during unfavorable periods, which probably determine a diapause period and a univoltine life cycle, which are peculiarities of the *S. polygama - C. rubra* system.
- The synchronization between development and seasonality confers the peculiarities to the *S. polygama - C. rubra* system in Mediterranean climate zone.

Keyboard: conical stem gall; univoltine; diapause; Mediterranean Region; life cycle

INTRODUCTION

Galls are abnormal plant structures developed under the stimuli of a gall-inducing animal, fungus, bacterium or virus, which governs tissue neoformation (Mani 1964; Oliveira & Isaias 2010a). The specific plant tissues differentiated at gall developmental site provide shelter, protection and nutrition to the gall inducing organisms and their descendants (Shorthouse *et al*. 2005). The ability to induce galls on plants is a specialized habit within the broad context of herbivorous insects (Raman 2011). The relationship between host plants and their galling herbivores is highly specific (Abrahamson & Weis 1997). This specificity is strongly linked to the choice of site, time of oviposition, feeding habits of the galling herbivore, local host plant abundance (Gonçalves-Alvim & Fernandes 2001), and the systems of recognition established with plant surface traits (Eigenbrode & Jetter 2002).

Particularly, the synchronization with host plant phenology is a critical event for galling herbivores, as time lag in synchronization will determine quality and quantity of available food resources (Kerslake & Hartley 1997; Yukawa 2000; Oliveira *et al*. 2016). Due to this requirement, there is a strong tendency in temperate regions among galling insects towards univoltinism (Weis *et al*. 1988; Hodkinson 2009), probably due to the availability of responsive tissues restricted to specific seasons of the year (Rohfritsch & Anthony 1992). However, multivoltine life cycles may occur in those parasites of evergreen plants; apparently, the availability of resources nd environmental conditions, determine this behavior (Carneiro *et al*. 2013; Carneiro *et al*. 2015).

The crucial morphogenetic changes for gall development involve anatomical and metabolic changes (Rohfritsch 1992). Accordingly, cell hypertrophy, tissue hyperplasia, inhibition of some developmental programs and cytological changes may occur (Mani 1964; Oliveira *et al.* 2011; Ferreira *et al*. 2017a). Even though galls are usually observed in plant vegetative organs (Araújo *et al.* 2011; Isaias *et al.* 2013; Guimarães *et al*. 2014; Mendonça *et al*. 2014), few studies particularly into the development of stem galls and their subsequent effects on this organ are conducted (Ferreira & Isaias 2013), in particular for plants from Mediterranean Region.

Schinus polygama (Cav.) (Cabrera) (Anacardiaceae) is an evergreen shrub, previously considered native to Argentina, Brazil and Chile (Rodríguez 2011), which hosts some gall morphotypes on its leaves and stems (Sáiz & Núñez 1997; Burckhardt & Basset 2000; Moreira *et al.* 2012; Dias *et al.* 2013a; Guedes *et al.* 2016). The galls induced by *Calophya* cf. *duvauae* (Scott) on leaf of *S. polygama*, have been previously described in Brazil (Dias

et al. 2013a, b). However, the taxonomy of genus *Schinus* is confused in South America and it is still under review. Probably *S. polygama* is distributed in Chile and in adjacent Argentina and *Schinus engleri* (Barkley) in Brazil (Burckdardt, D. personal communication). In response to this information, in this work it is considered that *C.* cf. *duvaudae* induces galls in leaves of *S. engleri* and not in the leaves of *S. polygama* as it has been considered by Dias *et al.* (2013a, b).

Usually, plants adjust their phenological events to local climatic conditions, which is followed by the associated galling organisms (Yukawa & Akimoto 2006). As *S. polygama* is a perennial species with potential responsive oviposition sites along the year, its association with gall inducing insects with multivoltine life cycles are expected. The galls induced by a multivoltine insect, *C.* cf. *duvauae* on leaves of *S. engleri* have been previously described in transitional zone between tropical and temperate climates of southern Brazil (Dias *et al.* 2013a, 2013b). Although phenological adjustments between the host plant and the galling insects have been studied in the Neotropic (see Oliveira *et al.* 2016), in the Mediterranean climate are yet to be investigated; especially the phenological events of the life cycles of calophyids and *S. polygama*.

Anatomical studies on galls induced by calophyids have been restricted to leaf galls (Oliveira & Isaias 2010a; Isaias *et al*. 2011; Dias *et al*. 2013a), and just recently anatomical and morphological characteristics of the conical stem gall on *S. polygama* have been described (Guedes *et al*. 2016). The conical gall morphotype is the most frequent in stems of *S. polygama* in the Mediterranean Region of southern Chile (Guedes *et al.* 2016). It is induced by *Calophya rubra* (Blanchard) (Hemiptera: Psylloidea), which has been described as a bivoltine species in Chile (Saiz & Núñez 1997; Burckhardt & Basset 2000).

In leaves of *S. engleri*, the feeding activity of a co-generic species, *C.* cf. *duvauae*, induces parenchyma homogenization and the neoformation of vascular bundles and trichomes (Dias *et al*. 2013a). Assuming the gall inducer's feeding behavior as the determinant factor for the anatomical peculiarities of the galls (Stone & Schönrogge 2003), it is expected that the processes of cell differentiation and redifferentiation induced by *C. rubra* and *C.* cf. *duvauae* in the stems of *S. polygama* and leaves of *S. engleri* should be similar. Accordingly, host organ should impose morphogenetical and phenological constraints, which should explain gall peculiarities. To assess these premises, we linked the phenological and anatomical development of the conical stem gall with the life cycle of *C. rubra*, foccusing on the following questions: (i) did distinct

climate zones drive distinct galling synchronization strategies in the same evergreen host plant species? And (ii) what are the traits of stem conical gall over the host stem morphogenetical potentialities? These questions should address the peculiarities of *S. polygama* - *C. rubra* system in comparison to a cogeneric galling insect, *C.* cf. *duvauae* over closely related host plant *S. engleri*.

MATERIALS AND METHODS

Sampling and collection

Current study was carried out in a population of *S. polygamus* in Chile, Biobío Region, Ñuble Province, Chillán Viejo, Chile, kilometer 4 (36°39'32"S 72°16'43"W, at 150 m.a.s.l), between July 2015 and March 2016. Plant identification was confirmed by specialists from the Department of Botany at University of Concepción (CONC). A voucher specimen was deposited in CONC under the accession number 180330. Meteorological data were obtained from Weatherbase (http://www.weatherbase.com).

The vegetative phenology was monitored from June 2015 to December 2016. Ten individuals of *S. polygamus* were randomly selected, based on the presence of axillary buds and new branches, which were visually inspected monthly. Ten branches were collected from each tree, stored in plastic bags, and transferred to the Laboratory of Natural Products Chemistry at the University of Concepción. There, each conical stem gall (CSG) (n= 5 for each individual, total of 50 galls) was dissected with a razor blade under stereomicroscope, and grouped considering the insect instar. Nymphs were collected and preserved in 70% ethanol and sent to the Naturhistorisches Museum of Switzerland (NHMB) for species identification. The insect vouchers are deposited in NHMB under accession numbers NMB-PSYLL0004268– NMB-PSYLL0004274 and NMB-PSYLL0004276– NMB-PSYLL0004282.

Sample fragments of non-galled stems (NGS) and CSG, at four developmental phases, were collected from individuals of *S. polygama* (n= 5) for anatomical, histometric and histochemical analyses. Galls in four developmental phases were sorted according to the instar. Growth and development, phase I (GDI): first instar nymphs; growth and developmental, phase II (GDII): second, third, and fourth instar nymphs; maturation phase (MP): fifth instar nymph; and senescent phase (SP): open gall without insect.

Anatomical and histometric analysis

After each sampling, fragments of NGS and CSG were fixed in 4% Karnovsky (2.5% glutaraldehyde and 4.5% formaldehyde in phosphate buffer in 0.1 M, modified to pH 7.2, O'Brien & McCully 1981) for 24 h, FAA (37% formaldehyde, glacial acetic acid, and 50% ethanol, 1:1:18, v/v, Johansen 1940), or 2.5% glutaraldehyde (Karnovsky 1965), and subsequently stored in 70% ethanol. Permanent slides were prepared by dehydrating tissue fragments in an n-butyl series, and embedding in Paraplast® (Kraus & Arduin 1997). Sections were produced using a rotary microtome (12-18 μm), the slides were stained with 0.5% astra blue and safranin (9:1 v/v, Bukatsch 1972), and mounted in clear varnish (Paiva *et al*. 2006).

Histometric and cytometric data were obtained from photomicrographs of NGS and mature CSG (n=5), using the AxioVision LE software (CarlZeiss MicroImaging, Jena, Germany). Five different sections were used for each organ and five measurements were taken for each section. In order to compare NGS and galls in MP, cortex thickness, height and width of cells in the NGS and in middle cortices of CSG were measured.

Histochemical analyses

Histochemical analyses were performed for the detection of polyphenols, lignins, suber and lipids, according to Ferreira *et al*. (2017b). Gall samples in GDI were fixed in Karnovsky's solution (Karnovsky 1965) and hand-sectioned. Stem and gall samples, in GDII and MP, were fixed in Karnovsky's solution, embedded in polyethylene glicol (PEG) (Ferreira *et al*. 2014, 2017b) and sectioned in rotary microtomes. Each section (20-40 μm) was tested with the following reagents: Sudan Black B and Sudan IV to detect suber and lipids (Jensen 1962), Maule's test for lignins, iron sulphate and 3% ferric chloride for polyphenols (Johansen 1940). Blank sections, without staining, were also mounted and analyzed for comparison. Digital images were obtained with an optical photomicroscope Zeiss® Primo Star.

Statistical analysis

Student's T test was used to compare NGS and CSG for each of the independent variables. Data normality was verified with the Shapiro-Wilk test. Levels of significance for all statistical analyses were carried out using InfoStat V. 2016, considering $p \leq 0.05$ (Rienzo *et al.* 2013).

RESULTS

Schinus polygama and gall description

Schinus polygama (Fig. 1A) individuals grow throughout the year, with sprouting peak during spring (September - November). Inflorescences were produced in the middle of the spring and fruiting occurred during the dry season (December to January). Four gall morphotypes can be observed on *S. polygama*: one bud gall and one fusiform stem gall induced by Lepidoptera: Cecidosidae (Fig. 1B, C), one globoid leaf gall, induced by *Calophya mammifex* (Burckhardt & Basset) (Psylloidea) (Fig. 1D) and a conical stem morphotype (Fig. 1E), the main focus of this study. This conical gall is induced by the first-instar nymphs of *C. rubra* in the young stems of *S. polygama*.

The CSG of *C. rubra* occur at the young stems (Fig. 1E), petioles (Fig. 1F), and occasionally leaves (Fig.1G). Galls at the beginning of GDI are small conical protuberances with a large amount of white trichomes emerging from the tip center (Fig. 1E). Conical stem gall coloration varies slightly, from green at the beginning of GDI (Fig. 1E) to brown in almost all gall development (Fig. 1H). Through GDII, trichomes turn coppery and disappear at the end of the MP. The GD and MP are characterized by an increase in gall size, and change in shape at the end of MP, when the gall turns from conical (Fig. 1E) to globose (Fig. 1H). At this time, the gall breaks in an apical cross-shaped openning (Fig. 1I). Grouped galls induced by *C. rubra* may occur, and in this case, they take varied and indefinite forms, and each gall shelters one chamber with a single galling insect; insects of distinct gall chambers are often in different instars. Senescent galls remain on the stems and the cross-shaped opening can be still observed (Fig. 1I).

Gall phenology

Gall growth and development started in November and lasted until October. Between December and July, only the first three instars are observed in galls, indicating a period of slow insect development due to diapause. The diapause period coincides with the driest months (December from February), the warmest (January), the wettest (June), and the coldest (July) (Fig. 2), typical of the Mediterranean climate of southern Chile. From August to November, coinciding with the peak of leaf sprouting, all instars were observed, with prevalence of the fourth and fifth instars. The presence of different instars of *C. rubra* during the sampled time indicates an asynchrony in the oviposition of the female. It appears that the species is univoltine. In early October, some

galls reached maturation, which lasted until December when the last senescent galls were registered. Gall maturation ocurred during the flowering stage of *S. polygama*, which begins in October and lasts until December.

Fig. 1. Galls on *Schinus polygama* (Anacardiaceae). A) Host plant in natural habitat. B) Stem branch with bud galls. C) Stem branch with fusiform galls. D) Mature leaf with several globoid galls. E-I) Conical galls induced by *Calophya rubra*: E) On a stem, at the beginning of growth and development, with white trichomes emerging from its tip center (arrow), F) On petioles, G) On the leaf (arrow), H) Mature globoid galls. I) Senescent gall with the cross-shaped opening. Scale bars: F: 2 mm; C, D, H: 5 mm; G: 0.5 cm; E: 1 cm; I: 1.5 cm; B: 10 cm. Fuente: Elaboración propia

Anatomical features of the host stem

The host stems may be divided into cortex, vascular system and pith (Fig. 3A), from the outer to the inner regions. Externally, the bark of *S. polygama* is flaky and gray, but in an anatomical cut phellogen and phelloderm could not be clearly discerned (Fig. 3B). The phellem consists of two or more layers of polygonal cells (Fig. 3B, C). The cortex is 7-22 layered (3C), with elongated and narrow cells that containing lipids and polyphenols (Fig. 3D, E). Secretory ducts and pericyclic fibers are distributed within the cortical parenchyma (Fig. 3C). The secondary xylem has vessel elements and numerous fibers (Fig. 3F). Vessel elements are

interspaced by radial parenchymatic cells and xylem fibers with lignified cell walls (Fig. 3F, G). Both secondary phloem and xylem parenchymatic cells contain polyphenols (Fig. 3G). The pith is cylindrical, with polygonal to globose cells and lignified walls (Fig. 3H).

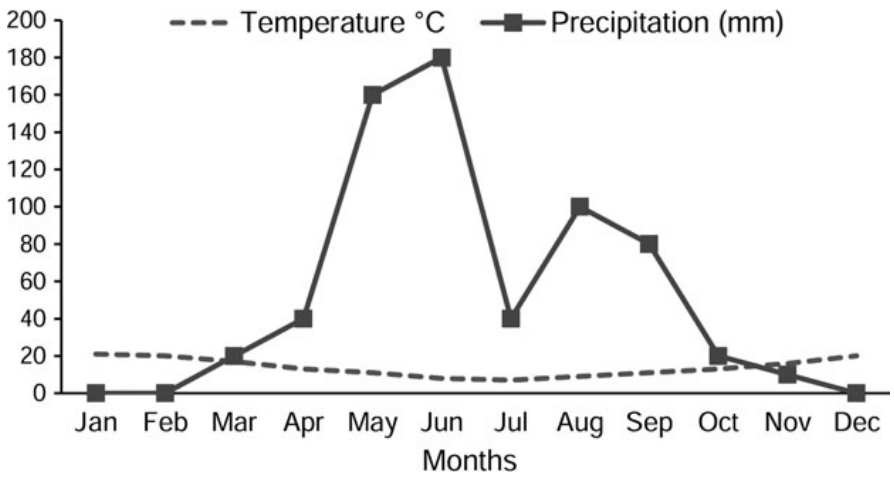

Fig. 2. Monthly record of temperature and precipitation average of Chillán, Chile, in the period 2010 to 2016 (Data from http://www.weatherbase.com). Fuente: Elaboración propia

Anatomical and histometrical features along gall development

The oviposition takes place through leaf gaps (parenchymatic space in the vascular cylinders of primary stems, occurring in the regions of the leaf trace divergence), in the stem cortex or in the parenchyma of the axillary bud (Fig. 4A, B). The beginning of the first instar nymph feeding stimulates the hyperplasia and hypertrophy of cortical cells (Fig. 4C). The enveloping cortical emergences then curve covering the nymph, which becomes enclosed inside the gall chamber. There is no tissue welding in the contact of the emergences, but only the overlapping by trichomes, which originate from modified epidermal cells (Fig. 4C). In GDI phase, three tissue layers - the inner, middle, and outer cortex - are observed in the gall wall, formed by the covering tissues (Fig 4C). Epidermis can still be observed during this phase (Fig. 4D), however, its replacement by phellem with deposition of suberin was detected (Fig. 4E). The outer cortex is continuous to the stem cortex (Fig. 4F). Middle cortex is formed by a parenchyma with large polygonal cells without phenols (Fig. 4G). New vascular cells redifferentiate within gall middle parenchyma and connect to those of the host organ, through radial parenchyma (Fig. 4H). Few neoformed vascular units are observed at the GDI, which formed from vascular bundles that develop to vascular cambium. Neoformed vascular units are just interspersed to cortical parenchyma, and surround the stem secondary vascular system and nymphal chamber to which it is oriented (Fig 4H). The inner cortex surrounds the nymphal

chamber (4I), and has periclinally elongated cells with phenols and lipids (Fig. 4J, K). Secretory ducts are rarely observed in gall developmental sites. The apical portion of the gall is not vascularized, and has isodiametric parenchyma cells.

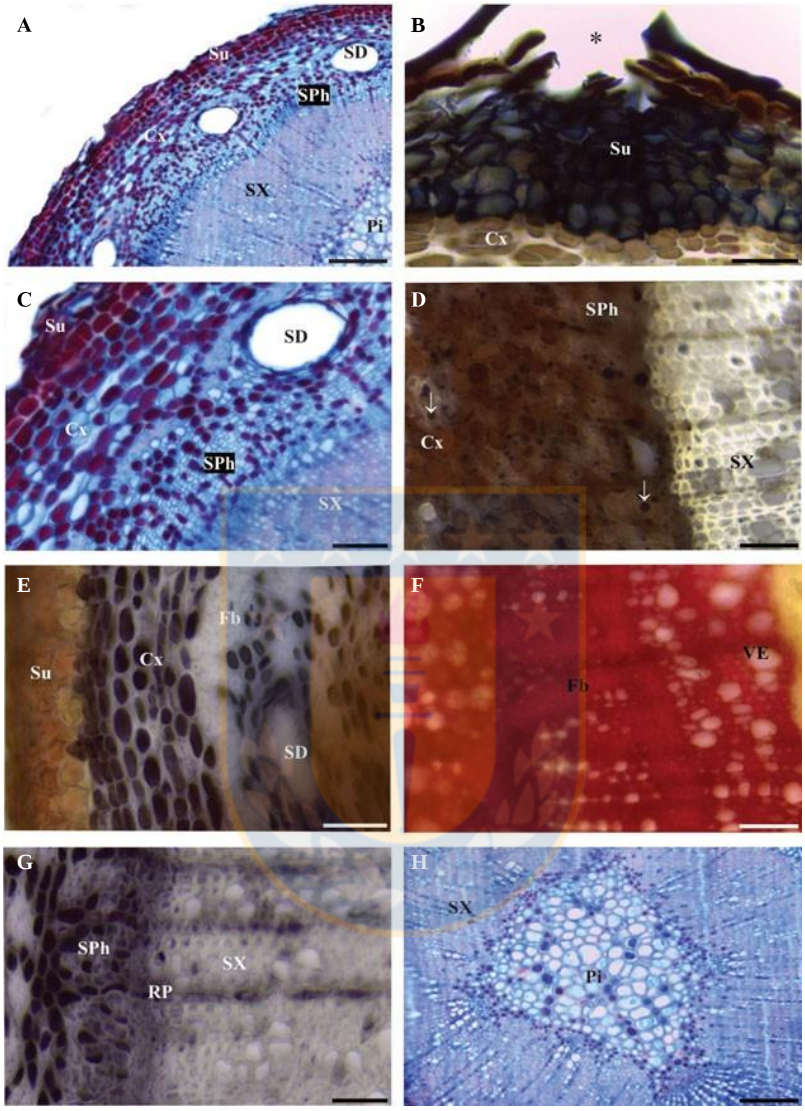

Fig. 3. Stem anatomy of *Schinus polygama* (Anacardiaceae). A) Cross section of young stem with secondary growth. B) Phellem layers with suber stained in black. C) Multilayered cortex and phellem. D-E) Cortex cells: D) Lipids droplets (arrows) in black, E) Phenolics in black. F) Fibers on secondary xylem in red. G) Phenolics in secondary phloem and xylem parenchyma cells stained in black. H) Rounded pith. Staining: A, C, H) Astra blue and safanin; B, D) Sudan black B; E, G) Iron(II) sulphate; F) Maule's reagent. Abbreviations: Cx: cortex, Fb: fibers, Pi: pith, RP: Radial parenchyma, SD: secretory ducts, SPh: secondary phloem, Su: suber, SX: secondary xylem, VE: Vessel elements, (*) lenticel. Scale bars: B-E, G: 50 µm; A, F, H: 200 µm. Fuente: Elaboración propia

The most relevant changes observed at the end of GDII are an increase in gall size and number of neoformed vascular units, eminently phloematic. The deposition of phenols increases in the inner cortex, and accumulate in some cells of the middle cortex (Fig. 4L). Lipids are present throughout gall parenchyma (Fig. 4M). Increase in vascular units is accompanied by the development of large vascular parenchyma cells in the middle cortex (Fig. 4N). Epidermis is totally replaced by the periderm, and deposition of suberin (phellem) is observed (Fig. 4O).

In the MP, phenolics accumulate in all three layers of gall cortex (Fig. 4P). At this phase, large vascular units with lipid-rich parenchymatic cells, without phenolics, are observed (Fig. 4Q, R). At the end of MP, the nymph of *C. rubra* completely occupies the nymphal chamber, and its terminalia is arranged towards the ostiolar opening. Presumably, the pressure exerted by the terminalia on the ostiolar opening, causes the cross-breaking of the gall. Then, the fully mature *C. rubra* adult emerge and the galls enter in the SP, when suberization occurs in the ostiolar opening, inner and middle cortex (Fig. 4S). Finally, senescent galls are totally wilted and dry, but remain attached to the stem.

The thickness of cortical layers significantly increases along gall development in comparison to the NGS (Fig. 5A). In addition, there is hypertrophy of the cells of the middle cortex, which increases diameter and height with regard to cortex cells of the NGS (Fig. 5B, C).

DISCUSSION

Phenological adjustments favoring the univoltine gall life cycle

Current analyses could diagnose a phenological adjustment between *S. polygama* and *C. rubra* in the population herein evaluated. It means that there is a synchrony; the insect reproductive maturation must match the most relevant host plant phenological stages for its development (Yukawa & Akimoto 2006; van Asch & Visser 2007). Currently, *C. rubra* time for oviposition coincides with *S. polygama* leaf flushing, which seems to be a successful strategy for its life cycle completion (Hodkinson 2009). Such synchrony has been previously observed for other Neotropical gall systems, such as *Copaifera langsdorffii* (Jacq.) (L.) - Cecidomyiidae (Oliveira *et al*. 2013), *Aspidosperma macrocarpon* (Mart) - *Pseudophacopteron longicaudatum* (Malenovský, Burckhardt, Queiroz, Isaias & Oliveira) (Phacopteronidae) (Castro *et al*. 2013), A*spidosperma australe* (Müll.Arg.) - *Pseudophacopteron aspidospermii* (Malenovský, Burckhardt, Queiroz, Isaias & Oliveira) (Phacopteronidae), and *Aspidosperma spruceanum* (Müll.Arg.) - Cecidomyiidae (Campos *et al*. 2010). The period of leaf flushing corresponds to

the preferential time for gall induction, because young tissues are more responsive to the galling insects' stimuli (Rohfritsch & Anthony 1992).

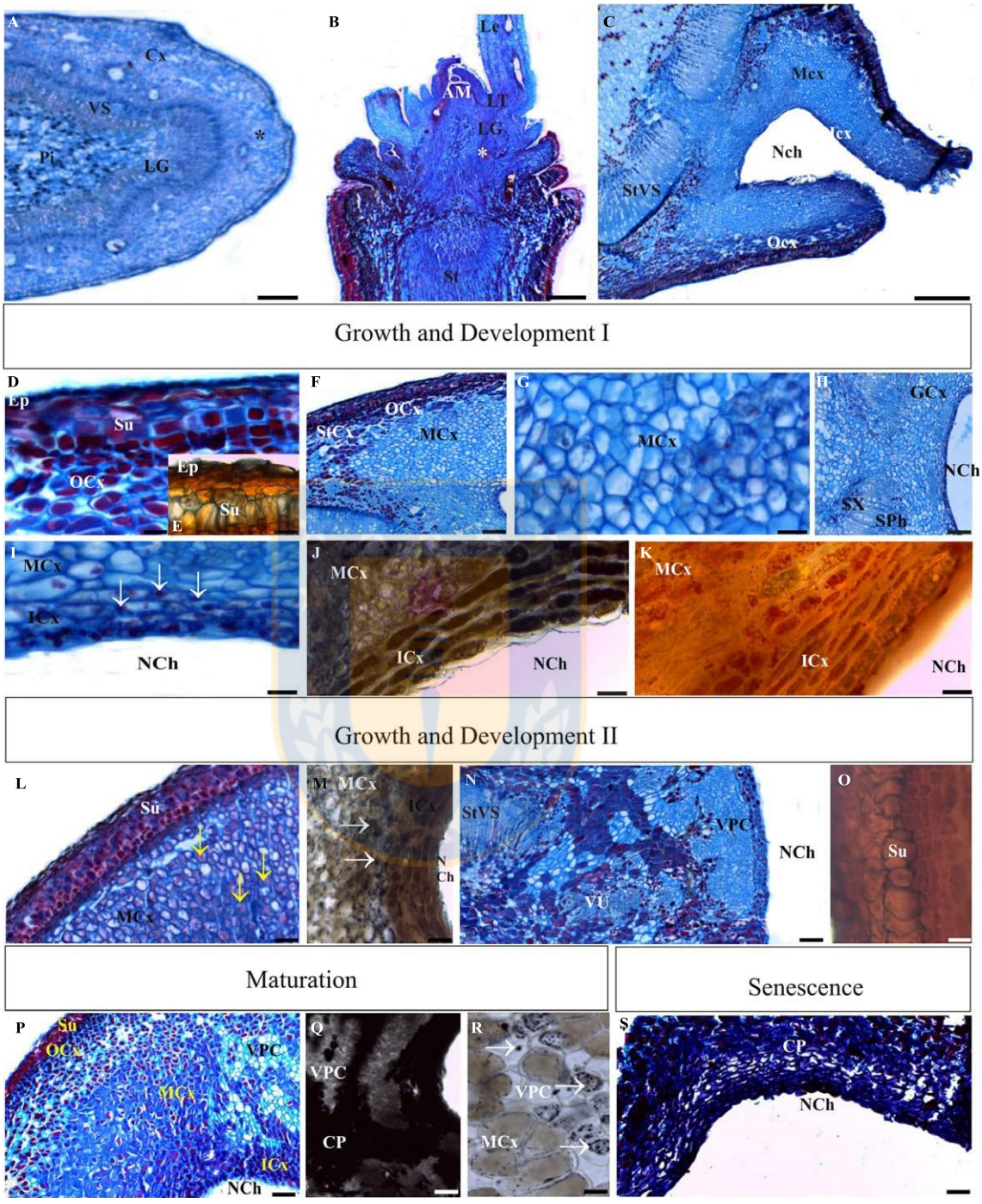

Fig. 4. Development of the galls of *Calophya rubra* (Psylloidea) on the stem of *Schinus polygama* (Anacardiaceae). A) Cross section of a young stem, showing establishment site of *C. rubra* (*). B) Longitudinal section of axillary bud, showing establishment site of *C. rubra* (*). C) Cross section of the young conical stem gall with tissue hyperplasia and cell hypertrophy. **D-K) Cross section of galls in GDI:** D) Phellem formation pushed the epidermis, E) Detail of phellem detected with suberin in black,

F) Continuum between the gall outer cortex and the stem cortex, G) Middle cortex with large polymorphic parenchyma cells, H) Neoformed vascular unit joining those of the stem, I-K) Inner cortex: I) Periclinally elongated cells, J) Phenolics in black, K) Lipids droplets in red. **L-O) Cross section of galls in GDII:** L) Middle cortex cells containing phenols (reddish), M) Lipids droplets in gall parenchyma in black (arrows), N) Basal region of the gall, highly vascularized and with vascular parenchyma cells, O) Suberin deposition detected in black. **P-R) Cross section of mature galls**: P) Apical region of gall with parenchyma cells containing phenolics (reddish), Q-R) Vascular parenchyma cells: Q) Negative reaction to polyphenols using iron(III) chloride reagent, R) Lipids detected in black. S) Phellem in the inner and middle cortex of a senescent gall. Staining: (A-D, F-I, L, N, P, S) astra blue and safranin; (E, M, O, R) Sudan black B; (J,Q) iron(III) chloride; (K) Sudan red B. Abbreviations: AM: apical meristem, CP: cortical parenchyma, Cx: cortex, Ep: epidermis, GCX: gall cortex, ICx: inner cortex, Le: leaf, LG: leaf gap, LT: leaf trace, MCx: middle cortex, NCh: nimphal chamber, OCx: outer cortex, Pi: pith, SPh: secondary phloem, StCx: stem cortex, StVS: stem vascular system, Su: suber, SX: secondary xylem, StVS: stem vascular system, VPC: vascular parenchyma cells VS: vascular system, VU: vascular unit. Scale bars: D, E, G, I-K, M: 50 µm; F, H, L, N-P, Q-S: 200 µm; A-C: 500 µm. Fuente: Elaboración propia

As sessile organisms, it is expected that plants adjust their phenological events to local climatic conditions, which is followed by the associated galling organisms (Yukawa & Akimoto 2006). For Psylloidea in the Neotropics, the availability of resources and environmental conditions favor multivoltine life cycles (Weis *et al.* 1988; Lima 2008; Dias *et al.* 2013a, b). In Southern Brazil, *C.* cf. *duvauae* establishes a multivoltine life cycle associated to *S. engleri* (Dias *et al*. 2013b). Nevertheless, the Mediterranean climate seems to impose a distinct phenological strategy for *C. rubra*, which established a univoltine life cycle with gall induction restricted to spring. Based on the fact that diapause provides the timing and synchronization mechanisms through which psyllids are able to survive unfavourable periods (Canard 2005; Hodkinson 2009), we propose that the climatic conditions of the Mediterranean Region limit *C. rubra* nymphs' activity during unfavourable periods, and could determine a diapause period and the univoltine life cycle.

For *C. rubra*, once diapause is broken, feeding and development resume in spring, with adult emergence soon afterwards, during the flowering period of *S. polygama*. This behavior has been observed among insects associated to temperate evergreen plants, such as *Strophingia ericae* (Curtis) on *Calluna vulgaris* (L.) (Hodkinson *et al*. 1999) and *Psylla buxi* (L.) on *Buxus* (Hodkinson 2009), and could imply in a period of adequate availability of host plant resources

(Yukawa & Akimoto 2006). In general, at the onset of reproduction, plants increase the allocation of resources to reproductive stems, resulting in an increase in the quality of phloem sap in flowering/fruiting parts (Salisbury & Ross 1992; Quental *et al*. 2005), which indirectly may determine the maturation of the CSG and the hatching of *C. rubra*.

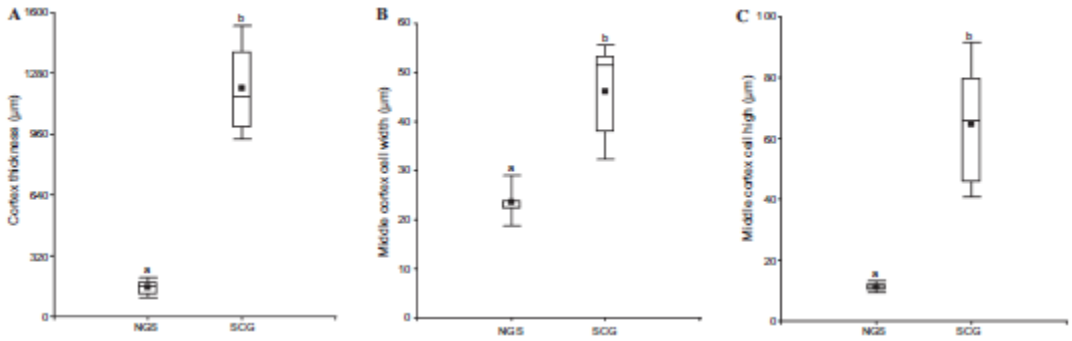

Fig. 5. Cytological and histometrical analyses of cross sections of non-galled stem (NGS) of *Schinus polygama* (Anacardiaceae) and mature conical stem galls (CSG) of *Calophya rubra* (Psylloidea). Bars with different letters are significantly different (Student's t; $P \leq 0.05$). Fuente: Elaboración propia

Traits of stem conical gall through its development

The site of the CSG establishment, the cortical parenchyma, has the most plastic of the plant cells, which retain the capacity to reassume meristematic activity and redifferentiate (Lev-Yadun 2003). Due to parenchyma cells potential large pool of cell responses to galling stimuli (Oliveira & Isaias 2010a; Ferreira & Isaias 2013), leading cell hypertrophy and hyperplasia, feeding sites for the nymphae and the major bunch of gall wall are produced. Currently, the gall wall is consequence of a curvature of the tissues around the nymphae, characterizing a covering gall (Shorthouse & Rohfritsch 1992), which develop throughout the proliferation of the epidermal and outer cortical cells after gall induction in stem surface (Shorthouse & Rohfritsch 1992, Isaias *et al*. 2014a), and may also have trichomes in the ostiolar opening.

Typical gall-inducing phloem-feeders are able to modify the organization of their main feeding site, the plant vascular tissues, toward an increment in food supply (Wool 2004; Álvarez 2011; Álvarez *et al*. 2014, 2016; Muñoz-Viveros *et al*. 2014; Ferreira *et al*. 2017a). The vascular units differentiated within gall cortex, mostly phloem elements and vascular parenchyma cells, guarantee the requirements of water and nutrients (Guedes *et al.* 2016) of *C. rubra*, since phloem is their food source (Wool *et al.* 1999). The increase in the number of vascular units during MP is a functional response to increased trophic activity of the inducer during this phase (Isaias *et*

al. 2014a), when it actively feeds on phloem cells (Kraus 2009). Stem galls have a well developed vascular parenchyma, probably because gall-inducing Psylloidea can feed on non-vascular tissue too, such as vascular parenchyma (Shorthouse & Rohfritsch 1992; Raman 2012). Also, the role of vascular parenchyma in the loading and unloading of sieve tubes is responsible for the photoasimilates transport and favor insect nutrition. Therefore, neoformed phloem cells are essential for inducer nourishment and for the maintenance of gall cellular machinery (Dias *et al*. 2013a).

The morphological changes in *C. rubra* galls on *S. polygama* are followed by alterations in color, shape, and increasing in size, which have been associated with the developmental stage of the gall and the age of the host organ by the time of oviposition, among other features (Isaias *et al*. 2014b). For CSG, the transition from conical to globoid shape has been associated with changes in the water status of the host plants (Sáiz & Nuñez 2000). Currently, the changes in shape have also been associated with the degree of gall maturation, and development of the galling insect, which reaches the fifth instar inside the gall.

Change in color is a natural process linked to stem aging, and it similarly occurs in galls, from green to brown. Along stem and gall maturation, there is a gradual decay of photosynthetic pigments in deeper tissues (Pilarski *et al*. 2007), which explains the color changes. Accordingly, color alteration in the CSG on *S. polygama* could be stimulated by variations in the balance between photosynthetic pigments and anthocyanin, as observed during the aging in other lignified plant species (Pilarski *et al*. 2007).

During late MP and SP, all tissues surrounding the nymphal chamber degrade as a result of the end of cell cycles. The peculiar trichomes of gall aperture are detached either by the pressure exerted by the insect terminalia on the ostiolar opening, which could facilitate the exit of the adult of *C. rubra* or by the end of gall-inducing insect feeding stimuli, as described for *S. engleri* - *C.* cf. *duvauae* (Dias *et al*. 2013b) and *Lantara camara* (L.) - *Aceria lantanae* (Cook) (Moura *et al*. 2009) systems.

Gall structural peculiarities regarding stem morphogenetical potential

Galling psyllids, such as *C. rubra*, feed by inserting their stylets into parenchyma or phloem cells (Hodkinson 2009), and induce minor modifications in their host plant tissues, thereby producing simple galls with high anatomical similarity to their host organs (Oliveira & Isaias 2010a). Nevertheless, on a quantitative basis, the CSG traits imply in gains over the NGS toward *C. rubra* success.

Qualitative analysis has elucidated gall developmental steps under the low plasticity of host stems (Formiga *et al*. 2015). As stems have low plasticity when compared to leaves, galls must develop under major morphogenetical constraints to deviate from the host organ ordinary pattern (NGS) (Formiga *et al*. 2015). The absence of alterations in the phellem, secretory ducts and pith are indicative traits of *S. polygama* stem that probably *C. rubra* cannot manipulate. In fact, the development of secretory ducts is usually conservative in gall development on distinct host plant species (Arduin & Kraus 2005; Dias *et al.* 2013a; Amorim *et al.* 2017; Ferreira *et al.* 2017a). Regarding the outer protective cell layers, the suberized cell walls of the phellem in CSG on *S. polygama* is a pre-existing ordinary developmental pathway of the plant secondary stems (Esau & de Morretes 1974), which may be positive for plant surviving in the hot and dry summer of the Mediterranean climate of Chile (Giorgi & Lionello 2008). Moreover, suberization and lignification confer physiologically important plant-environment interfaces, for suber and lignin may act as barriers that limit water and nutrient transport, and protect plants from the invasion of pathogens (Franke & Schreiber 2007). Likewise, the presence of lignins in perivascular fibers, secondary xylem and pith parenchyma of NGS, is important for plant support as well as for waterproofing (Boerjan *et al*. 2003), as suberization and lignification in cell walls may affect the radial water transport (Franke & Schreiber 2007). Another peculiarity of the CSG cells is the non-lignification of the perivascular fibers and secondary xylem, which may favor the feeding of *C. rubra*. Accordingly, the stem gall on *S. polygama* combines the stimuli of *C. rubra* and the morphogenetic constraints of the host stems, which result in the CSG.

In the Neotropics, psyllid galls are generally parenchymatic (Isaias *et al*. 2011; 2014b; Dias *et al*. 2013a; Formiga *et al*. 2015). Particularly, the feeding activity of *C.* cf. *duvuaue* induces parenchyma homogenization, and the neoformation of few vascular bundles and trichomes in leaves of *S. engleri* (Dias *et al*. 2013a). As a peculiarity, the CSG induced by *C. rubra* on *S. polygama* seem to be structurally more complex than the leaf galls induced by *C.* cf. *duvuaue*. Non-homogenous and highly vascularized hyperplasic parenchyma, neoformation of vascular units and vascular parenchyma cells are the major differences with the leaf galls induced by *C.* cf. *duvuaue.*

The accumulation patterns of primary and secondary metabolites in gall tissue compartments is commonly related both to gall inducing taxa and feeding behavior (Bragança *et al*. 2017). In the CSG, polyphenols accumulation in the outer compartment seems to vary

during gall development, due to the influence of the biotic stress of gall induction over their biosynthesis (Trabelsi *et al*. 2012). The major detected polyphenol in the CSG on *S. polygama*, the pyrogallol, has been considered to act in chemical defense against natural enemies (Guedes *et al*. 2016). Nevertheless, polyphenols may also play important roles in developmental, physiological and structural processes (Hattenschwiler & Vitousek 2000; Lattanzio *et al*. 2006).

In the inner compartment of CSG, lipids accumulate, which implies in a similarity with other galls induced by phloem-sucking insects (Oliveira *et al*. 2006; Oliveira & Isaias 2010b). However, while for these systems lipids accumulation have been related to the maintenance of gall structure, we herein assume an indirect participation in *C. rubra* nutrition, since it accumulation was observed in the phloem cells. Fatty acid precursors related to the increment of the nutritional value have been detected in the CSG tissues (Guedes *et al*. 2016), which is in accordance to the histochemical detection of lipids in the outer and inner tissue compartments in a centrifugal gradient along the CSG development. These high energetic metabolites must not be directly used by *C. rubra*, but their location should be related to the loading and unloading of sieve tubes, the actual feeding site of *C. rubra*. Current results of phenols and lipids accumulation in both NGS and CSG could indicate that *C. rubra* is favored by the presence of these metabolites in the host stems of *S. polygama*. Then, *C. rubra* could overpotentize lipid and phenol synthesis pathways to favor gall nutrition, maintenance and/or protection.

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CAPÍTULO III

Factors influencing the morphogenesis of galls induced by *Calophya mammifex* (Calophyidae) on *Schinus polygama* (Anacardiaceae) leaves

Article under Review in Botany

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Abstract

Environment, plant and gall-inducing insect genotypes are key factors in determining the morphogenesis of galls. However, the exact roles of these factors have not been clarified. We used anatomical and histochemical methods to evaluate dterminant factors in the final structure of galls induced by *Calophya mammifex* on *Schinus polygama* leaves, under the Mediterranean climatic conditions of southern Chile. Also, we compared mature galls with those induced by the congeneric *C. rubra* on the same host plant. *Calophya mammifex* develops a univoltine life cycle and a diapause period in the Mediterranean climatic conditions of southern Chile. Morphogenetic and histochemical leaf patterns were altered by *C. mammifex* feeding activity. For the first time, two specialized tissue compartments, a nutritive-like tissue and a common storage tissue, are reported for Calophyidae-induced galls in the Mediterranean region. Galls induced by *C. mammifex* and *C. rubra* have sufficient anatomical and histochemical alterations to be diagnosed as complex structures, whose distinction in vascular system differentiation implies structural constraints imposed by host plant organs.

Keywords: calophyids; gall; histochemistry; morphogenetic potentialities; Mediterranean climate; univoltinism

Introduction

Gall inducers are biotic factors capable of altering the morphogenetic fate of host plant cells, generating specific gall phenotypes for each host plant (Isaias and Oliveira 2014). *Schinus polygama* (Cav.) (Cabrera) (Anacardiaceae) responds differently to four different gall-inducing insects – two Hemiptera (Calophyidae) and two Lepidoptera (Guedes et al. 2018). *Calophya mammifex* (Burckhardt & Basset) (Calophyidae) induces globoid leaf galls, the most common morphotype, whereas *Calophya rubra* (Blanchard) (Calophyidae) induces conical stem galls on *S. polygama* (Burckhardt and Basset 2000; Guedes et al. 2018). Insects with similar feeding habits, such as the calophyids, are expected to influence host plant tissues similarly (Rohfritsch 1992). Moreover, plant responses to gall inducer feeding habits should also be considered, for they are specific and specialized (Meyer 1987). Galls are host plant cell build-ups, and their development must be constrained by host plant organ and cell morphogenetic potentialities (Isaias and Oliveira 2014), to consequently determine whether galls may develop complex or simple structures (Formiga et al. 2015).

Histological, histochemical, and histometric alterations, as well as the differentiation of specialized storage cells and tissues, imply distinct levels of structural complexity among galls (Ferreira et al. 2017a). Galls with little organization and tissue differentiation, several layers of parenchymatic cells, and no metabolic storage tissues are considered simple galls (Rohfritsch and Anthony 1992; Isaias et al. 2014a; Ferreira et al. 2017a). Gall complexity may be attributed to the vascular bundle development, and primary metabolite storage (Ferreira et al. 2017a), features that confer an intermediate level of structural complexity to Calophyidae-induced galls (*C.* cf. *duvauae* and *C. rubra*) (Dias et al. 2013a; Guedes et al. 2018).

Although dense trichomes, large nymphal chambers, and tissue hyperplasia have been described in *C. mammifex* mature globoid leaf galls on *S. polygama* (Guedes et al. 2016), their anatomical development remains unknown. Phenological, anatomical and histochemical peculiarities of the *S. polygama – C. rubra* system associated with Mediterranean climatic conditions of southern Chile have been recently studied (Guedes et al. 2018), and could be extended to the *S. polygama – C. mammifex* system, as evaluated in this study. Therefore, the current study model is non-galled *S. polygama* leaves, as control-organs, and galls induced by *C. rubra* (Guedes et al. 2018), a congeneric species of *C. mammifex*, for comparative analysis of structural alterations.

Although synergistic interactions between environment, host-plant genotype and inducer genotype can determine specific gall phenotype (Weis et al. 1988; Abrahamson and Weiss 1997), their roles in determining gall morphology is not clearly understood (Moura et al. 2008). The *S. polygama* superhost and *C. mammifex/C. rubra*-induced galls constitute an appropriate model to study the role of host organ constraints on gall development and on the extended phenotypes of galling herbivores. The following questions are addressed: (i) What are the diagnostic features of *C. mammifex* leaf galls on *S. polygama*? (ii) Should galls induced by *C. mammifex* and *C. rubra* have similarities? And (iii) Are there morphogenetic constraints in host leaves expressed on *C. mammifex*-induced galls?

Materials and methods

Sampling and collection site

A *S. polygama* population located in the Mediterranean climate of Chile, in Bío-bío Region, Ñuble Province, at kilometre 4 on the Itata highway (36°39'32"S, 72°16'43"W at 150 m.a.s.l) was studied The Mediterranean climate of southern Chile has cold, wet winters (June – August) and hot, dry summers (December – February) (Giorgi and Lionello 2008). Meteorological data were obtained from Weatherbase (http://www.weatherbase.com). During the study period, July 2015 and March 2016, the highest temperatures were recorded in January 2016 (36 °C), and the lowest in June, July and August 2015 (-3 °C, -1 °C and -3 °C, respectively). The month with the highest average precipitation was June (185.4 mm), and the month with the lowest average precipitation was February (2.5 mm).

For vegetative phenology, we randomly marked ten *S. polygama* individuals and observed monthly. Leaf sprouting, senescent leaves and leaf fall were evaluated visually. We collected branches with and without galls from each tree monthly, which were stored in plastic bags and transferred to the Laboratory of Natural Product Chemistry at the Universidad de Concepción. On each branch, we assessed twenty leaves (n =20) with galls by direct observation, and registered gall position as follows: apex, mid-portion, basal regions, leaf margin, and leaf lamina.

We randomly marked five trees for anatomical and cyto-histometric analysis, and collected five (n =5) non-galled mature leaves (NGL) and globoid leaf galls (GLG) from each tree at growth and development (GD), maturation (MP), and senescence (SP) stages (n =5 per developmental stage). Leaf galls were sorted according to insect instar (Guedes et al. 2018), as

follows: GD – first to fourth instar; MP – fifth instar; and SP – open gall and absent gall-inducer. In addition, conical stem galls (CSG) in maturation phase (n =5) were collected for comparison with GLG. Also, some GLG were dissected with a razor blade under the stereomicroscope and grouped according to insect instar. Nymphs were collected and preserved in 70 % ethanol, then sent to Daniel Burckhardt at the Natural History Museum of Switzerland (NHMB) for species identification. Several voucher specimens were deposited in NHMB under accession numbers NMB-PSYLL0004288 – NMB-PSYLL0004293.

Anatomical and cyto-histometric analysis

Following each collection, the NGL, GLG and CSG samples (n =5) were fixed in 4% Karnovsky (2.5% glutaraldehyde and 4.5% formaldehyde in phosphate buffer 0.1 M, modified to pH 7.2; O'Brien & McCully 1981), 2.5 % glutaraldehyde (Karnovsky 1965), or FAA (37 % formaldehyde, glacial acetic acid, and 50 % ethanol, 1:1:18 v/v/v), and subsequently stored in 70 % ethanol. Fixed samples were dehydrated in n-butyl series (Johansen 1940), embedded in Paraplast® (Kraus and Arduin 1997), sectioned (12-18 µm) with a rotary microtome (Leica 2035 BIOCUT), stained with 0.5 % astra blue and safranin (9:1 v/v) (Bukatsch 1972) and mounted with clear varnish (Paiva et al. 2006). The histological slides were observed and photographed using a light microscope (Leica® DM500) coupled with a digital camera (Leica® ICC50 HD).

Histometric and cytometric data were obtained from NGL, GLG and CSG photomicrographs, using AxioVision LE software (CarlZeiss MicroImaging, Jena, Germany). For measurements, five different sections from each sample were used, and three different cell and tissue measurements from each section were taken. To compare NGL and GLG, measurements adaxial surface cuticle thickness, adaxial and abaxial epidermal cell thickness, and the number of mesophyll cell layers were measured. In addition, parenchyma, abaxial and adaxial epidermal cell areas, in transverse sections of both NGL and GLG were measured. To compare GLG and CSG, the number of vascular bundles, the number of parenchyma cell layers in a transverse gall section, and parenchyma tissue thickness in gall walls were measured. Parenchymatous cell area and nymphal chamber area were also measured.

Histochemical analysis

For histochemical reactions, fresh samples of mature NGL and GLG (n =5) were fixed in 2.5 % glutaraldehyde and 4.5 % formaldehyde (4 % Karnovsky, 0.1 M, pH 7.2) (O'Brien and McCully 1981) for 24 h, embedded in polyethylene glycol (PEG), and sectioned (20-40 µm)

using a rotary microtome (Leica 2035 BIOCUT) (Ferreira et al. 2014; Ferreira et al. 2017b). Each section was submitted to histochemical tests for starch (Lugol's reagent, Johansen 1940), reducing sugars (Fehling's reagent, Sass 1951), lipids (Sudan IV and Sudan red B, Brundett et al. 1991), and total proteins (mercuric bromophenol blue solution and acetic acid, Baker 1958). Treated sections were mounted on glass slides with 50 % glycerin or water (Kraus and Arduin 1997), observed, and photographed with a light microscope (Leica® DM500) coupled with a digital camera (Leica® ICC50 HD), then compared with blank sections.

Statistical analysis

Student's T-test was used to compare NGL to GLG, and GLG to CSG for each independent variable. Data normality was verified with the Shapiro-Wilk test. Differences were significant at a probability of 5 % (p <0.05). Statistical analysis was performed using InfoStat software (v.2013) (Rienzo et al. 2013).

Results

Phenology and morphological description of host organ and gall morphotype

Schinus polygama leaves are simple, elliptic, and usually glabrous with a conspicuous midrib (Fig. 1A, B). The first instar of *C. mammifex* (Hemiptera: Calophyidae) induces galls on the adaxial surface of the leaf lamina, generally near the midrib of young *S. polygama* leaves (Table 1). These galls are globoid, mostly located in the median and basal portions of the leaf, and less frequently in the apical region (Table 1).

At the beginning of GD, galling insects form a globular projection on the abaxial surface of leaf lamina, with white trichomes that close the gall aperture (Fig. 1A, C). GLG are randomly distributed; number and colour can vary on the same leaf lamina (Fig. 1D), regardless of insect developmental stage. The abaxial surface can be red, green, or intermediate tones, while the adaxial surface generally remains green. Also, red and green galls are observed on the same leaf lamina (Fig. 1D). Senescent galls are dark brown and open to the abaxial surface (Fig. 1E) due to pressure exerted by adult terminalia.

Table 1. *Calophya mammifex* (Hemiptera: Calophyidae) gall position on *Schinus polygama* (Anacardiaceae) leaves

Position of galls on the leaf lamina	Total galls	(%)
Apex	66	14.9
Mid portion	211	47.5
Base	167	37.6
Leaf margin	100	22.5
Leaf inner	344	77.5
Total Galls	**444**	-

Gall induction begins in mid-spring (October), and the first galls are observed in the growth and development phase at the end of spring in Southern Chile (November) (Fig. 2). From mid-summer to late winter (January to August), only immature *C. mammifex* instars (I, II and III) are observed, indicating a diapause period in insect development. During the spring (September to November), galls open and the adults emerge, completing an annual life cycle. *Schinus polygama* is an evergreen plant, with little phenophase demarcation. A leaf-flushing peak occurs during spring, and leaves with senescent galls fall from the host plant in late spring (November) (Fig. 2).

Anatomical features of non-galled leaves and leaf galls

Mature *S. polygama* leaves are amphistomatic, with a smooth cuticle and uni-stratified epidermis (Fig. 3A, B). Leaf lamina are bifacial with two layers of palisade parenchyma facing the adaxial surface and a variable number of spongy parenchyma cell layers facing the abaxial surface (Fig. 3A). The spongy parenchyma cells are loosely organized, and both palisade and spongy parenchyma have idioblasts with crystal druses (Fig. 3B). Secretory ducts associate to vascular tissues, and are included in phloem parenchyma (Fig. 3A).

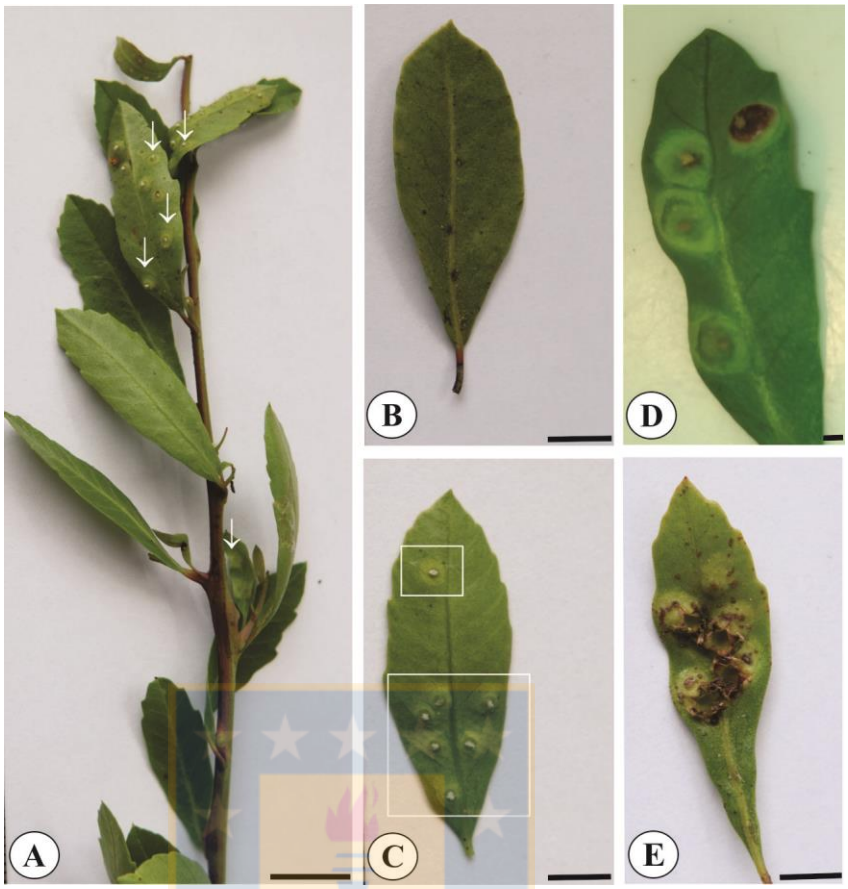

Figure 1. Non-galled leaves and *Calophya mammifex* leaf galls on *Schinus polygama*. A) Branch with young leaves and galls at the beginning of growth and development phase (white arrows). B) Mature leaf with conspicuous midrib. C) Abaxial surface of leaf lamina with abundant galls at the beginning of growth and development stage (circle) closed by white trichomes. D) Leaf lamina with abundant galls of different colors in final stage of growth and development. E) Leaf lamina with open senescent galls. Bars: 1cm. Fuente: Elaboración propia

First instar nymph feeding activity induces a globoid gall with an ample chamber, sheltering a single gall inducer (Fig. 3C). Gall establishment occurs in the mesophyll, and a homogenous parenchyma is formed by hyperplasia and cell hypertrophy. Gall walls can be divided into an abaxial parenchymatous portion and an adaxial vascularized portion (Fig. 3C). The abaxial portion is formed of 7–10 non-vascularized cell layers with periclinal elongation (Fig. 3C). The abaxial projection terminates with the gall opening, from whence the adult emerges. The gall opening is covered by lignified multicellular uniseriate trichomes (Fig. 3C). The adaxial portion can be divided into three tissue compartments: outer (OC), median (MC), and inner compartments (IC) (Fig. 3D), derived from the adaxial, median, and abaxial layers of the leaf lamina ground meristem. The IC is formed of 4–5 non-vascularized cell layers with periclinal

elongation and a uni-stratified epidermis forming the nymphal chamber outline (Fig. E). In the MC, neoformed vascular bundles occur, with well-developed vascular parenchyma (Fig. 3F). Vascular bundles are numerous and the phloem is oriented towards the nymphal chamber (Fig. 3G). A single layered epidermis with thick cuticle delimits the gall wall (Fig. 3 H).

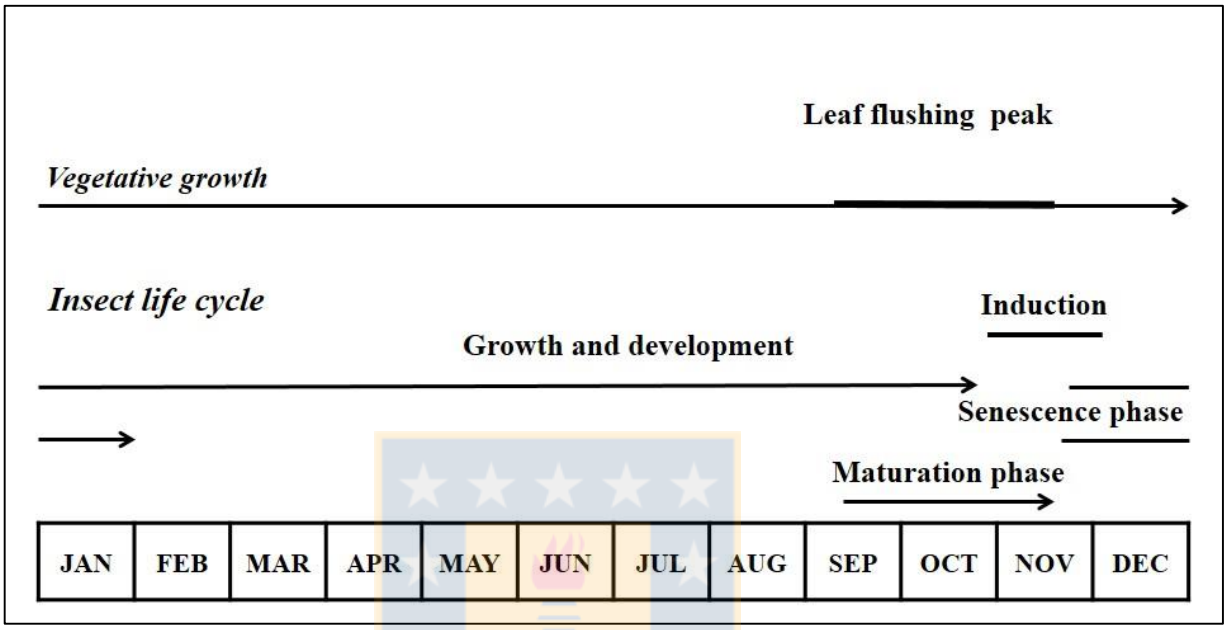

Figure 2. Univoltine life cycle of *Calophya mammifex* on *Schinus polygama* leaves. Gall induction occurs during the leaf-flushing peak (October to November). Growth and development stage ranges from November until mid-September, when galls enter maturation stage, which lasts until mid-November. The senescence stage goes from October until November, when leaves with senescent galls fall from the host plant. Fuente: Elaboración propia

During GD, galls increase in size due to continuous divisions of parenchyma cells. In the MP, a large number of vascular bundles are observed. The gall opening begins at the end of maturation, facilitating the adult's exit. The SP begins when the gall opens fully and the insect escapes (Fig. 3I). During this phase, the apical portion degrades rapidly, tissues are disorganized, and in the gall opening, suberization and trichome decay occur. Druses abound in the adaxial portion (Fig. 3D, E), and are numerous towards the abaxial portion throughout senescence (Fig. 3J).

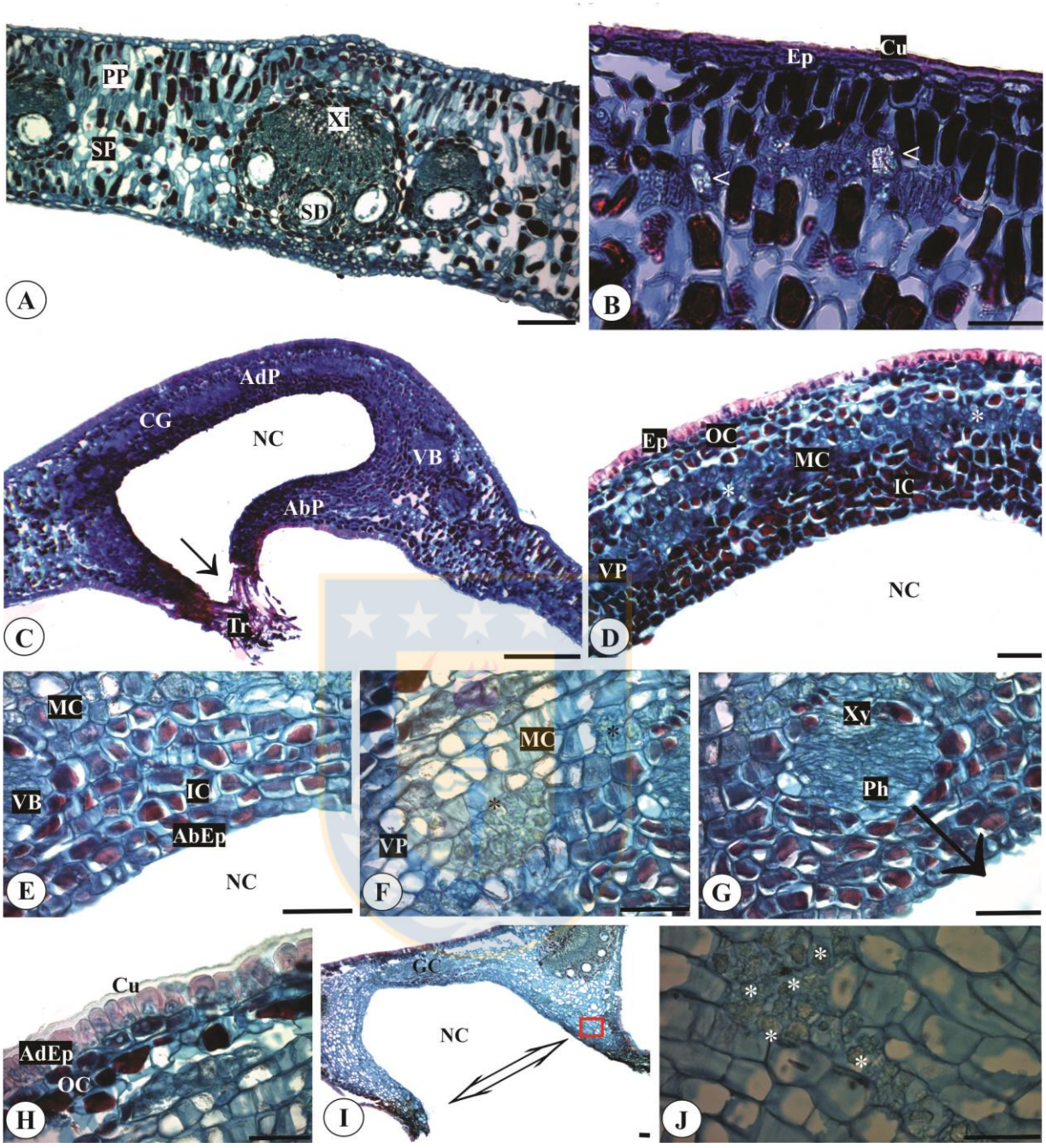

Figure 3. Leaf and gall anatomy. A-B) Mature *Schinus polygama* leaf. A) Bifacial leaf lamina with palisade parenchyma facing the adaxial surface and spongy parenchyma facing the abaxial surface. B) Adaxial epidermal surface with druses (head arrows). C-J) Leaf gall induced by *Calophya mammifex*. C) Mature gall with large nymphal chamber (arrow indicates gall opening). D) Gall cortex limited by adaxial and abaxial epidermis and divided into three compartments. E) Inner compartment with multi-layered cells of periclinal elongation and uniseriate abaxial epidermis limiting the nymphal chamber. F) Well-developed vascular parenchyma in the median compartment with abundant druses (asterisks). G) A vascular bundle in the median compartment with phloem oriented toward the nymphal chamber (arrow). H) Outer compartment limited by uniseriate epidermis with thick cuticle. I) Senescent gall with degraded,

disorganized and suberized tissues in abaxial portion (double arrow indicates separation of GO). J) Detail of red rectangle in I shows abundant druses on abaxial portion of the senescent gall. Abbreviation: AbEp: abaxial epidermis, AdEp: adaxial epidermis, AbP: abaxial portion, AdP: adaxial portion, Cu: cuticle, Ep: epidermis, GC: gall cortex, IC: inner compartment, LL: leaf lamina, MC: median compartment, NC: nymphal chamber, OC: outer compartment, GO: gall opening, Ph: phloem, PP: palisade parenchyma, SD: secretory ducts, SP: spongy parenchyma, VB: vascular bundle, VP: vascular parenchyma, Xy: xylem. Druses (*). Bars: 50 µm (B), 500 µm (C), 1 mm (A, D, I), 5 mm (E-H, J). Fuente: Elaboración propia

Histochemical profiles of leaves and galls

Starch grains were detected throughout the chlorophyllous parenchyma (Fig. 4A, B) in non-galled leaves, but they were not detected in mature galls. Reducing sugars were detected in the mesophyll, bundle sheath cells, radial parenchyma (Fig. 4C), and in the outer and inner tissue compartments, and perivascular parenchyma of galls (Fig. 4D, E). Lipid droplets were detected in the chlorophyllous parenchyma, vascular bundles, and secretory ducts in NGL, yet they were not as abundant in the epidermal cells (Fig. 4F). In GLG, lipids were detected both in outer and median compartments (Fig. 4G), as well as in phloem parenchyma cells (Fig. 4H). Proteins were observed in the spongy and vascular parenchyma of leaves (Fig. 4I). In GLG, proteins were mostly detected in median compartment cell walls (Fig. 4J).

Quantitative comparison

Every cytometric and histometric measurement was different between NGL and mature GLG, except for the abaxial epidermal cell area (Table 2). Adaxial cuticle thickness, adaxial and abaxial epidermal cell thickness, adaxial epidermal cell area, number of cortical cell layers, and parenchymatous cell area were all greater in mature galls than in NGL (Table 2). Abaxial epidermal cell area was similar in NLG and GLG (Table 2).

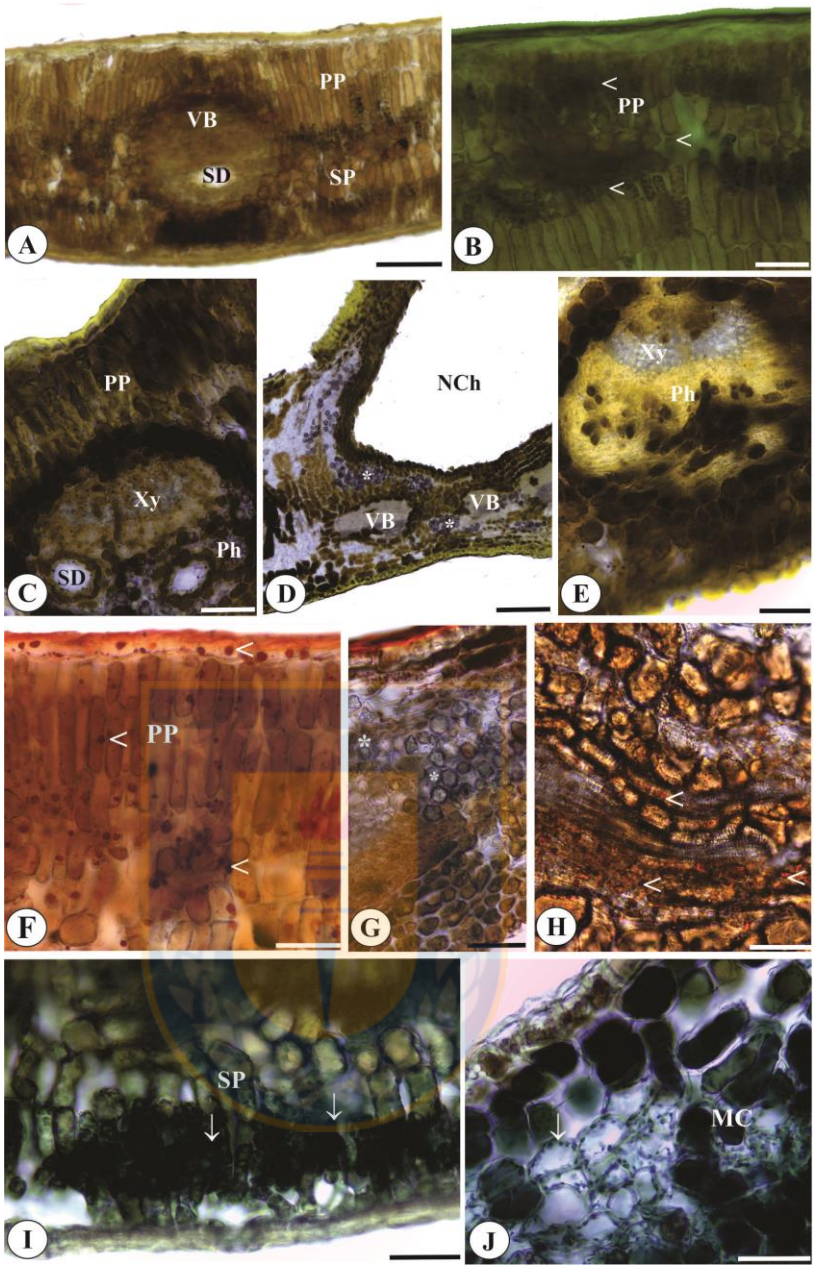

Figure 4. Histolocalization of metabolites in *Schinus polygama* leaves and *Calophya mammifex* galls. A-B) Reaction of Lugol's reagent to starch in leaf tissues, A) around vascular bundle, B) in chlorophyllous parenchyma (arrowheads). C-E) Reactions of Felhing's reagent to reducing sugars, C) in leaf chlorophyllous parenchyma, endoderm, phloem and secretory ducts, and D) in inner compartment, surrounding the vascular bundles of the gall. E) Detail of vascular bundle in the gall, with reducing sugars in phloem, endoderm and cortical cells. F) Reaction of Sudan IV to lipids in leaf chlorophyllous parenchyma (arrowheads). G-H) Reaction of Sudan red B to lipids in the gall, F) in outer and inner compartments, and G) in neoformed vascular bundles (arrowheads). I-J) Reaction to bromophenol blue evidencing proteins, I) in spongy leaf parenchyma (arrow), J) in gall median compartment (arrow). Abbreviation: MC: median cortex, NCh: nymphal chamber, Ph: phloem, PP: palisade parenchyma, SD:

secretory ducts, SP: spongy parenchyma, VB: vascular bundle, Xy: xylem. Druses (*). Bars: 50 µm (B, E-H), 200 µm (A, C, D, I, J). Fuente: Elaboración propia

Table 2. Cytometry and histometry of non-galled leaves (NGL) and mature globoid leaf galls (GLG) induced by *Calophya mammifex* (Hemiptera: Calophyidae) on *Schinus polygama* (Anacardiaceae)

Parameters	**GLG**	**CSG**	**P- value**
Cell layers (n)	14.8 ± 2.3 b	29.3 ± 2.8 a	0.0001
Gall cortex thickness (µm)	307.8 ± 54.3 b	1200.7 ± 256.3 a	0.0016
Parenchymatic cell area (µm²)	543.7 ± 65.9b	3217.8 ± 1685.1 a	0.0239
Vascular bundles/Vascular unit (n) in the median region of the gall	3.4 ± 0.4 b	7.4 ± 1.1 a	0.0001
Nymphal chamber area (mm²)	0.722 ± 0.556 a	2.060 ± 1.309 a	0.1007

The two mature gall morphotypes differed in some parameters (Table 3). The number of cell layers and the thickness of gall cortex were significantly greater in CSG than in GLG (Table 3). Parenchymatous cell area was also greater in CSG when compared to GLG (Table 3). Stem galls had more vascular units per transverse median section than GLG vascular bundles (Table 3). Nymphal chamber area was not significantly different between gall morphotypes (Table 3).

Table 3 Cyto-histometric comparison between globoid leaf galls (GLG) and conical stem galls (CSG) on *Schinus polygama* (Anacardiaceae)

Parameter	**NGL**	**GLG**	**P- value**
Adaxial cuticule thickness (µm)	6.1 ± 1.3 b	8.9 ± 1.8 a	0.0001
Adaxial epidermis thickness (µm)	16.3 ± 2.2 b	27.0 ± 6.8 a	0.0001
Adaxial epidermal cell area (µm²)	360.2 ± 86.5 b	498.4 ± 153.9 a	0.0003
Parenchyma cell layers (n)	9.3 ± 1.0 b	14.9 ± 2.8 a	0.0001
Parenchymatic cell area (µm²)	426.5 ± 145.9 b	535.5 ± 133.9 a	0.0194
Abaxial epidermis thickness (µm)	13.7 ± 2.3 b	16.5 ± 2.9 a	0.0014
Abaxial epidermal cell area (µm²)	338.6 ± 98.9 a	333.6 ± 97.0 a	0.8582

Discussion

The phenological features of *C. mammifex*-induced galls follow the expected pattern for Mediterranean climatic conditions of southern Chile. Some anatomical and histochemical

features follow peculiarities determined by gall-inducing calophyid feeding habits. Accordingly, some gall features are determined by developmental constraints imposed by *S. polygama* host organs. In stem galls, the increased vascular unit differentiation may be related to the physiological and anatomical potential of stems, with cambial and procambial meristematic regions.

Diagnostic features of Calophya mammifex galls

Schinus polygama is an evergreen species with available oviposition sites throughout the year (Guedes et al. 2018). Nevertheless, *C. mammifex* induces galls only during the spring, probably due to unfavourable environmental conditions during summer, autumn and winter in the Mediterranean climate of southern Chile. This climate has cold, wet winters and hot, dry summers (Giorgi and Lionello 2008), which can determine the diapause period and univoltine life cycle of *C. mammifex*, as previously described for *C. rubra* in the same climatic conditions (Guedes et al. 2018). Galling insect reproductive success seems to be determined by environmental conditions and host-plant characteristics (Fernandes and Price 1992; Yukawa and Akimoto 2006). Multivoltine life cycles seem to occur due to subtropical and tropical climate conditions for other psylloid galls on evergreen plants (Dias et al. 2013b; Lima 2008), and have been associated to the constant availability of resources (Carneiro and Isaias 2015). However, in seasonal climates such as the Mediterranean climate of southern Chile, inducers such as *C. mammifex* and *C. rubra* must adapt their life cycles to periods with favourable conditions (Guedes et al. 2018).

Immediately after the rainy season, temperatures increase in Mediterranean climates, which triggers a leaf-flushing peak. Such conditions seem to favour diapause breaking, adult *C. mammifex* emergence, and the induction of a new gall cycle on young leaves. For most systems, this leaf-flushing period corresponds to the preferential time for gall induction (Abrahamson et al. 1991; Gonçalves-Alvim and Fernandes 2001), since young leaves seem to be more responsive to galling insect stimuli (Rohfritsch and Anthony 1992), and could guarantee the quality and quantity of available resources for insects (Yukawa 2000; Yukawa and Akimoto 2006). In addition, the selection of oviposition site is important for gall development, as it can determine nutrient allocation (Price and Roininen 1993) and improve sink-strength generated by the gall (Larson and Whitham 1997). *Calophya mammifex* preferentially induce galls in the median portion of leaves, as other Psylloidea (Weis et al. 1988; Ferreira et al. 1990; Dias et al. 2013b) and Cecidomyiidae (Formiga et al. 2009).

Morphogenetic constraints of host leaves on C. mammifex galls

The decription of the leaf anatomy of *S. polygama* coincides with that of Dias et al. (2013a) for *S. engleri* (reported as *S. polygamus*), a closely related species from southern Brazil. Dorsiventral mesophyll with druses and the presence of secretory structures immersed in phloem parenchyma are common features among *Schinus* (Blanco 2004; Nascimento-Silva et al. 2011).

Regarding the dermal system, *C. mammifex* influence has been constrained to the cuticle of gall development sites, and trichome differentiation in gall apertures. The thick cuticle of globoid leaf galls (GLG) implies a greater investment in protection against water loss, since cuticle reflects solar rays and maintains leaf tissue temperatures (Fahn 1992), and conditions outside the gall are highly desiccating (Ramløv et al. 2000), particularly considering that spring and summer are very dry in Mediterranean climate regions (Acevedo et al. 1999). The re-differentiation of trichomes in gall aperture has also been associated with insect protection, against natural enemies as well as unfavourable environmental conditions (Guedes et al. 2016).

The histochemical profile of globoid leaf galls also reveals high constraints of host leaf metabolism once the histochemical detection of the metabolites is very similar between host leaves and galls, except for the absence of starch in galls. *Calophya mammifex*-induced galls probably have a weak capacity to synthesize photoassimilates, and function primarily as sink of resource (Weis et al. 1988; Raman et al. 2006; Álvarez et al. 2009). The accumulation of reducing sugars, proteins, and lipids in gall phloem and vascular parenchyma indicates high metabolic activity and the re-differentiation of a nutritive-like tissue (Ferreira et al. 2017a). Lipids and proteins were also detected in gall outer tissue compartments (OC), indicating their function as common storage tissues (Ferreira et al. 2017a). The accumulation of primary metabolites and druses in galls indicates a redirection of non-galled host organ patterns (Schonrogge et al. 1998), and it has been reported for Psylloidea galls in Neotropical climates (Oliveira et al. 2006; Oliveira et al. 2010; Isaias et al. 2011; Carneiro et al. 2014a, b; Malenovský et al. 2015). In Mediterranean conditions, such accumulation is a product of the peculiar reduced gall metabolism throughout summer, autumn and winter.

Peculiarities of C. mamifex-induced galls vs. C. rubra-induced galls

Cell hypertrophy and tissue hyperplasia, as observed in *C. mammifex*-induced galls, are the most common cellular processes in gall development (Mani 1964). The larger number of cell

layers and higher cells in stem galls induced by *C. rubra* than in leaf galls induced by *C. mammifex* may be explained by the activation of vascular procambium and cambium during stem gall development (Mani 1964; Guedes et al. 2018). The differential rates of hyperplasia and cell hypertrophy in distinct tissue compartments determine the distinct gall shapes (Isaias and Oliveira 2014).

The reorganization of plant vascular tissues during gall development (Oliveira et al. 2016) facilitates nutrient translocation and access to host-plant nutrients by the galling insects (Wool et al. 1999). Therefore, the neoformation of vascular bundles and a well-developed vascular parenchyma is expected for sap-feeding insects (Shorthouse and Rohfritsch 1992; Wool et al. 1999; Raman 2011), such as *C. mammifex*, which has two feeding sites, the phloem – its main food source – and the vascular parenchyma. The differentiation of new vascular tissues in GLG and CSG (Guedes et al. 2018) – mostly phloem – is directed to the nymphal chamber and grows like a mantle around the abaxial portion. The orientation of the phloem portions toward the nymphal chamber is similar to the common orientation of vascular bundles in leaves (Álvarez et al. 2009; Ferreira et al. 2017a), and favours the feeding process, as the stylets of the inducing insect can suck phloem sap without crossing the xylem.

In woody perennials like *S. polygama*, the auxin produced by growing buds in spring stimulates procambium activation in a basipetal direction (Taiz and Zeiger 2006). This stimulus may explain the neoformation of vascular units by procambium activation in CSG, which is the most conspicuous difference between leaf and stem galls in *S. polygama*,. This peculiarity is evidence that the host organ's morphogenetic potential determines gall anatomy.

Despite the quantitative differences in tissue layers and vascular tissues, there are numerous convergences between GLG and CSG. Many anatomical characteristics of the two gall morphotypes are likely conserved traits in Psylloidea, particularly in Calophyidae-induced galls (*C. mammifex*, *C. rubra*, and *C.* cf. *duvauae*), where three general features are convergent, and independent of host organs. First, the three morphotypes are open galls, with overlapping trichomes covering gall apertures; second, the nymphal chambers are large, even when hosting a single small inducing-insect; and third, Calophyidae-induced galls are highly parenchymatic and vascularized, with abundant development of phloem and vascular parenchyma.

Recently, gall complexity was evaluated by the occurrence of one or several anatomical features such as cell hypertrophy, tissue hyperplasia, vascular bundle hypertrophy, cell re-differentiation, histochemical changes, and manipulation of meristematic activity (Ferreira et al.

2017a). Considering the anatomical and histochemical features, *C. mammifex* and *C. rubra* may be considered complex galls, contrary to the literature on Hemipteran galls (Rohfritsch 1992; Oliveira and Isaias 2010a).

Main conclusions

Environmental conditions in the Mediterranean climate of southern Chile probably determine a univoltine life cycle and a diapause period for *C. mammifex* development on *S. polygama*, as well as the anatomical and histochemical features of its galls. For the first time, we detected energetic metabolites in a nutritive-like tissue and in a common storage tissue in galls in Mediterranean climate conditions, which may be related to the nutritional improvement of galls. Galls induced by *C. mammifex* and *C. rubra* have sufficient anatomical and histochemical peculiarities to be diagnosed as complex structures, whose distinction in vascular system differentiation implies structural constraints imposed by host plant organs.

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CAPITULO IV

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

Article under Review in Journal of Chemoecology

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Abstract

The expression of plant secondary metabolism pathways is strongly controlled in plants both time and space. Although the variation of secondary metabolites, such as phenolics and lignins, is largely observed in gall-inducing insects when compared to their host non-galled organs, only a few datasets recording such variation are available. Accordingly, a few of these datasets evaluate the relative importance of phenolics spatiotemporal variability under the influence of gall developmental stages and the original composition of the host organs. To face this knowledge gap, we determined the histochemical sites of polyphenol and lignin accumulation, and also, the polyphenol contents in three developmental stages of two calophyid galls and their correspondent host organs. Current results indicate that the compartmentalization of phenolics and lignins on *S. polygama* follows a generic pattern in the two-calophyid galls, accumulating in the outer and inner tissue compartments, and non-accumulation in the median tissue compartment due to its nutritive profile. Also, the concentration of phenolics has opposite dynamics in temporal scale, decreasing in leaf galls and increasing in stem galls from maturation toward senescence. Moreover, the distinct concentration of phenolics in non-galled host organs and both galls indicated that the influence of *C. rubra* and *C. mammifex* over the host plant metabolic potentiality reinforces the extended phenotype of galling insects.

Key Words- Calophyids, compartmentalization, development stage, gall, lignins, polyphenols

INTRODUCTION

Plants, as sessile organisms, develop physical and chemical strategies to protect themselves against abiotic and biotic stresses (Wink 1997). Usually, plants synthesize a wide spectrum of secondary metabolites, particularly phenolic compounds, in response to the attack of galling insects (Tooker and Helms 2014). Polyphenols were usually interpreted as defensive metabolites, acting as herbivore deterrents, growth inhibitors and toxins against herbivores (Nyman and Julkunen-Tiitto 2000; Close and McArthur 2002; Lattanzio et al. 2006). Recently, polyphenols have been associated with antioxidant and photoprotective mechanisms, which explains their increase in plant tissues under abiotic stress and attack by pathogens and predators (Close and McArthur 2002). Therefore, the occurrence of phenolics and flavonoid derivatives has been related to reactive oxygen species (ROS) dissipation in high oxidative stress conditions caused by the gall-inducing organisms (Isaias et al. 2015; Oliveira et al. 2017). Also, phenolics may be involved in indol-3-acetic acid (IAA) metabolism, and consequently influence cell hypertrophy at gall developmental sites (Hori 1992; Bedetti et al. 2014a).

Despite the toxicity of phenolics, their concentrations usually increase in galls (Cornell 1983; Abrahamson et al. 1991; Hartley, 1998; Nyman and Julkunen-Tiitto 2000; Motta et al. 2005; Formiga et al. 2009; Agudelo et al. 2013), probably because specialist herbivores, as galling insects, are adapted to their toxicity (Carmona et al. 2011) and develop mechanisms of detoxification (Lattanzio et al. 2006; Taiz and Zeiger 2006; Després et al. 2007). However, some galls may contain lower concentrations of phenolic compounds than their host organs (Hartley 1998; Stone and Schonrogge 2003). More than a matter of static variation in levels, the expression of plant secondary pathways may be strongly controlled both in spatial and temporal scales (Moore et al. 2013), which has been observed in some galls (Nyman and Julkunen-Tiitto 2000; Dias et al. 2013; Isaias and Oliveira 2014; Bragança et al. 2017; Guedes et al. 2018a, b; Isaias et al. 2018). Spatiotemporal variation in phenolic levels may be consequence of plant responses to environmental factors, plant physiology and developmental stages (Herms and Mattson 1992; Kause et al. 1999; Wink 2003, 2013).

Current model of study, *Schinus* is often heavily attacked by galling calophyids (Psylloidea: Calophyidae: Calophya) (Burckhardt and Basset 2000). The *Schinus* spp.-*Calophya* spp. systems have been previously studied from the anatomical and phenological perspective in Brazil and Chile (Dias et al. 20113a, b; Guedes et al. 2016, 2018a, b). In Brazil, *Calophya* cf. *duvauae* Scott is the inducer of leaf galls on *Schinus engleri* Barkley (= *S. polygamus*); in its

galls a progressive accumulation of phenolics were observed (Dias et al. 2013a). *Schinus polygama* (Cav.) Cabrera is a common species in the flora of Chile (Rodríguez 2011), and hosts at least two gall morphotypes induced by calophyids: one induced by *Calophya rubra* Blanchard (Psylloidea: Calophydae) on stems and one induced by *Calophya mammifex* Burckhardt and Basset (Psylloidea: Calophydae) on leaves (Guedes et al. 2018a). Both galling calophyids manipulate their host-plant organ morphogenesis and the general chemical composition of gall tissues, which was investigated by chromatographic profiles (Guedes et al. 2016). Such investigations of stem and leaf galls reveal that the most abundant compound in both galls is the tannin pyrogallol (Guedes et al. 2016).

Calophyids are phloem-sucking insects that feed on vascular tissues, mostly phloem, which are neoformed in the median cell layers (= tissue compartment *sensu* Bragança et al. 2017) of gall walls (Guedes et al. 2018a, b). Also, in both leaf and stem gall morphotypes the compartmentalization of nutritive primary metabolites has been observed in the median tissue compartments, where calophyids feed (Guedes et al. 2018a). Based on previous studies, it is expected that phenolic compounds should accumulate in tissue compartments not involved in gall nutritive profile to avoid the contact of the insects feeding apparatus with unpalatable phenolics. Also, the levels of phenolic compounds in leaves and stem galls could be influenced by the original composition of host organs and gall development stage. Therefore, taking the superhost *S. polygama* and galls induced by *C. rubra* and *C. mammifex* as models of study, we expect to advance on the knowledge of the little explored context of spatiotemporal variation of phenolics in galls. We assume that once the phenotypes of gall inducers extend to the physiological level in gall developmental sites, the influence of the two-cogeneric galling insects over the potential of the same host plant will set lights on the understanding of the amplitude of the extended phenotype of gall inducers over the secondary metabolism of host plants. Our main questions are: (i) Does the compartmentalization of secondary metabolites follow a generic or a specific pattern under galling insect influence? (ii) Do the levels of phenolics vary in galls in accordance to the patterns of their host organs? (iii) Is there any convergence in spatiotemporal variation in the levels of phenolics in the cogeneric calophyid galls?

MATERIAL AND METHODS

Collection of Plant Material. Five trees of *S. polygama* were marked in an area of sclerophyllous forest in southern Chile (36°39'32"S 72°16'43"W at 152 m.a.s.l). On October and November 2016, samples of non-galled leaves (NGL) and globoid leaf galls (GLG) were collected from

plant, and the same node level; and non-galled stems (NGS) and conical stem galls (CSG) were collected from the same internode level to guarantee their similar physiological age. All organs were collected at three developmental stages. The developmental stages of NGL were classified as follows: stage I (young fully expanded leaves, before turning into dark green); stage II (mature leaves, about eight months old); and stage III (old leaves, about one year old). The developmental stages of NGS were classified as follows: stage I (young stems, before turning brown); stage II (mature brown stems about eight months old); stage III (old dark brown stems about one-year old). For galls, the three developmental stages corresponded to the instar nymphs (Guedes et al. 2018a): stage I (growth and development (GD) - with first, second, third and fourth instar nymphs); stage II (maturation phase (MP) - with fifth instar nymph; and stage III (senescent phase (SP) - empty galls).

Histochemical Analyses. For histochemical analyses, fresh fragments of NGL, NGS, GLG and CSG at the three developmental stages were fixed in ferrous sulfate and formalin (37% formaldehyde and 0.1 g L^{-1} Iron(II) sulfate heptahydrate) (Johansen 1940) for 72 hours for polyphenol detection, and in Karnovsky's solution (2.5% glutaraldehyde and 4.5% formaldehyde in 0.1 mM phosphate buffer) (O'Brien and McCully 1981) for 24h for lignin detection (Ferreira et al. 2017a). The samples were washed in distilled water, embedded in polyethylene glycol 6000 (PEG), and sectioned in a rotary microtome (30-40 µm) (Ferreira et al. 2017a). For detection of lignins, the sections were submitted to Maule's reagent (Patten et al. 2007). The sections were observed under light microscope (Leica DM500, Wetzlar, Germany), photographed (Leica ICC50 HD) and compared to its respective blank section.

Sample Preparation and Extraction of Phenols. Samples of NGS and NGL, and of CSG and GLG, previously dissected to remove the insects, at three development stages were oven dried at 30°C for one week. 200 mg of each sample from each plant (n = 10) were pulverized and macerated in 10 mL of 80% methanol (8:2 v/v) at room temperature in darkness. After 24 hours, the extracts were filtered through the Whatman® No 1 filter paper and stored at 4°C for further experiments. Previous to the analyses, the extracts were concentrated at a reduced pressure with a rotary evaporator until completely dry. Subsequently, the crude extracts were resuspended in 10 mL 80% methanol (8:2 v/v) and analyzed for total phenolic contents using spectrophotometric methods.

Total Polyphenol Contents. Total polyphenol contents were determined with the Folin-Ciocalteu reagent, according to Attard (2013), with slight modifications. 10 µL of diluted

extracts were pipetted into wells of a microtitre plate (MTP), 100 µL of Folin-Ciocalteu reagent was added, and then the plate was shaken for 30 seconds and incubated for 5 minutes. Subsequently, 80 µL of sodium carbonate were added. The plate was shaken on the MTP reader for 30 seconds, incubated at 40°C for 30 minutes, and then read at 765 nm. Solutions between 0 and 500 mg L^{-1} of gallic acid were used as standards to generate the calibration curve. The results were expressed as gallic acid equivalent (GAE) per milligrams of dry weight (µg of GAE mg^{-1} of dry weight). All samples and standards were measured in triplicate against water blank.

Statistical Analyses. The average values of all treatments (NGL, GLG, NGS and CSG) were compared using *Analyses of Variance* (ANOVA) followed by *Tukey's post-test* in InfoStat software (v.2013) (Rienzo et al. 2013). Differences were considered to be significant at 5% probability ($p < 0.05$).

RESULTS

Sites of Accumulation of Lignins. Lignins were detected in light brown in epidermal cells and in xylem of NGL (Fig. 1a-b). In GLG in growth and development phase (GD), lignins were not detected. In GLG in maturation phase (MP), lignins stained orange in trichomes, and in the adaxial and abaxial epidermal cells (Fig. 1c), and light brown in vessel elements (Fig. 1d). In GLG in senescence phase (SP), lignins were detected in brownish-orange in the adaxial and abaxial epidermal cells (Fig. 1e) and in light brown color in vessel elements. In NGS, lignins stained orange in phelloderm, and magenta in perivascular fibers, secondary xylem, and pith parenchyma cells (Fig. 1f). In CSG in GD, lignins were not detected. However, in CSG in MP, lignins stained orange in phellem cells (Fig.1g) and magenta/orange in the scarce differentiated xylem cells (Fig. 1h). In CSG in SP, lignins were scarcely detected in xylem and phelloderm.

Sites of Accumulation of Polyphenols. Polyphenols were detected in epidermis, chlorophyll parenchyma (Fig. 2a), vascular parenchyma and cells of the duct sheath of NGL (Fig. 2b). In GD galls, the polyphenols accumulated in outer epidermis (of abaxial and adaxial gall walls), and both in outer and inner compartments, in the abaxial epidermal ordinary cells and trichomes (Fig 2c). Polyphenols accumulated more scarcely in the median compartment (Fig 2c). During the MP, phenolics strongly accumulated in outer and inner compartments, abaxial epidermal ordinary cells, trichomes, and more scarcely in outer epidermal cells (of adaxial and abaxial gall walls) and median compartment (Fig 2d), but they were undetectable in vascular cells and idioblasts with druses (Fig 2e). In the SP, the accumulation of phenolics followed the same

patterns of compartmentalization of the MP, except for the tissues in the abaxial portion of gall wall and the trichomes, which degraded during senescence (Fig. 2f).

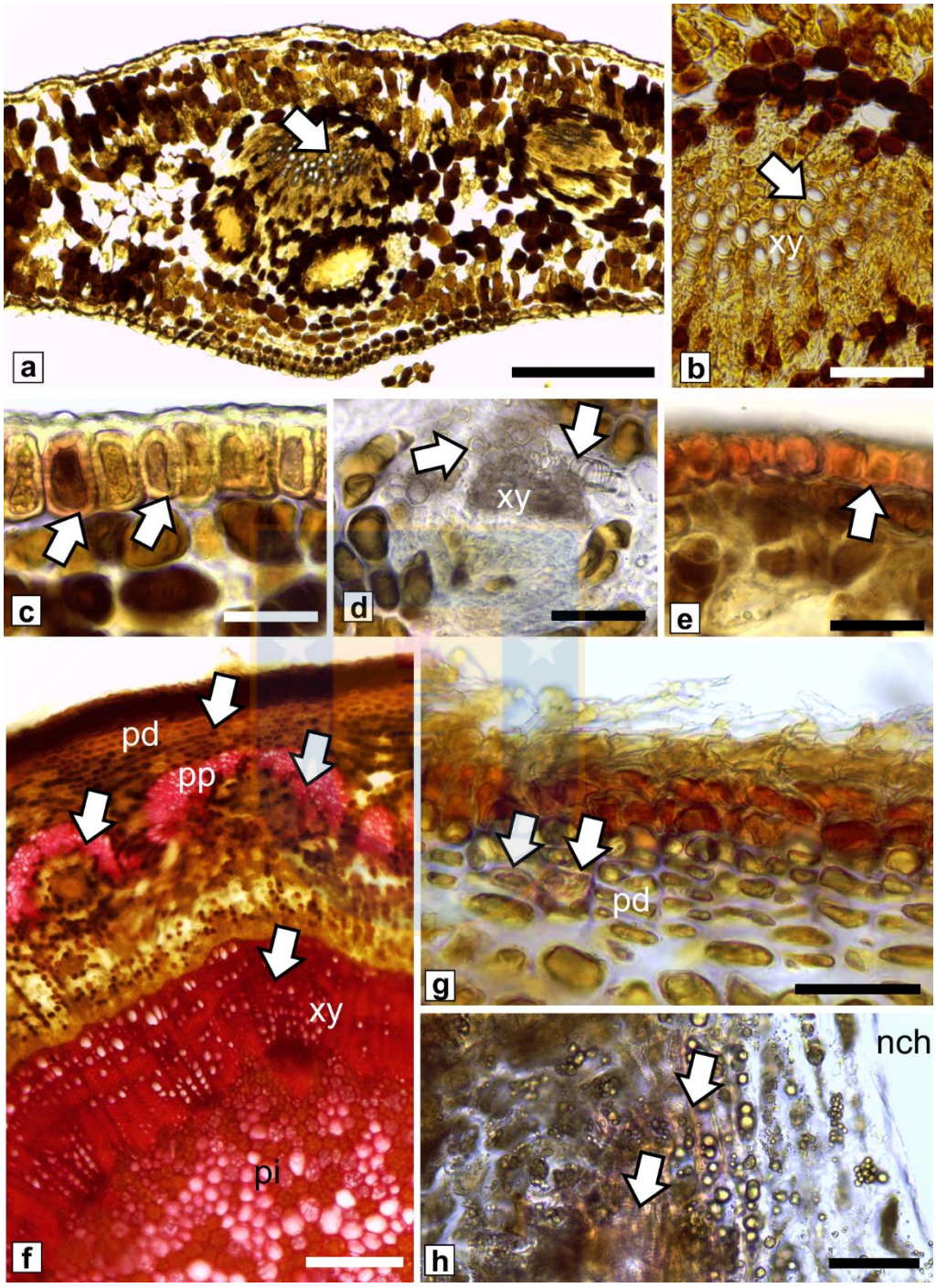


Fig. 1 Histolocalization of lignins with Maule's reagent in host organs and calophyid gall on *Schinus polygama* at three developmental stages. Arrows indicated sites of lignins accumulation: **a.** in the midrib of the leaf; **b.** in the xylem of vascular bundle (detail, light brown); **c-e.** in leaf gall induced by *Calophya mammifex*: **c.** in adaxial epidermis (orange color), and **d.** in vessel elements of xylem of mature galls (light brown); **e.** in the adaxial epidermis of senescent gall (brownish-orange color); **f.** in phelloderm,

perivascular fibers, secondary xylem, and pith parenchyma cells of mature stem (magenta color); **g.** in phellem cells (orange), and **h.** in xylem formed in the vascular units of mature stem gall induce by *Calophya rubra* (magenta/orange). Abbreviations. *pd:* phelloderm, *pp*: perivascular fiber, *nch*: nymphal chamber, *pi*: pith, *xy*: xylem. Bars: 50 µm (b, e, g, h), 200 µm (a, f). Fuente: Elaboración propia

In NGS, phenolics strongly accumulated in epidermal and cortical parenchyma cells, and in cells of phloem parenchyma, duct sheath cells, xylem radial parenchyma, and in some idioblasts in pith parenchyma (Fig. 3a). In stems gall in GD, polyphenols accumulated moderately in gall cortex (Fig. 3b), epidermis, outer and median compartment cells, but more intensely in cells of the inner compartment (Fig. 3c). In MP, phenolics strongly accumulated in phellem, outer and inner compartments, and moderately accumulated in some median parenchyma cells (Fig. 3d, e). During the SP, phenolics accumulated in cells of phellem, outer and median compartment (Fig. 3f).

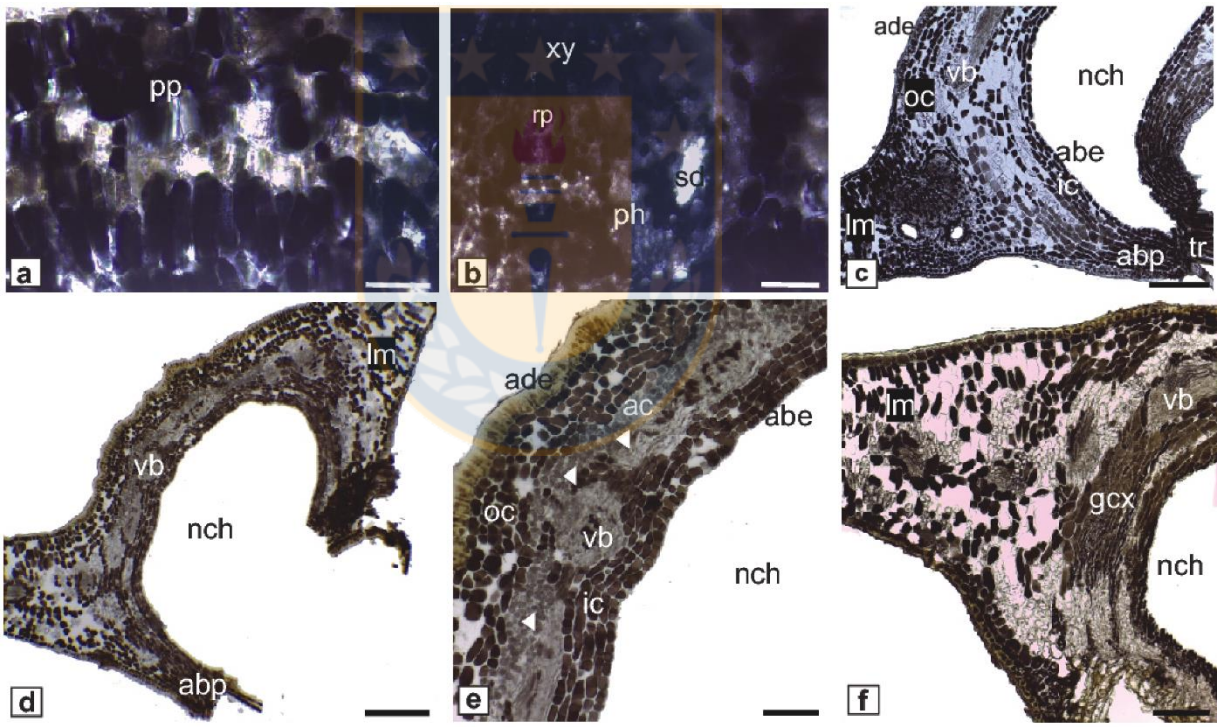


Fig. 2 Histolocalization of polyphenols with ferrous sulfate and formalin in non-galled leaves and globoid leaves gall induced by *Calophya mammifex* (Psylloidea: Calophydae) on *Schinus polygama* (Anacardiaceae). Black and dark brown coloration in the cells indicates positive reaction for polyphenols. **a-b.** Non-galled leaf, **a.** leaf mesophyll, **b.** Midrib; **c-d.** Detection of polyphenols through developmental stages of gall, **c.** Young leaf gall (Stage I), **d.** Mature leaf gall (Stage II), **e.** Detail of the adaxial region of the mature gall (arrowheads indicate the presence of idioblasts with druses), **f.** Senescent gall (Stage III) with degraded, disorganized and suberized tissues in inner compartment and

abaxial portion. Abbreviations. *abe*: abaxial epidermis, *ade*: adaxial epidermis, *abp*: abaxial portion, *gc*: gall cortex, *ic*: inner compartment, *lm*: leaf mesophyll, *mc*: median compartment, *nch*: nymphal chamber, *oc*: outer compartment, *ph*: phloem, *pp*: palisade parenchyma, *rp*: parenchyma rays, *sd*: secretory ducts, *tr*: trichomes, *vb*: vascular bundle, *xy*: xylem. Bars: 50 µm (a, b), 200 µm (c, e, f) 500 µm (d). Fuente: Elaboración propia

Polyphenol Contents. The phenolic content of non-galled leaves was lower than that of non-galled stems in stage I, but temporally the concentration of phenolics increased in both organs in stage II, and decreased in stage III (Fig. 4a-b).

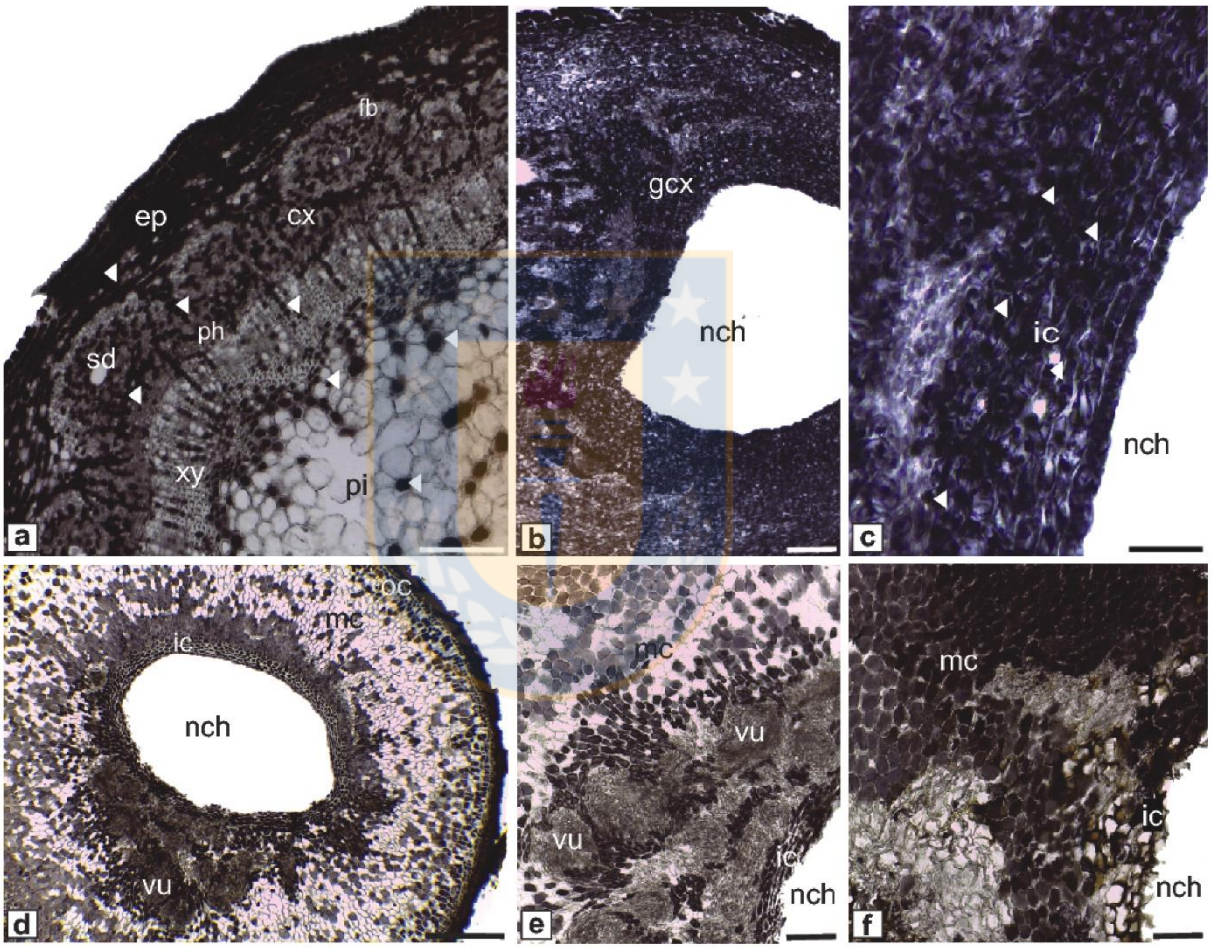


Fig. 3 Histolocalization of polyphenols with ferrous sulfate and formalin in non-galled stems and conical stem galls induced by *Calophya rubra* (Psylloidea: Calophydae) on *Schinus polygama* (Anacardiaceae). Black and dark brown coloration in the cells indicates positive reaction for polyphenols. **a.** Non-galled stem (arrowheads indicate sites of polyphenol accumulation); **b-f.** Detection of polyphenols through developmental stages of gall, **b.** Young stem gall (Stage I), **c.** Detail of the adaxial region of the young gall (arrowheads indicate the presence of polyphenols), **d.** Mature stem gall (Stage II), **e.** Detail of the inner and outer compartment of the mature gall, **f.** Senescent gall with degraded, disorganized and suberized tissues in the inner compartment. Abbreviations. e*p:* epidermis, *fb*: fiber, *cx*: cortex, *gcx*: gall

cortex, *ic*: inner compartment, *mc*: median compartment, *nch*: nymphal chamber, *oc*: outer compartment, *ph*: phloem, *pi*: pith, *sd*: secretory ducts, *vu*: vascular unit, *xy*: xylem. Bars: 50 µm (c), 200 µm (a, b e, f) 500 µm (d). Fuente: Elaboración propia

The concentration of phenolics in globoid leaf galls (GLG) was similar in stage I to the non-galled leaves (NGL), decreased 33.6% in stage II, and kept this level in stage III. Temporally, phenolic contents were similar in young and mature galls, and decreased in senescent galls (Fig.4a). The concentration of phenolics in conical stem galls (GSG) in relation to the non-galled stems (NGS) was 37.5% and 60.8% lower in stages I and II respectively, but it was 89.0% higher in stage III. Temporally, phenolic contents were similar in young and mature galls, and increased 63.9% in senescent galls (Fig. 4b).

Leaf (GLG) and stem galls (CSG) had different concentrations of phenolics in the three stages of development. Conical stem galls had lower concentrations of phenolics than GLG in stages I and II, but higher concentration of phenolics in stage III (Fig. 4c).

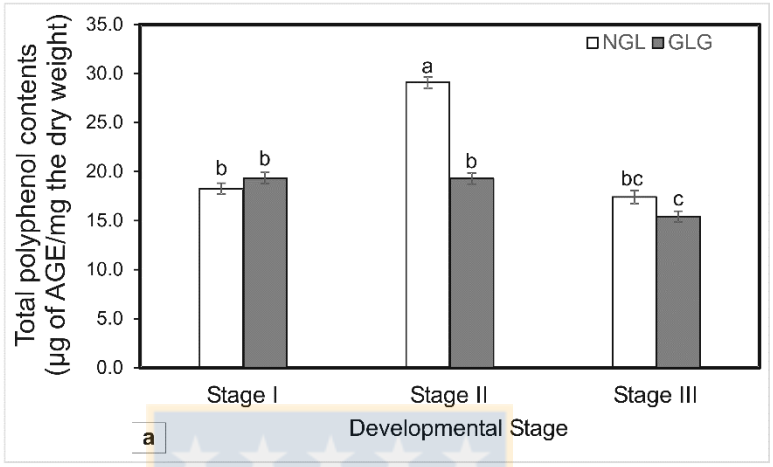
DISCUSSION

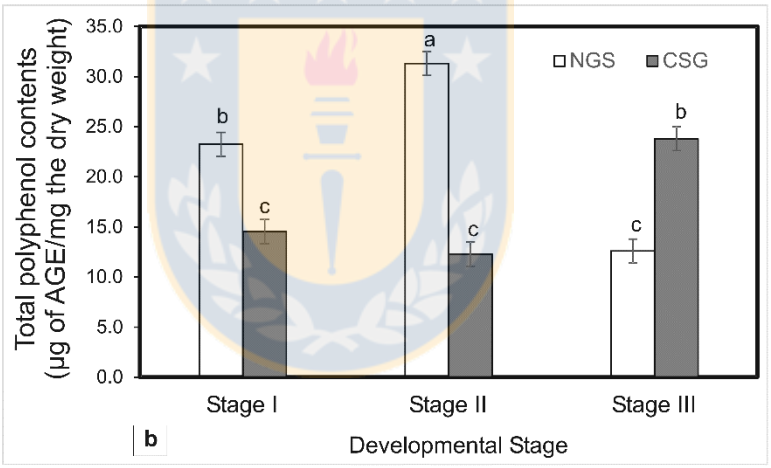
The Compartimentalization of Phenolics is Linked to Insect Genera

Lignins are phenolic biopolymers generated by radical coupling of hydroxycinnamyl alcohols, monolignols, named guaiacyl, syringyl, and *p*-hydroxyphenyl units (Wang et al. 2013). Syringyl propane lignins always stain red, and guaiacyl and hydroxyphenyl lignins stain brown by the Maule's reactive (Ferreira et al. 2017a). The orange or brownish-orange reactions indicate intermediary levels of syringyl and coniferyl/coumaryl alcohols lignins (Van Cutsem et al. 2011; Ferreira et al. 2017a). The staining of lignins with Maule`s reactive developed different colors in non-galled host organs and galls, indicating distinct types of lignin precursors in cell walls (Van Cutsem et al. 2011). The guaiacyl lignin predominates in the xylem both in NGL and GLG, but the distinctive orange and brownish-orange coloration in GLG indicates the possible reallocation of syringyl and hydroxyl-coumaroyl lignin precursors to gall developmental site. In addition, in NGS and CSG the syringyl units predominate in perivascular fibers and xylem, but syringyl and hydroxyl-coumaroyl lignin were detected in phelloderm.

The typical lignin of angiosperms is guaiacyl-syringyl lignin (Higuchi 1985), however the metabolism of lignins is very plastic (Lu et al. 2010), and the amount and type of lignin normally present in tissue and organ can change when plants are subjected to biotic or abiotic stresses (Moura et al. 2010; Neutelings 2011). Both galls have similar lignin profile with the detection

of syringyl and hydroxyl-coumaroyl lignin. The increase of syringyl lignins in cell walls has been observed in plants stressed by pathogens (Bishop 2002). Therefore, the galling stimuli of *C. rubra* and *C. mammifex* influence the synthesis of lignin types in gall tissues, whose biological significance is yet to be understood.





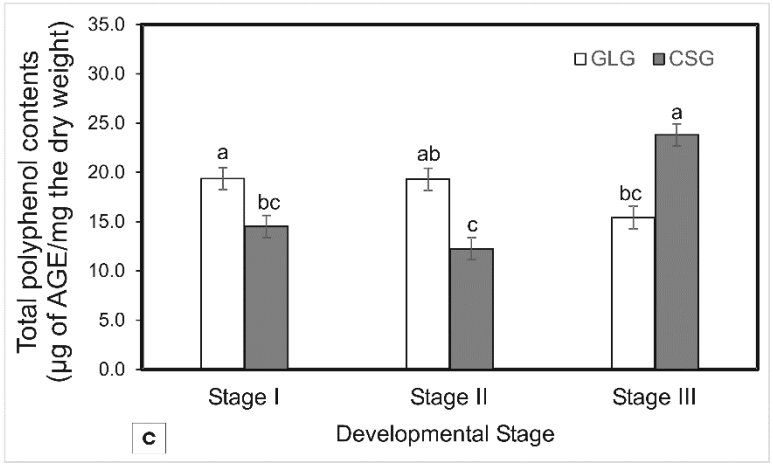


Fig. 4 Polyphenol detection in host organs and calophyids gall on *Schinus polygama* at three developmental stages. Non-galled leaves (NGLs) and non-galled stems (NGSs): Stage I. Young leaves and stem. Stage II. Mature leaves and stems about eight months old. Stage III. Old leaves and stems about one year old. Globoid leaf galls (GLGs) and Conical stem galls (CSGs): Stage I. Growth and development (GD): first, second, third and fourth instar nymphs. Stage II. Maturation phase (MP): fifth instar nymph. Stage III. Senescent phase (SP): recently open gall without insect. **A.** Polyphenol contents in NLG and GLG. **B.** Polyphenol contents in NSG and CSG. **C.** Polyphenol contents in GLG and CSG. Bars indicate the means and standard deviations of 10 individual trees. Different letters mean differences between treatments at $p < 0.05$. Fuente: Elaboración propia

Generally, cells with lignified walls such as fibers and lignins are related to structural stability of the gall and provide a favorable microenvironment for larval development (Motta et al. 2005). However, the restriction of lignins deposition to small amounts in cell wall of tracheal elements and dermal system in calophyid galls indicates an impairment of lignification in leaf and stem galls induced by these sucking insects, as is common for some calophyid galls (Dias et al. 2013a). Such impairment is probably consequence of the redirection of lignin precursors as vacuolar phenolic compounds to the vacuoles of the epidermal, cortical and vascular parenchyma cells. The redirection of lignin precursors has been observed under various biotic stresses, such as pathogen attack (Wang et al. 2013), and is demonstrated here for the first time in insect galls.

Lignification is important to reinforce cell walls for water conduction, mechanical support and defence of the plant (Wang et al. 2013). Currently, the sites of lignin accumulation in both gall morphotypes indicate that for calophyid galls the synthesis of lignins is related more to water conduction and defence than to mechanical support. Finally, the non-lignification of cell layers in the inner and median gall compartments may favour the insertion of the stylets of *C. rubra* and *C. mammifex* (Guedes et al. 2018).

While the outer and inner tissue compartments accumulated phenolics, the accumulation of primary metabolites, mainly reducing sugars, proteins, and lipids, in median tissue compartment in both globoid leaf gall and conical stem gall indicated the development of a nutritive-like tissue in median compartment (Guedes et al. 2018). The primary metabolites, mainly lipids and proteins, accumulated in the outer compartments in a common storage tissue, may function in gall structural maintenance, as proposed for other insect (Oliveira et al. 2010; Ferreira and Isaias 2013, 2014) and nematode galls (Ferreira et al. 2017b). Additionaly, the outer compartment may

function as a protective layer against the attack of natural enemies and parasitoids due the polyphenol and lignins accumulation.

As phloem suckers, *C. mammifex* and *C. rubra* feed on the median gall compartment, a peculiarity of the *S. polygama*-calophyid systems, where new vascular tissues and well-developed vascular parenchyma cells rich in primary metabolites differentiate (Guedes et al. 2018). The absence of polyphenols in the phloem and vascular parenchyma could prevent inducers from coming into contact with unpalatable secondary metabolites, which has been observed in Cecidomyiidae galls (Bragança et al. 2017). Moreover, the calophyids mouth apparatus must cross the phenolic-rich parenchyma cells of the inner compartment, which may indicate that despite the potential toxicity of plant phenols (Róstas et al. 2013); their saliva may readily degrade phenols (Hori 1992). Such chemical adaptation to remove phenolics from their digestive systems (Lattanzio et al. 2006; Taiz and Zeiger 2006; Després et al. 2007) probably occurs in *C. rubra* and *C. mammifex*. These two-calophyid species induce galls where the compartmentalization of phenolics and lignins follows a generic pattern, accumulating similarly in the outer and inner tissue compartments.

The Spatiotemporal Dynamics of Phenolics Differs in Host Organs and Galls

The secondary chemistry can change considerably during ontogenetic development and plant growth (Donaldson et al. 2006; Neilson et al. 2013). During periods of intense plant growth, such as from young to maturation stage, there is a high demand for resources (Patrick 1988; Cipollini and Redman 1999; Van Dam et al. 2001), and consequently, plant investment in defence should be low. Currently, the low levels of phenolics in young non-galled host organs indicated a probable tradeoff between growth and defence (Herms and Mattson 1992). This tradeoff seems to have worked out for gall establishment because it could be beneficial for the establishment of the nymphs, due to the easier expected adaptation to lower toxic chemical levels (Harborne and Grayer 1993; Berenbaum and Zangerl 1999).

The high levels of phenolics in mature host organs do not impair gall maturation. Probably, the increase of polyphenol contents in stage II of non-galled host organs could be consequence of an increased concentration of high-molecular-weight polymers, such as lignin and others polyphenols (Herman and Watson1992). As gall tissue matures, metabolic processes changed as also expected for plant organs in general (Jones 1999). The low concentration of phenolics in both mature galls indicated a phenolic-reduced environment for calophyid galls development. However, for leaf and stem galls, there was no significant increase in polyphenols from stage I

to stage II, probably because of the impairment of the differentiation of fibers and sclereids in gall cortex, both under tissue manipulation by *C. rubra* and *C. mammifex*, as previously reported (Guedes et al. 2018).

Contrary to the expected pattern, the highest polyphenol accumulation was detected in senescent CSG, and should avoid fungi or other pathogens invasion, once the gall developmental sites remain attached to the host plant. The protective reaction, by cell wall suberisation, occurs in the abaxial portion, ostiolar opening and inner compartment (Guedes et al. 2018), and forms an impermeable and protective barrier (Kolattukudy et al. 2001). Suberization involves the deposition of various lipid polymers and lignin-like phenolics in cell wall, being considered important apoplastic barriers against infections and water loss (Borg-Olivier and Monties 1993). This feature should be indicative of the role of phenolics in protecting the site of stem galls from fungi invasion (Franke and Schreiber 2007).

Even though the content of phenolic compounds usually increases in gall tissues (Cornell 1983; Abrahamson et al. 1991; Hartley 1998; Nyman and Julkunen-Tiitto 2000; Motta et al. 2005; Formiga et al. 2009; Agudelo et al. 2013), the phenolic contents can be maintained or reduced in other galls (Hartley 1998; Stone and Schonrogge 2003). For both gall mophotypes on *S. polygama*, polyphenol levels decrease in developmental site compared to host organs. However, while polyphenol levels in leaf galls are very similar to those observed in non-galled leaves, in stem galls polyphenol levels were lower than in non-galled stems, except during gall senescence. This behavior in the levels of phenols in the calophyid galls could be indicative of metabolic constraints imposed by the host organ.

On a qualitative perspective, pyrogallol was detected both in stem and leaf galls due to the galling stimuli of *C. rubra* and *C. mammifex*, respectively (Guedes et al. 2016), despite the galling stimuli of distinct inducers commonly result in different chemical composition in gall tissues produced in the same host plant (Hartley 1998). Current analyses indicate that on a temporal perspective, polyphenols may have independent chemical quantitative profile in host organs and galls. Along gall development, the secondary metabolism of *S. polygama* should be restrictive due to substrate and/or energy limited, and the synthesis of phenolic compounds should demand high energetic costs for the host plants (Gulmon and Mooney 1986). Accordingly, on temporal bases, the concentration of phenolics is distinct both in host non-galled stems and leaves, and have opposite dynamics, decreasing in leaf galls and increasing in stem galls from maturation toward senescence.

Due to the oxidative stress generated during gall development (Soares et al. 2000; Oliveira et al. 2011a, b), phenolics accumulation may also help the maintenance of gall structure (Bedetti et al. 2014b). Accordingly, polyphenol accumulation in the inner and outer compartments of *C. rubra* and C. *mammifex* galls could be related to cell expansion and division. Phenolics in galls have recently been related to indol-3-acetic acid metabolism, and consequently influence cell hypertrophy and hyperplasia (Bedetti et al. 2014b; Bedetti et al. 2017; Carneiro et al. 2017), and in ROS dissipation (Isaias et al. 2015), which is yet to be tested in *S. polygama*-calophyid galls.

Despite the secondary role attributed to the defensive function of phenolics in gall systems (Close and McArthur 2002; Bedetti et al. 2014b), the detection of phenolics and lignins in outer tissues in both gall morphotypes, led us to assume their relation both to gall development and to mechanical defence in *S. polygama-Calophya* spp. systems. In addition, the detection of pyrogallol both in globoid leaf galls and in conical stem galls has been related to insecticide properties (Balasubramanian et al. 2014), which supports that phenolics on *C. rubra* and *C. mammifex* galls can enhance their chemical protection against natural enemies.

In the Neotropics, the variations in phenolic contents during gall cycles were associated to water and light radiation stresses (Formiga et al. 2009; Detoni et al. 2011). However, current analyses have indicated that polyphenol contents in galls also depend on the developmental stage of the calophyids and on the metabolic peculiarities of host organs. The synthesis of phenols, as a potential chemical defence, is neither constant throughout a plant's lifetime nor is expressed evenly throughout the plant's body (Zangerl and Rutledge 1996).

The detection of phenolics and lignins was spatially similar in both gall morphotypes, being impaired in the median tissue compartment, due to its nutritive profile. Moreover, the distinct concentration of phenolics in non-galled host organs and both galls indicated gall inducing species influence over the host plant metabolic potentiality and reinforces the extended phenotype of *C. rubra* and *C. mammifex* on *S. polygama*.

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DISCUSIÓN GENERAL

El fenotipo de las agallas está determinado por la interacción entre el medio ambiente, el genotipo de la planta huésped y el del inductor (Weis &Abrahansom 1986); sin embargo, el papel de dichos factores en la determinación de la morfología de la agalla no ha sido explorado suficientemente (Moura et al. 2008). En este sentido, el estudio de las agallas inducidas por *C. rubra* y *C. mammifex* en los tallos y hojas del super-hospedero *S. polygama* ha contribuido a esclarecer la influencia de los mencionados factores sobre las características morfo-anatómicas, histoquímicas y fenológicas de ambos morfotipos de agallas.

Los procesos más comunes en las agallas de hemípteros son la hiperplasia tisular, hipertrofia celular, y la neoformación de elementos vasculares y tricomas (Mani 1964), lo que se pudo corroborar en las agallas globoides de hoja y cónicas de tallo inducidas en *S. polygama*. Estas características son el resultado de la potenciación de los patrones morfogenéticos de los órganos hospederos bajo la influencia del aparato succionador de los inductores calófidos (Isaias & Oliveria 2014), por lo que se consideran convergencias determinadas por el hábito alimenticio de los inductores (Isaias et al. 2014a). No obstante, se evidenciaron diferencias en las características anatómicas de ambos morfotipos de agallas, lo que puede deberse a las limitaciones morfogenéticas impuestas por los órganos hospederos al desarrollo de las agallas (Formiga et al. 2015).

Los insectos succionadores del floema, como *C. rubra* y *C. mammifex*, se alimentan en el floema (Wool et al. 1999) o en el parénquima vascular (Shorthouse & Rohfritsch 1992; Raman 2011), por lo que la neoformación de tejido y parénquima vascular en las agallas de estos insectos es una característica esperada. En el compartimento medio de ambos morfotipos de agallas se observó la formación de tejido vascular y de parénquima vascular bien desarrollado. La rediferenciación de estos nuevos tejidos se debe a la plasticidad del parénquima donde se establecen ambos inductores, el cual retiene la capacidad de reasumir la actividad meristemática y rediferenciarse para formar tejidos especializados (Lev-Yadun 2003). El nuevo tejido vascular formado, mayormente floema, se localiza en la región basal de ambas agallas, y está dirigido directamente hacia la cámara ninfal. Así se favorece el proceso de alimentación, debido a que el estilete de los inductores puede succionar la savia del floema sin necesidad de cruzar el xilema. El proceso de alimentación se ve también favorecido por la ausencia de células lignificadas en los compartimentos internos de las agallas.

El establecimiento de *C. rubra* en el córtex de los tallos promueve una fuerte activación del procambium y cambium vascular, y da origen a la formación de unidades vasculares en las agallas de tallo. Estas células cambiales se pueden modificar para que los rayos medulares se agranden y promuevan la proliferación celular en esta región, lo que resulta en una masa o parénquima continuo (hiperplasia) (Kolodziejek et al. 2003). De esta manera se induce mayor hiperplasia en las agallas de tallos que en las agallas de hojas. Probablemente la gran hiperplasia de la agalla de tallo determina la forma cónica de la misma (Isaias & Oliveira 2014). Tales características diferenciales de las agallas de tallo son una fuerte evidencia de la influencia del potencial morfogenético del órgano hospedero sobre la anatomía de las agallas.

El desarrollo de algunas de las características anatómicas y morfológicas de las agallas se han relacionado con mecanismos de protección contra factores ambientales (Stone & Sch**ö**nrogge 2003) y enemigos naturales (Oliveira et al. 2006; Álvarez et al. 2009). El desarrollo de tricomas, y de tejidos hiplerplásicos (parénquima y epidermis) en ambos morfotipos de agallas pudieran ser características desarrolladas bajo la influencia que ejerce el medioambiente y los enemigos naturales en el desarrollo de la agalla. La presencia de tricomas en hojas y tallos de *S. polygama* es poco frecuente (Dias et al. 2013a), por lo que la diferenciación de tricomas en las agallas pueden representar una sobrepotencialización del patrón morfogenético ordinario de los tejidos de las hojas y los tallos. Estos procesos de diferenciación celular pueden estar determinados por factores internos y externos, mayormente ambientales, que fuerzan a las células a la rediferenciación para asumir nuevas funciones en las agallas (Lev-Yadun 2003).

Las condiciones climáticas ejercen una fuerte influencia en la fenología de las plantas; las que ajustan su fenología a los eventos locales del clima, igual que los insectos gallícolas asociados (Yukawa & Akimoto 2006). Para los insectos gallícolas la sincronía con la fenología de la planta hospedero es un evento crítico, ya que determina la calidad y cantidad de los recursos alimenticios disponibles (Kerslake & Hartley 1997; Yukawa 2000; Oliveira et al. 2016). A pesar de que *S. polygama* es una especie siempre verde, con crecimiento vegetativo durante todo el año, la inducción de agallas por *C. rubra* y *C. mammifex* se produce solamente durante la primavera. Ambas especies de insectos tienen un ciclo de vida univoltino, y un período de diapausa durante el verano e invierno, lo que parece ser una estrategia de estas especies para escapar de las condiciones desfavorables del clima durante este período de tiempo. Aunque el éxito reproductivo de los insectos gallícolas está determinado por la interacción sinérgica de las condiciones ambientales y características de la planta hospedero (Fernandes & Price 1992), al

parecer en climas estacionales, como en el Mediterráneo, las condiciones climáticas pueden ser exclusivas para el desarrollo del inductor. En especies de plantas siempre verde de climas subtropicales se reportan ciclos de vida multivoltinos para especies de psílidos gallícolas asociadas (Lima 2008; Dias et al 2013a, b), lo que ha sido relacionado con la disponibilidad de recursos para la oviposición y alimentación, más que con las condiciones climáticas (Carneiro & Isaias 2015).

Calophya rubra y *C. mammifex* no solo alteraron la estructura de sus respectivos órganos hospederos, sino también la composición y distribución de sus metabolitos primarios y secundarios. En ambos morfotipos de agallas se detectaron dos compartimentos especializados de almacenamiento de lípidos: uno en el compartimento medio (parénquima vascular y células del floema) y otro en el compartimiento externo de la agalla. Especialmente, en el parénquima vascular de las agallas de hojas, también se encontró acumulación de proteínas y azúcares reductores; característico de los tejidos similares a nutritivos (*sensu* Ferreira et al. 2017). En el compartimiento externo de las agallas de hoja, además de lípidos, se localizaron proteínas en los denominados tejidos de almacenamiento común (Ferreira et al. 2017). La acumulación de metabolitos primarios en agallas de psílidos se ha reportado previamente en el Neotrópico (Oliveira et al. 2006; Isaias et al. 2011; Oliveira & Isaias 2010a; Malenovsky' et al. 2015, Carneiro et al. 2014; Carneiro & Isaias 2015), sin embargo es la primera vez que se da a conocer para el Mediterráneo.

De igual manera, el perfil de metabolitos secundarios (MS) de los órganos hospederos fue alterado por la acción de *C. rubra* y *C. mammifex*. Particularmente el perfil cromatográfico (obtenido por GC-MS) de los órganos hospederos no se mantiene en las agallas. Aunque los compuestos predominantes en *S. polygama* fueron terpenos, mayormente sesquiterpenos, estos no se detectaron en las agallas. En ambos morfotipos de agallas predominaron los compuestos fenólicos.

La expresión de síntesis de MS en plantas está fuertemente controlada en el tiempo y espacio (Moore et al. 2014). Tal comportamiento se reveló mediante análisis histoquímico y espectográfico en los órganos hospederos y sus respectivas agallas. La detección de compuestos fenólicos y ligninas fue espacialmente similar en ambos morfotipos de agallas, y se vio afectada en el tejido del compartimento medio debido a su perfil nutritivo. Los fenoles totales se acumulan mayormente en los compartimentos internos y externos de las agallas, mientras que las ligninas fueron redireccionadas solamente al sistema dérmico de las agallas y en pocas

cantidades en el xilema del tejido vascular neoformado. Los sitios de acumulación de lignina en ambos morfotipos de agallas indican que para las agallas de calófidos la síntesis de ligninas está más relacionada con la conducción de agua y defensa que con el soporte mecánico. Finalmente, la no lignificación de las capas celulares en los compartimentos medio e interno de las agallas puede favorecer la inserción de los estiles de *C. rubra* y *C. mammifex* en los tejidos del compartimento medio de las agallas, donde se alimentan.

Por su parte, la ausencia de fenoles en el floema y el sistema vascular de las agallas (compartimento medio) y la acumulación de los polifenoles en los compartimentos externo e interno de las agallas puede ser un mecanismo para prevenir que los inductores entren en contacto con compuestos fenólicos no palatables y/o tóxicos (Bragança et al. 2017). Por tanto, los fenoles depositados en el compartimento externo podrían actuar como capa protectora contra enemigos naturales y factores climáticos (Abrahamson & Weis 1997). Sin embargo, los estiletes de *C. rubra* y *C. mammifex* deben atravesar las células, ricas en fenoles, del compartimento interno, lo que puede indicar que a pesar de la toxicidad potencial de los polifenoles de las plantas (Róstas et al. 2013); la saliva de los insectos hervíboros especialistas puede degradar fácilmente dichos fenoles (Hori 1992). Tal adaptación química para eliminar compuestos fenólicos del sistema digestivo de los insectos hervíboros especialistas (Lattanzio et al. 2006; Taiz & Zeiger 2006; Després et al. 2007) probablemente esté presente en *C. rubra* y *C. mammifex*. En estas dos especies de calófidos la compartimentación de compuestos fenólicos y ligninas sigue un patrón genérico, acumulándose de manera similar en los tejidos de los compartimentos externo e interno de las agallas.

En las agallas de hoja y tallos inducidas en *S. polygama* los niveles de polifenoles disminuyeron en los sitios de desarrollo de la agalla en comparación con los órganos hospederos. Sin embargo, aunque los niveles de polifenoles en las agallas de las hojas son muy similares a los observados en las hojas sin agallas, en las agallas del tallo los niveles de polifenoles fueron más bajos que en los tallos sin agallas, excepto durante la senescencia de las agallas. Este comportamiento en los niveles de fenoles en las agallas de calófidos podría ser indicativo de restricciones metabólicas impuestas por el órgano hospedero. Por tanto, la concentración de compuestos fenólicos es temporalmente distintiva para cada órgano hospedero, y para las agallas tuvo una dinámica opuesta, disminuyeron en las agallas de hoja y aumentaron en las agallas del tallo desde la maduración hacia la senescencia.

En el Neotrópico se ha observado que la variación de la concentración de polifenoles en agallas ha estado asociada al estrés hídrico y a la radiación lumínica (Formiga et al. 2009; Detoni et al. 2011). No obstante, en las agallas de calófidos las oscilaciones en los niveles de fenoles también dependen de la etapa de desarrollo de la agalla y de las peculiaridades metabólicas de los órganos hospederos. Debido al estrés oxidativo generado durante el desarrollo de las agallas (Soares et al. 2000; Oliveira et al. 2011a, b), la acumulación de polifenoles también puede contribuir al mantenimiento de la estructura de la agalla (Bedetti et al. 2014). En consecuencia, la acumulación de polifenoles en los compartimentos interno y externo de las agallas de *C. rubra* y *C. mammifex* podría estar relacionada con la expansión y división celular. Sin embargo, a pesar del papel secundario atribuido a la función defensiva de los compuestos fenólicos en los sistemas gallícolas (Close & McArthur 2002; Bedetti et al. 2014b), la detección de compuestos fenólicos y ligninas en los tejidos externos en ambos morfotipos de agallas, nos lleva a suponer que ambos MS intervienen en el desarrollo y defensa mecánica en el sistema *S. polygama*-*Calophya* spp.

Las evidencias recopiladas a través del desarrollo de este trabajo confirman que las agallas de calófidos son un fenotipo extendido de sus inductores. A pesar de ello, los órganos hospederos ejercen influencia y restricciones al desarrollo anatómico y metabólico de las agallas. De igual manera, se revela que el clima Mediterráneo ejerce una marcada influencia en la morfología y química de las agallas; así como en el ciclo de vida de los insectos. Las características anatómicas, histoquímicas y fenológicas de las agallas de *C. mammifex* y *C. rubra* refuerzan su valor adaptativo en términos nutritivos, microambientales y protectores, previamente descrito por Price et al. (1987). Las agallas de calófidos son estructuras complejas, que se desarrollan bajo las restricciones impuestas por los órganos hospederos, la influencia del hábito succionador de floema de los inductores y las condiciones climáticas del Mediterráneo del centro-sur de Chile.

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