



UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS
INSTITUTO DE ECOLOGÍA
BIOLOGÍA EVOLUTIVA

**Cambios en las trayectorias ontogenéticas de la defensa de las
plantas cuando una especie oportunista invade un mutualismo**

TESIS

(POR ARTÍCULO CIENTÍFICO)

QUE PARA OPTAR POR EL GRADO DE:
MAESTRA EN CIENCIAS BIOLÓGICAS

PRESENTA:

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MÉXICO, CD. MX.

JULIO 2019



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OFICIO CPCB/696/2019

Asunto: Oficio de Jurado para Examen de Grado.

M en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
Presente

Me permito informar a usted, que el Subcomité de Ecología y Biología Evolutiva, en su sesión ordinaria del día 8 de abril de 2019, aprobó el siguiente jurado para el examen de grado de **MAESTRA EN CIENCIAS BIOLÓGICAS** en el campo de conocimiento de **Biología Evolutiva** de la alumno **FONSECA ROMERO MITZI ALEJANDRA** con número de cuenta: **309009841**, por la modalidad de graduación de **tesis por artículo científico**, con la tesis titulada: "Changes in the ontogenetic strategies in plant defense when exploiters invade a plant-ant mutualism", producto del proyecto realizado en la maestría que lleva por título: "**CAMBIOS EN LAS TRAYECTORIAS ONTOGENÉTICAS DE LA DEFENSA DE LAS PLANTAS CUANDO UNA ESPECIE OPORTUNISTA INVADE UN MUTUALISMO**", bajo la dirección de la DRA. KARINA BOEGE PARÉ, quedando integrado de la siguiente manera:

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Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, Cd. Mx., a. 10 de junio de 2019

DR. ADOLFO GERARDO NAVARRO SIGÜENZA
COORDINADOR DEL PROGRAMA



Agradecimientos institucionales

Agradezco a la Universidad Nacional Autónoma de México, así como al Posgrado en Ciencias Biológicas, por permitirme ingresar a la Maestría en Ciencias Biológicas.

Agradezco al Consejo Nacional de Ciencia y Tecnología (CONACYT) por el apoyo económico con número 775654, que me permitió realizar mis estudios en el programa y mi investigación. Al igual que el financiamiento del Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica –UNAM (PAPIIT-IN211314).

Agradezco especialmente a mi tutora Dra. Karina Paré y a mi comité tutor Dra. Ek del Val de Gortaria y Dr. Juan Enrique Fornoni Agnelli, por haberme acompañado durante la realización de este proyecto y su valiosa contribución para que pudiera culminar el mismo.

Agradecimientos personales

Agradezco a mi jurado Dr. Pedro Luis Valverde Padilla, Dr. Johnattan Hernández Cumplido, Dra. Sonia Vázquez Santana y Dr. Martin Heil por su apoyo y valiosos comentarios que contribuyeron a la realización de esta investigación.

Quiero agradecer de manera muy especial a Rosario Razo y Omar Hernández y por su invaluable apoyo en campo, amabilidad y comentarios que me ayudaron a realizar este trabajo. De igual manera agradezco a Rubén Pérez Ishiwara por su apoyo en campo.

A todos mis compañeros del Laboratorio de Interacción Planta-Animal, gracias por compañerismo y comentarios. En particular quiero agradecer a Xoch y Sofi por su paciencia, consejos y gran ayuda.

A mis padres, hermanos y sobrinos por apoyarme siempre, enseñarme tanto, echarme porras y motivarme a seguir creciendo cada día y llegar más lejos. Son el pilar de mi vida. Los amo.

A mi abuelita, aunque no llegó a ver la culminación de este trabajo, es parte importante de mi vida y quien siempre nos enseñó a tener amor y respeto por la naturaleza y la vida. Siempre estás en mi corazón abue.

A mis tíos por sus enseñanzas, consejos y entusiasmo que me impulsa a seguir.

A mis amigos por estar en las buenas y en las malas, por sus locuras y alegría que transmiten y que siempre me motiva para dar lo mejor de mí.

Y un agradecimiento especial a José de Jesús por ser un gran apoyo, compañero y soporte que me motiva a darlo todo, y que me llena de alegría con sus locuras y ocurrencias. Sin duda eres parte fundamental que me impulsó a terminar.

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Resumen

Comúnmente los mutualismos son invadidos por especies oportunistas. Como la inversión de recursos en recompensas sin obtener los beneficios correspondientes puede incrementar el costo de la interacción para los mutualistas, las plantas pueden ajustar facultativamente sus fenotipos para evitar pérdidas energéticas y reducir costos reproductivos. En particular, las plantas mirmecófitas que son colonizadas por hormigas explotadoras pueden modificar facultativamente la producción de recompensas. Sin embargo, no existen evidencias sobre cómo las trayectorias ontogenéticas de las defensas pueden ajustarse en función de la especie de hormiga que coloniza las plantas mirmecófitas.

El objetivo de este estudio fue evaluar si las trayectorias ontogenéticas de defensas directas e indirectas cambian en función de la presencia de hormigas mutualistas o explotadoras. Con este fin, se cuantificaron los atributos de la defensa directa (potencial cianogénico y grosor foliar) e indirecta (contenido de azúcar en néctar extrafloral), así como el daño foliar causado por herbívoros, en distintos estadios ontogenéticos de *Vachellia hindsii* colonizados por hormigas mutualistas (*Pseudomyrmex ferrugineus*) o explotadoras (*Pseudomyrmex gracilis*).

Independientemente de la especie de hormiga que coloniza las plantas mirmecófitas, éstas expresaron defensas alternas durante su desarrollo. Mientras las defensas químicas disminuyeron, las defensas físicas y bióticas aumentaron en función de la edad de las plantas. Sin embargo, las trayectorias ontogenéticas en las defensas se modificaron en función de la presencia de especies explotadoras. En presencia de éstas, la cantidad de recompensas producidas en los nectarios extraflorales disminuyó, mientras que las defensas directas, como el grosor foliar o el potencial cianogénico aumentaron.

Por otra parte, observamos un porcentaje de daño foliar por herbívoros similar en los distintos estadios ontogenéticos de *V. hindsii*, lo cual puede estar regulado por la alternancia ontogenética en las defensas directas e indirectas. En el caso particular del estadio juvenil, la presencia de hormigas mutualistas reduce el ataque de herbívoros, en comparación con plantas colonizadas con hormigas explotadoras. Probablemente como una defensa efectiva ante una mayor producción de recompensas.

Con este estudio se demostró que en el sistema mutualista *V. hindsii*-*Pseudomyrmex*, las plantas pueden modificar la expresión de defensas directas e indirectas una vez que son colonizadas por organismos explotadores. Este trabajo constituye el primer reporte de que las estrategias ontogenéticas pueden cambiar en función del contexto ecológico en que las plantas crecen, en particular, las hormigas que las colonizan.

Abstract

Frequently mutualisms are invaded by opportunistic species. Because the investment of resources in rewards without obtaining the corresponding benefits can increase the interaction costs in the mutualism, plants can facultatively adjust their phenotypes to avoid energy losses and reduce reproductive costs. In particular, the myrmecophytic plants colonized by exploiting ants can facultatively modify the production of rewards and other defenses. However, there is no evidence that ontogenetic trajectories of defenses can be adjusted as a function of the ant species colonizing myrmecophytic plants.

The aim of this study was to assess the differences in the ontogenetic trajectories of direct and indirect defenses between plants colonized either by mutualistic or exploiting ant species. The attributes of direct (cyanogenic potential and leaf thickness) and indirect (sugar content in extrafloral nectar) defenses and leaf damage caused by herbivories were assessed at four ontogenetic stages of *Vachellia hindsii* colonized by mutualistic (*Pseudomyrmex ferrugineus*) or exploiting (*Pseudomyrmex gracilis*) ants.

Regardless of the identity of ants colonizing myrmecophytic plants, these expressed alternative defenses during their development. While chemical defenses decreased, physic and biotic defenses increased as a function of plants age. However, the ontogenetic trajectories in the defenses were modified according to the presence of exploiting species. In the presence of these, the amount of rewards produced in extrafloral nectaries decreased, while direct defenses, such as leaf thickness or cyanogenic potential, increased.

On the other hand, we observed a similar percentage of foliar damage by herbivores in the different ontogenetic stages of *V. hindsii*, which can be regulated by the ontogenetic

alternation in the direct and indirect defenses. In the particular case of the juvenile stage, the presence of mutualistic ants seems to reduce the attack of herbivores, in comparison with plants colonized with exploiting ants. Probably as an effective defense against a greater production of rewards.

With this study, we showed that in the mutualistic system *V. hindsii*-*Pseudomyrmex*, plants can modify the expression of direct and indirect defenses once they are colonized by exploitative organisms. This work is the first report that ontogenetic strategies can change depending on the ecological context in which plants grow, in particular, the ants that colonize them.

Introducción General

La evolución de las especies y el mantenimiento de las interacciones cooperativas ha sido de gran interés en los estudios de Ecología Evolutiva. Es común que las interacciones mutualistas sean invadidas por especies oportunistas que resultan en la explotación de al menos una de las especies (Bronstein, 2001; Heil et al., 2009a; Kautz et al., 2012). Esto, a su vez, podría generar una desestabilización del mutualismo (Ferrière et al., 2007; Kautz et al., 2009a; Heil, 2013) y promover alteraciones en la expresión de atributos involucrados en la interacción mutualista (Bronstein, 2001; Kautz et al., 2009).

La presencia de explotadores se ha reportado en diversos sistemas mutualistas defensivos, en donde un organismo ofrece recompensas a cambio de ser protegido tanto del medio biótico como abiótico (Bronstein, 2001). Por ejemplo, en la asociación de plantas y hormigas defensoras, éstas pueden expresar distintos grados de agresividad y efectividad al defender a su planta hospedera (Palmer y Brody, 2007). Por ende, la explotación se genera en distintas situaciones, como: el consumir las recompensas sin proporcionar una defensa efectiva contra herbívoros e incluso, llegar a consumir a su hospedero (Sakata, 1994; Stanton et al., 1999); una defensa poco efectiva del tejido reproductivo (Palmer y Brody, 2007), que pudiera ser producto de la intervención de un tercer interactuante (como los homópteros) (Gaume et al., 1998); o, en el peor de los casos, llegar a esterilizar a la planta hospedera con el fin de minimizar la competencia con hormigas dominantes (Yu y Pierce, 1998). Estas interacciones mutualistas y antagónicas han brindado excelentes oportunidades para comprender la dinámica de las interacciones cooperativas y para identificar los mecanismos mediante los cuales la selección natural favorece las estrategias

para evitar la explotación por parte de especies oportunistas, a través de la expresión de respuestas plásticas (Bronstein, 2001; Heil, et al., 2010, Kautz et al., 2012).

Las especies mutualistas afectadas por explotadores pueden expresar cierto grado de plasticidad fenotípica para evitar o minimizar los efectos negativos de la explotación. Por ejemplo, cuando las especies oportunistas consumen las recompensas de las flores sin proporcionar el servicio de polinización, las plantas pueden aumentar el grosor de los tejidos (es decir, corolas y cálices) como un mecanismo de resistencia, impidiendo que oportunistas roben las recompensas (Inouye, 1983; Irwin, et al., 2010), producir concentraciones más altas de metabolitos secundarios tanto en néctar como en tejido floral (Adler, 2000; Maloof, 2001) o disminuir la calidad y cantidad de néctar (Orona-Tamayo y Heil, 2013; González-Teuber et al., 2012). En el caso de las plantas mirmecófitas, pueden reducir la producción de recompensas para las hormigas (Agrawal, 2001; Heil, 2009b). Sin embargo, no se ha estudiado cómo la presencia de especies explotadoras puede modificar los patrones de expresión de atributos defensivos alternativos durante el desarrollo de las plantas para evitar el daño por herbívoros.

La capacidad de modificar la expresión de rasgos de recompensa en función de la presencia de especies oportunistas puede ser particularmente importante en la conformación de las estrategias ontogenéticas defensivas de las plantas. Estas estrategias pueden ser influenciadas tanto por limitaciones internas (i.e. restricciones arquitectónicas, disponibilidad de recursos y su prioridad en distintas funciones como crecimiento, reproducción y defensa; Stamp, 2003; Boege y Marquis, 2005; Barton y Koricheva, 2010; Quintero et al., 2013; Ochoa-López et al., 2015), como factores externos (i.e. daño por herbívoros y patógenos; Warner y Cushman, 2002; del-Val y Dirzo, 2003; Barrett y

Agrawal, 2004; Thomas et al., 2010). En el caso particular de plantas mirmecófitas, estos factores están involucrados en la producción de recompensas para la atracción de hormigas a lo largo de la ontogenia. En particular, el mutualismo con hormigas defensoras se establece sólo hasta que las plantas adquieren un tamaño adecuado para ofrecer suficientes recompensas, y se intensifica conforme las plantas crecen (del-Val y Dirzo, 2003; Clark et al., 2012; Quintero et al., 2013). Las plántulas, al ser particularmente vulnerables a ser atacadas por herbívoros, deberían expresar defensas alternativas para reducir el daño (del-Val & Dirzo, 2003; Ochoa-López et al., 2015). Una vez que las plantas alcanzan el tamaño necesario para establecer el mutualismo, la producción de atributos de recompensa se incrementa (Quintero et al., 2013), mientras que las defensas directas deberían disminuir (Siegler y Ebinger, 1987; Siegler, 1991; Boege y Marquis, 2005; Ochoa-López et al., 2015). De esta manera, el ajuste facultativo de estrategias produce trayectorias ontogenéticas de las defensas directas e indirectas en direcciones opuestas. Mientras las defensas directas disminuyen, las indirectas incrementan conforme las plantas crecen.

Sin embargo, cuando las plantas han adquirido el tamaño y vigor necesario para producir defensas indirectas, pero son colonizadas por hormigas explotadoras, no obtienen beneficios de la producción de recompensas. Esto puede representar una pérdida energética y derivar en un incremento en el daño foliar ante la falta de protección efectiva por las hormigas (Heil et al., 2009b; Kautz et al., 2012). En este contexto, ante la presencia de especies explotadoras, podría esperarse que la expresión de defensas directas no disminuya durante la ontogenia de las plantas y se minimice la producción de recompensas. En este caso, la expresión de defensas directas tendría que mantenerse constante durante el desarrollo de las plantas o con trayectorias ontogenéticas en la misma dirección que las

defensas indirectas. Si las plantas no tienen la capacidad de realizar un ajuste en sus defensas, se esperaría un incremento en el daño foliar en estadios juvenil y adulto. Este es el primer reporte sobre cómo las trayectorias ontogenéticas de defensas alternativas de plantas mirmecófitas, cambian en función de las hormigas que las colonizan.

El objetivo de este estudio fue determinar si plantas mirmecófitas expresan trayectorias ontogenéticas alternativas en la defensa cuando un organismo oportunista interviene en el sistema mutualista. Con este fin, medimos y comparamos atributos de la defensa directa (potencial cianogénico y grosor foliar) e indirecta (contenido de azúcar en el néctar extrafloral) en distintos estadios ontogenéticos de *Vachellia hindsii* colonizados por hormigas mutualistas (*Pseudomyrmex ferrugineus*) o explotadoras (*Pseudomyrmex gracilis*). El estudio de la intervención de organismos antagonistas en las trayectorias ontogenéticas en la defensa de plantas mirmecófitas ofrece una oportunidad para entender cómo los rasgos defensivos pueden ser alterados por el contexto ecológico en el que interacciones entre especies ocurren, incluyendo la ontogenia de las plantas como una fuente de variación importante de la expresión de diferentes atributos fenotípicos.

Changes in the ontogenetic strategies in plant defense when opportunistic ants invade a plant-ant mutualism

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Abstract

When opportunistic species invade a mutualism, plants can facultatively adjust their phenotypes to avoid energy losses and reduce reproductive costs. An opportunistic or exploiting species is one that consumes rewards provided by a mutualistic system without returning any benefit. In particular, the myrmecophytic plants colonized by opportunistic ants can facultatively modify the production of rewards and other defenses. To explore if this facultative response can alter the ontogenetic trajectories in plant defense, we estimated direct and indirect defenses at four ontogenetic stages of the myrmecophytic plant *Vachellia hindsii*, in the presence of mutualistic or opportunistic ant partners.

The ontogenetic trajectories in the production of hydrocyanic acid, leaf thickness and sugar in the extrafloral nectar were modified according to the presence of either mutualistic or opportunistic ant species. We observed that, during the development of the plants, the production of the cyanogenic potential decreased while leaf thickness and the production of sugar in EFN increased. The magnitude of these ontogenetic changes, however, varied as a function of the identity of the colonizing ants. When colonized by opportunistic ants, plant produced more direct defenses and reduced their reward for patrolling ants.

The ontogenetic trajectories in direct and indirect defenses can be adjusted according to the ant species colonizing myrmecophytic plants, and this could be a mechanism to reduce the fitness costs associated to exploitation interactions, reducing the energy losses involved in the production of EFN and assuring alternative defense strategies.

Keywords: exploitation, ontogenetic trajectories, plant–herbivore interactions, *Pseudomyrmex*, *Vachellia*.

Introduction

How species evolve and maintain cooperative interactions has been of great interest in evolutionary ecology studies. Because these interactions involve the exchange of resources and services, they are commonly exploited by opportunistic species. This exploitation implies, for one of the species, that they invest resources in rewards without obtaining the corresponding benefits. This often increases the costs of the interaction and can lead to the destabilization of the mutualism (Ferrière et al. 2007; Kautz et al. 2009; Heil 2013). For example, in plant-pollinator mutualisms, opportunistic species consume flower rewards without transporting plant gametes and, in the worst case, destroying them (Gómez and Espalader 1998; Maloof and Inouye 2000). Nutritional mutualisms can be also invaded by opportunistic species, as is the case for the association between mycorrhizal fungi and plants, which are often exploited by parasitic fungal species. These fungi consume carbon delivered by plants without providing nutrients to their hosts (Smith and Smith 1996). In the case of defensive mutualisms, such as the association between plants and ants (del-Val and Dirzo 2004), it is common to find ant species with different degrees of aggressiveness, deriving in either an effective defense against herbivores, or the exploitation of plant rewards without providing the defensive service. These mutualistic and antagonistic interactions have provided excellent opportunities to understand the dynamics of cooperative interactions and to identify the mechanisms by which natural selection favors strategies to avoid the exploitation by opportunistic species, through the expression of plastic responses (Bronstein 2001; Heil et al. 2009b; Kautz et al. 2012).

Mutualistic species affected by exploiters can express a certain degree of phenotypic plasticity to avoid or minimize the negative effects of the exploitation. For example, when opportunistic species exploit flower rewards, plants can increase the thickness of tissues (i.e. corollas and calyces; Inouye 1983; Irwin et al. 2010), produce higher concentrations of secondary metabolites in both nectar and floral tissue (Adler 2000; Maloof 2001) or decrease the quality and quantity of nectar (Orona-Tamayo and Heil 2013; González-Teuber et al. 2012). In the case of myrmecophytic plants, they can reduce the production of rewards for ants (Agrawal 2001; Heil 2009b). What remains unclear in these cases is if, in the absence of effective ant defenders, plants also respond with alternative direct defenses to avoid herbivore damage.

The ability to change the expression of rewarding traits as a function of the presence of opportunistic species should be particularly important in shaping the ontogenetic defensive strategies of plants. These strategies allow organisms to deal with different selective forces across their development, and can determine their lifetime fitness (Boege et al. in press). For example, ontogenetic changes in the expression of defenses are ubiquitous for most plant species (Barton and Boege 2017). In the case of myrmecophytic plants, architectural restrictions, availability of resources and functional priorities (i.e. growth, reproduction and defense) promote an increase in the expression of rewards for ants as they develop (Stamp 2003; Boege and Marquis 2005; Barton and Koricheva 2010; Quintero et al. 2013; Ochoa-López et al. 2015). In fact, the mutualism is established only once plants can offer sufficient rewards to maintain their ant colonies (del-Val and Dirzo 2003; Clark et al. 2012; Quintero et al. 2013). Before this stage, young plants rely on alternative defenses to reduce damage by herbivores (del-Val and Dirzo 2003; Ochoa-López et al. 2015). Once

they reach the size to establish a mutualism with defending ants, the expression of direct defensive traits decreases (Siegler and Ebinger 1987; Siegler 1991; Boege and Marquis 2005; Ochoa-López et al. 2015). These facultative adjustments, in turn, produce ontogenetic trajectories of direct and indirect defenses with opposite directions (Fig. 1a). These ontogenetic shifts in the expression of direct and indirect defenses has been reported in at least one myrmecophyllic species, *Turnera velutina* (Ochoa-López et al. 2015; Ochoa-López et al. 2018).

The presence of opportunistic ant species, however, is likely to change these ontogenetic strategies. When plants acquire the size and vigor to produce enough rewards, but do not obtain benefits from the opportunistic ants, they cannot rely on this indirect defense. In the absence of an effective ant protection, herbivore damage should increase. In this context, the expression of direct defenses is expected to remain constant or even increase during plant development, whereas a reduction in resource allocation to indirect defenses should be observed (Figure 1b). Although plants can facultatively reduce reward production when colonized by opportunistic ants (Heil et al. 2009b), there is no empirical evidence that ontogenetic trajectories of defenses can be adjusted as a function of the ant species (i.e. mutualist or opportunist) colonizing myrmecophytic plants. The aim of this study was to assess the differences in the ontogenetic trajectories of direct and indirect defenses between plants colonized either by mutualistic or opportunistic ant species. We quantified attributes of direct (cyanogenic potential and leaf thickness) and indirect (sugar content in extrafloral nectar) defenses at four ontogenetic stages of *Vachellia hindsii* colonized by mutualistic (*Pseudomyrmex ferrugineus*) or opportunistic (*Pseudomyrmex gracilis*) ants.

Materials and methods

Study system

This study was conducted in Oaxaca, Mexico, near the Manialtepec Lagoon (15 ° 55'N, 097 ° 09'W, 25 m.a.s.l.) The climate is warm sub-humid with a rainy season in summer, an annual precipitation between 800 - 2000 mm and a mean annual temperature between 22 and 28 ° C (INEGI, 2008). *Vachellia hindsii* (Leguminosae) (formerly *Acacia hindsii*; Miller & Seigler, 2012) is a myrmecophytic plant that grows in tropical dry forests of México and Central America (Rico-Gray 2001). It produces hollow thorns, extrafloral nectaries in the rachis of the leaves, food bodies at the tip of leaflets, known as Beltian bodies. All these traits are considered an indirect defense, as they attract patrolling ants that deter herbivores from their host plants (Rico-Gray 2001; Bronstein et al. 2006; Clement et al. 2008). *V. hindsii* leaves display chemical defensive traits such as secondary metabolites (e.g. cyanogenic glycosides, condensed tannins) and/or defensive proteins (Seigler and Ebinger 1987; Seigler 1991). At least four species of ants with different degree of aggressiveness can coexist in *V. hindsii* (Heil 2013). The degree of aggressiveness is determined by the defensive behavior against herbivores of each species (following Palmer 2004; 2010; Kautz 2012; Heil 2013). *Pseudomyrmex ferrugineus* is the most frequent and aggressive mutualistic ant present in 55-60% of the plants (rated on an ordinal scale by Heil 2013, following Clement et al. 2008). The opportunistic ant *Pseudomyrmex gracilis* can occupy 26-30% of the plants and has the lowest aggressiveness (non-defending ants). Two other mutualistic species with intermediate levels of aggressiveness occupy the remaining 10% of the plants, *Pseudomyrmex mixtecus* and *Crematogaster sp.* (Ward and Downie 2005; Heil et al. 2009b; Heil 2013).

Measurement of defense attributes

To assess the ontogenetic trajectories of direct and indirect defenses in the presence of mutualistic (*P. ferrugineus*) and opportunistic (*P. gracilis*) ants, 121 individuals of *V. hindsii* from four ontogenetic stages were selected ($N= 32$ seedlings (< 15 cm height without swollen thorns), 23 young juvenile individuals (15-100 cm height, with at least one swollen thorn), 40 old juvenile individuals (101-200 cm height with fully developed swollen thorns) and 26 adult plants, (> 201 cm height, with reproductive structures).

Whereas seedlings and young juvenile plants were randomly selected from the studied population, old juvenile and adult plants were chosen based on the ant species present in the plant (old juveniles: $N = 20$ for each ant species, adult plants: $N= 16$ colonized by mutualistic ants; 10 colonized by opportunistic ants). For all individuals, we measured plant height, stem diameter at the base and registered the presence of spines, Beltian bodies and ants, specifying their identity. Physical, chemical and biotic defenses were estimated as described next.

We measured leaf thickness of the third fully expanded apical leaf of each plant using an electronic micrometer (Fred V. Fowler Co., Inc). Three measurements were taken in different parts of one leaflet avoiding the mid vein, and the mean value for thickness was calculated. Thickness has been previously used as a proxy of leaf toughness (Pérez-Harguindeguy et al. 2013), which is considered a defense with consistent effects against herbivore damage to a greater extent than other direct defenses (Carmona et al. 2011).

In the case of chemical defenses, we assessed the cyanogenic potential of plant tissue using a colorimetric test with sodium picrate to estimate hydrocyanic acid (HCN) concentration (Schappert and Shore 1995). Two leaflets of equivalent size were cut from

the collected leaves, placed in 1.5 ml Eppendorf tubes and stored in a cooler with ice until further processing. One leaflet was used for determination of weight. A second leaflet was macerated inside the Eppendorf tube with 7 μ l of chloroform. A strip of 2.5 cm \times 1 cm filter paper previously soaked in a solution of picric acid (0.5%) and Na₂CO₃ (5%), was placed in each tube with macerated leaves avoiding direct contact with the plant tissue. The tubes were incubated at room temperature in the dark for 24 hours. After this period, a 6 mm diameter circle of the filter paper was cut and placed in a new Eppendorf tube with 1 ml of alcohol. To quantify HCN concentration, three 200 μ l samples were taken from each tube and read at 590 nm in an ELISA spectrophotometer (BioTek®: ELx808, USA). The estimation of μ g of HCN in the sample was calculated as $\mu g HCN * g^{-1} = (Abs\ 590\ nm - 0.036865)/0.0001236) * g^{-1}$ dry weight. This formula was obtained from a regression analysis of a series of solutions with different HCN concentrations and their absorbance at 590 nm (Ochoa-López et al. 2015). Dry weight was obtained from the second collected leaflet, which was dried at room temperature until no changes in weight were recorded.

To evaluate the investment of plants in biotic defenses at different ontogenetic stages, we quantified the content of sugar in the extrafloral nectar (EFN) following the protocols described in Heil et al. (2004) and Hernández-Zepeda et al. (2018). First, the hollow thorns of a branch were cut, and ants were removed. To prevent access by nectar and ant robbers, the branch was isolated with a mesh bag sealed with plasticized wires and Tangle trap (a sticky resin that prevents ants from passing, The Tangle Foot Cooperation www.planetnatural.com/product/tree-tanglefoot-insect-barrier/). After 24 hours, three leaves of each individual were collected for the extraction of EFN (because seedlings do

not produce extrafloral nectaries, we only used young juvenile, old juvenile and adult plants). The nectar of all extrafloral nectaries of each leaf was collected with a micropipette with 5µl of distilled water. The diluted nectar was placed in a refractometer to estimate sugar concentration in Brix degrees. Nectar was then reabsorbed using 10µl microcapillars, and its volume was quantified measuring the length of the liquid column in the capillary using a caliper. Sugar content in the EFN was estimated as $mg * g^{-1} (Brix * \mu l / 100) * g^{-1} dry weight$ (Damián et al. 2018). To obtain dry weight values, each leaf was dried at room temperature until no changes in weight were recorded.

Characterization of ontogenetic trajectories in plant defenses

To evaluate if the simultaneous expression of defensive traits changed along plant development, we used a multivariate analysis of variance (MANOVA) for plants colonized by *P. ferrugineus* or by *P. gracilis* (i.e., an independent analysis was performed for plants colonized by each ant specie, but data of seedlings and young juvenile plants with no ants, were used the same for both data sets). The model included ontogenetic stage as the independent variable and each defense (leaf thickness, HCN concentration and sugar content in EFN) as the dependent variables. Afterwards, one-way analyses of variance (ANOVA) were performed for each defensive trait for each type of associated ant. The models included ontogenetic stage as the independent variable and each defense as the dependent variable. To determine the differences in the expression of defenses among stages, independent Tukey HSD post-hoc tests were performed for each defense.

Changes in the expression of defenses as a function of ant identity

Because not all ontogenetic stages had ants, we could not perform a two-way ANOVA to evaluate differences in the expression of defenses as a function of the colonizing ant species. Instead, we performed independent t-test analyses for old juvenile and adult plants, which were the ontogenetic stages associated with either mutualistic or opportunistic ant species. One independent analysis was performed for each plant defense, including ant species as an independent variable. To simultaneously visualize the ontogenetic trajectories in the three defensive traits, values of each trait were standardized as $Z = (\bar{X}_i - \bar{X}) / (\text{sd of } X)$, where \bar{X}_i is the average of the defensive trait at the i^{th} ontogenetic stage and \bar{X} is the average of that trait across all ontogenetic stages (Ochoa-López et al. 2015).

Leaf damage by herbivores

To estimate the percentage of accumulated leaf damage caused by herbivores, an herbivory index was used (Domínguez and Dirzo 1995) modified by Sánchez Galván and Rico-Gray (2011). Leaves of seedlings ($N = 3$ leaves / plant), young juvenile ($N = 5$ leaves / plant), old juvenile and adult individuals ($N = 10$ leaves / plant) were collected. Leaf damage was visually classified into six categories of damage: 0 = 0% (without leaf damage), 1 = 1-6%, 2 = 7-12%, 3 = 13-25%, 4 = 26-50%, 5 = from 50% - 100%. Leaf damage index was then calculated as $HI = (\sum(Hi)(i)) / n$, where: Hi = number of leaflets in category (i), i = damage category (0 - 5) and n = total number of leaflets in the leaf. To estimate differences in leaf damage by herbivores across plant ontogeny the herbivory index was back-transformed to the mean value of the percentage of damaged at each category. A one-way ANOVA was performed considering the ontogenetic stage as the independent variable and the percentage of damage as the dependent variable. To determine differences in leaf damage as a function of the colonizing ant species, independent Tukey HSD post-hoc tests

were performed for old juvenile and adult plants. The identity of ants (mutualist or opportunist) was considered as the independent variable and leaf damage as the dependent variable. All statistical analyses were performed using the R version 3.5 program (R Core Team, 2018).

Results

Ontogenetic trajectories in physical, chemical and biotic defense

Overall, we found a significant effect of plant ontogeny on the simultaneous expression of plant defenses when colonized by either ant species (mutualistic ants: *Wilks' λ* = 0.357, *P* = 0.002; opportunistic ants: *Wilks' λ* = 0.285, *P* = 0.03). In particular, we found that, leaf thickness was two-fold thicker in adult plants than in seedlings, both in the presence of mutualistic ($F_{3,83} = 26.45$, $P < 0.001$) and opportunistic ants ($F_{3,54} = 53.82$, $P < 0.001$; Fig. 2a). In contrast, the concentration of HCN was five times greater in seedlings than in old juvenile and adult plants, both in the presence of mutualistic ($F_{3,79} = 12.32$, $P < 0.001$) and opportunistic ants ($F_{3,60} = 8.04$, $P < 0.001$; Fig. 2b). The production of sugar in the extrafloral nectar was up to three-fold greater in juvenile plants colonized by mutualistic ants than in young juvenile and adult plants ($F_{3,37} = 11.81$, $P < 0.001$), but not in the presence of opportunistic ants ($F_{3,26} = 0.5$, $P = 0.613$; Fig. 2c).

Changes in the expression of defenses as a function of the colonizing ant species

In old juvenile plants colonized by *P. gracilis*, leaf thickness was 1.4 times greater than in plants colonized by the mutualistic ant *P. ferrugineus* ($t = -6.14$, $P < 0.001$, Fig. 2a). At this same stage, sugar content in EFN was three times lower in the presence of opportunistic ants than in the presence of the mutualists ($t = 6.158$, $P < 0.001$; Fig. 2c).

Plants at the adult stage produced 1.6 times more HCN in the presence of opportunistic ants than when colonized by mutualistic ants ($t = -2.202$, $P = 0.055$; Fig. 2b). As a result of these differences, the ontogenetic changes of the three defensive traits were of different magnitudes according to the identity of the ants associated with each ontogenetic stage of plants (Fig. 3a and b).

Leaf herbivore damage

Leaf damage caused by herbivores ranged between 7 and 25% of the total leaf area. No differences were observed among ontogenetic stages colonized by either mutualistic ($F_{3,34} = 0.36$, $P = 0.78$) and opportunistic ants ($F_{3,26} = 0.64$, $P = 0.59$). However, old juvenile plants colonized by opportunistic ants had greater leaf damage than plants colonized by mutualistic ants ($t = -2.667$, $P = 0.018$; Fig. 4).

Discussion

We report that ontogenetic patterns in the expression of physical, chemical and biotic defenses in a myrmecophytic plant can be modified as a function of the presence of opportunistic ant species. As predicted, we found that, regardless of the identity of colonizing ants, *V. hindsii* plants expressed greater direct defenses at the younger stages, until they reached the stage in which they are able to host a functional colony of ants and started producing indirect defensive traits. In particular, we found ontogenetic trajectories with opposite directions for the expression of chemical defenses (HCN) and sugar in EFN of leaves. However, we found that the magnitude of these ontogenetic changes was influenced by the identity of the ants colonizing old juvenile and adult plants. Old juvenile plants colonized by opportunistic ants invested less resources in extrafloral nectar than

plants colonized by mutualistic ants. In adult plants, physical (leaf thickness) and chemical (HCN) defenses were greater in the presence of opportunistic ants. This is the first study to report what seems to be a facultative ability to modify the ontogenetic trajectories in plant defense, as the result of the presence of opportunistic species invading a plant-ant mutualism.

Changes in the expression of defenses across plant ontogeny

Our results show that plants of *V. hindsii* can modify the expression of their direct and indirect defenses across their ontogeny. This ontogenetic switches in defensive strategies could be promoted by trade-offs implied in the simultaneous expression of different defensive traits (Boege et al. 2007) and/or by developmental or anatomical constraints (Quintero et al. 2013; Villamil et al. 2013).

Consistent with our findings, previous studies on *V. hindsii* have already reported higher concentrations of cyanogenic glycosides in early than in later ontogenetic stages (Seigler and Ebinger 1987; Seigler 1991). These mechanisms represent a possible alternative defense until plants acquire sufficient biomass to offer enough resources for an effective biotic defense, as suggested by our results showing an increase in the sugar produced in EFN as plants develop (Barton and Koricheva 2010; Barton and Hanley 2013; Quintero et al. 2013). Alternate ontogenetic trajectories of different plant defenses have also been reported in *Turnera velutina* (Ochoa-López et al. 2015). In this species, whereas the cyanogenic potential and tolerance decrease, trichome density and sugar in EFN increase along plant ontogeny (Ochoa-López et al. 2015). This evidence suggests that myrmecophytic species can express particular ontogenetic strategies to optimize the expression of different plant defenses as a function of their adaptive value (Ochoa-López et

al. unpublished). For example, the constancy in herbivore damage observed at different ontogenetic stages of *V. hindsii* suggests that the ontogenetic alternation of the direct and indirect defenses promotes an equivalent efficiency of both strategies, when expressed at different ontogenetic stages.

Changes in ontogenetic strategies as a function of the identity of colonizing ants

Our results suggest that *V. hindsii* plants have the ability to facultatively modify the expression of their direct and indirect defenses across their ontogeny, as a function of the colonizing ant species. In the presence of opportunistic species, the amount of sugar produced in extrafloral nectar (indirect defense) was lower, while the expression of direct defenses, such as leaf thickness and cyanogenic potential was greater than in the presence of mutualistic ant species. We suggest that this could be a mechanism to avoid the energetic loss caused by nectar consumption by opportunistic ants (Bronstein 2001) and to guarantee the defense of the tissues with alternative defense mechanisms (direct defenses). These plastic responses, in turn, produced different ontogenetic trajectories in plant indirect and direct defenses (Fig. 3).

The mechanisms by which plants responded to the presence of opportunistic species remains unclear and await further investigation. Most plants commonly respond to the attack by herbivores increasing the production of secondary metabolites (Janzen 1967; Agrawal 2001; Heil 2007, 2010) and/or leaf thickness (increasing the concentration of lignin) (Schädler et al. 2003). Hence, one possibility is that increased leaf damage after being colonized by non-aggressive ants triggered the induction of direct defenses. Indeed, we found that at least in plants at their old juvenile stage, the presence of opportunistic ants increased herbivore damage, in comparison with plants colonized with mutualistic ants.

This pattern is consistent with previous studies of exploitation in myrmecophytic plants of the same genus, *Acacia cornigera* (Sánchez-Galván and Rico-Gray 2011), as well as in the absence of defending ants in *V. hindsii* (González-Teuber et al. 2012).

Alternatively, a direct recognition of the ants colonizing the myrmecophytic plants could trigger the adjustment in the expression of both direct and indirect defensive traits (Razo-Bellman and Heil unpublished). For example, several studies have reported that, in the presence of effective defensive ants, the production of EFN is of high quality, with greater contents of sugar and amino acids (Heil 2005, 2011; González-Teuber et al. 2012; Heil 2013). Otherwise, when plants are colonized by opportunistic ants, the quality of EFN decreases, reducing energy losses caused by the consumption of antagonistic organisms (Agrawal and Rutter 1998; Heil et al. 2005; Heil et al. 2009a). Accordingly, we also found that plants colonized by mutualistic ants produced a greater amount of sugar in the EFN. Because the production of EFN implies energy costs for the plant, its adaptive value relies on the benefits obtained by ant patrolling (Agrawal and Rutter 1998; Heil et al. 2005; González-Teuber et al. 2012; Heil 2013; Quintero et al. 2013; Heil 2015; Hernández-Zepeda et al. 2018). However, the mechanisms by which plants recognize and respond to the presence of opportunistic species remain unclear and this await further investigation.

Conclusion

We demonstrated that in the mutualistic system *V. hindsii*-*Pseudomyrmex*, plants can modify the expression of their direct and indirect defenses once they are colonized by opportunistic organisms. Although ontogenetic trajectories in plant defense have been widely described in many species, this is the first report that those ontogenetic changes can vary as a function of the ecological context in which plants grow, in this case, the identity

of ants colonizing the plants. If indeed, plants have the ability to facultatively change their ontogenetic trajectories, this could represent a mechanism to reduce energy losses implied in the production of rewards when there is an exploitation event. The inclusion of antagonistic organisms (such as opportunistic ants), as well as the ontogeny of myrmecophytic plants, can reveal underestimated sources of variation in plant defense, and allow a better understanding of the defensive strategies that plants express to deal with herbivores and opportunistic species.

Acknowledgments

The authors thank Rubén Pérez Ishiwara for logistic support during fieldwork. M.F. was supported by a fellowship from Consejo Nacional de Ciencia y Tecnología (775654). This article is a requirement for the first author MFR to obtain her Master's degree in Biological Sciences (Ecology) of the Posgrado en Ciencias Biológicas from the Universidad Nacional Autónoma de México.

Author contributions

MFR, JF, EV and KB conceived the ideas and designed methodology; MFR conducted field work and analyzed the data. MFR and KB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Funding

This work was funded by Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica -UNAM grant (PAPIIT-IN211314) to K.B.

Conflict of interests

None of the authors have any conflict of interests associated with this work.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data accessibility

Upon acceptance, we intend to upload our data set into a public database repository and provide the respective DOI to enable readers to locate archived data from our paper.

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Figure 1. Predicted ontogenetic strategies in the production of direct (solid lines) and indirect defenses (dashed lines) in the presence of A) mutualistic ants, which promote ontogenetic trajectories of direct and indirect defenses with opposite directions, and B) exploiter ants that should promote a continuous production of direct defenses and only a subtle increase of indirect defenses along ontogeny.

Figure 2. Ontogenetic trajectories of three defenses of *A. hindisii* when colonized by *P. ferrugineus* (mutualist, solid line) and *P. gracilis* (exploiter, dashed line) ants. A) Leaf toughness, B) Cyanogenic potential and C) Sugar in extrafloral nectar. Different letters indicate significant differences among stages. * indicates significant differences between mutualists and exploiters at each ontogenetic stage. Sample sizes used for each defense and stage were as follow: Leaf thickness: 20 seedlings, 32 young juvenile, 39 old juvenile, and 26 adult plants; HCN: 28 seedlings, 20 young juvenile, 39 old juvenile, and 25 adult plants; Sugar in EFN: 0 seedlings, 10 young juvenile, 21 old juvenile and 14 adult plants).

Figure 3. Ontogenetic strategies of *V. hindsii* described by chemical defense (cyanogenic potential, solid line), physical defense (leaf thickness, dotted line) and biotic defense (sugar content in EFN, dashed line), depending on the identity of A) mutualist and B) exploiter ants.

Figure 4. Mean leaf damage (\pm standard error) registered in *V. hindsii* in plants inhabited by *P. ferrugineus* (mutualist) and *P. gracilis* (exploiter) at different ontogenetic stages. * indicates significant differences between mutualist and opportunist at the old juvenile stage (N= 10 seedlings, 11 young juvenile, 21 old juvenile, and 14 adult plants).

Figure 1.

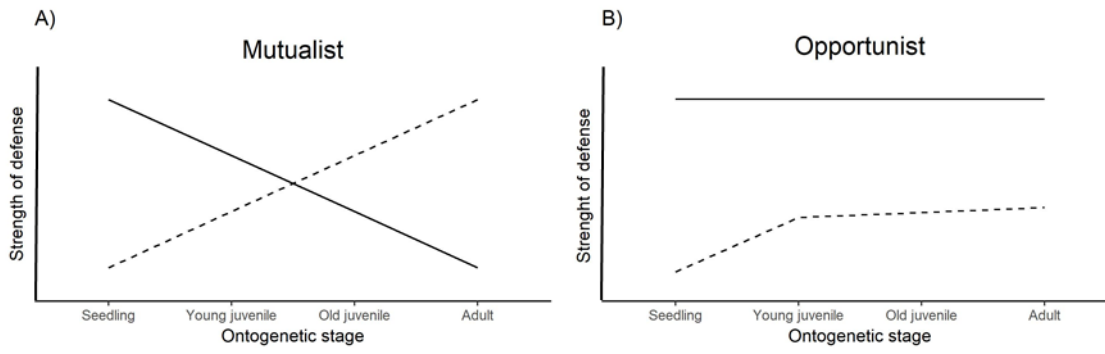


Figure 2.

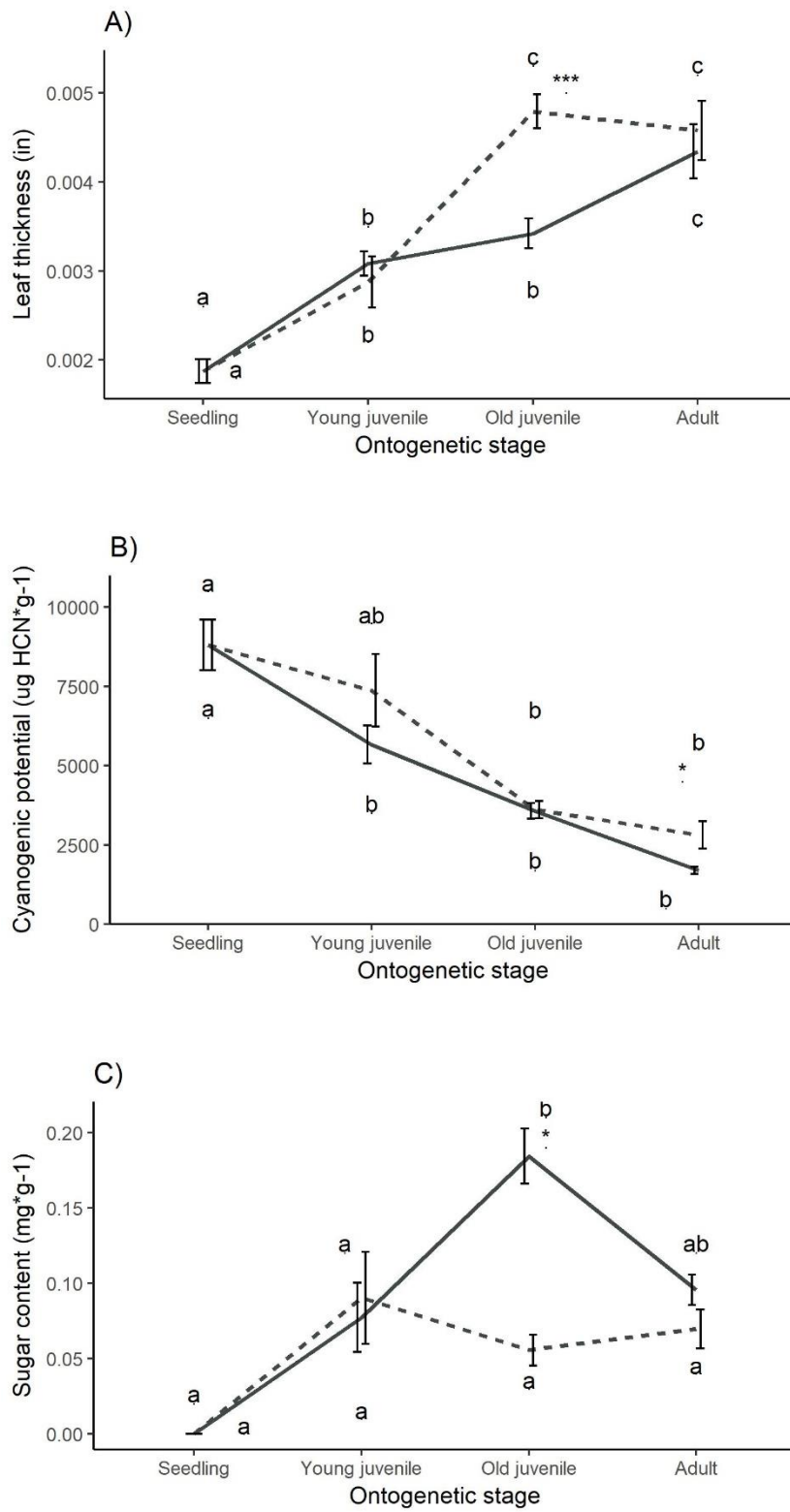


Figure 3.

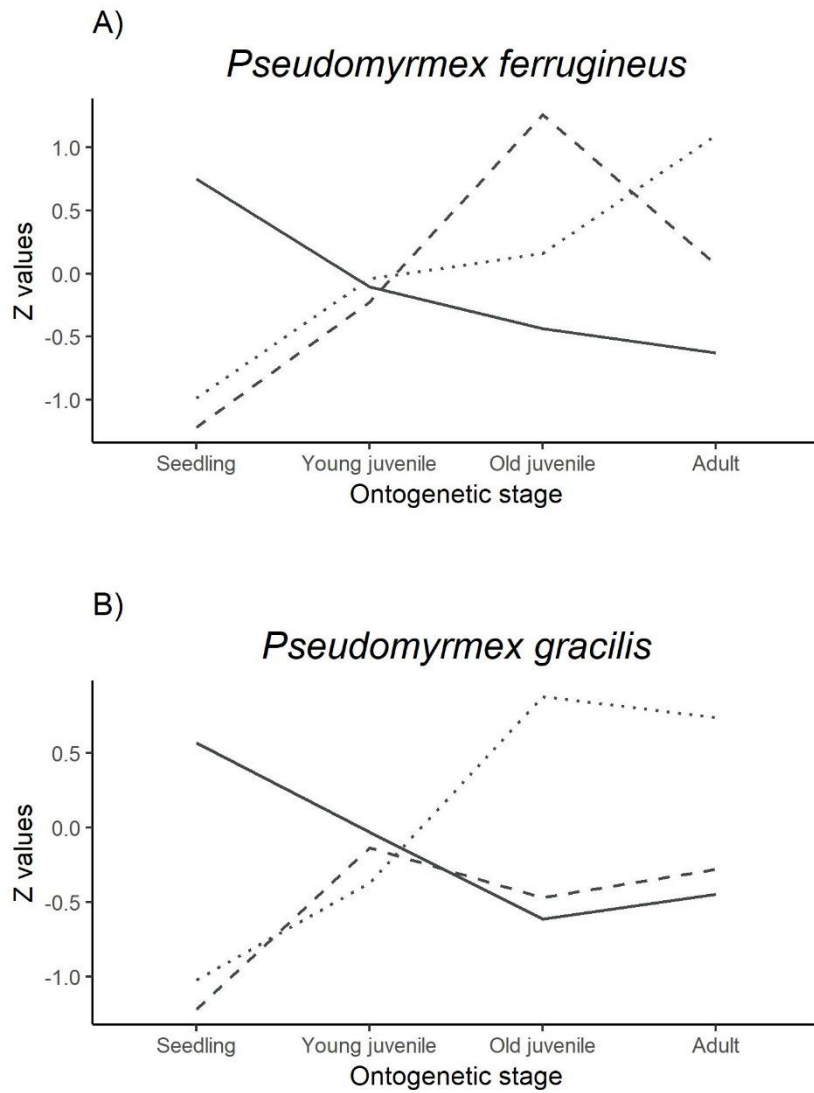
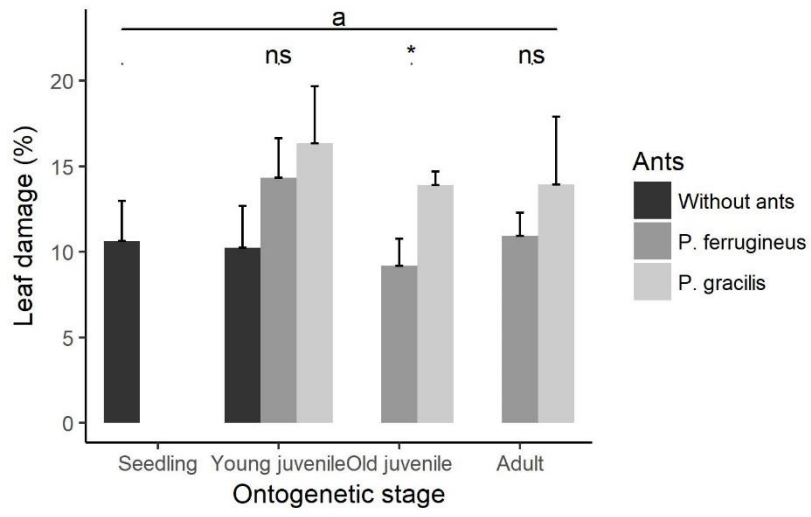


Figure 4



Discusión General

Los resultados de este trabajo sugieren que los patrones ontogenéticos en la defensa de las plantas pueden modificarse en función de la presencia de especies oportunistas en sistemas mirmecófitos. Como patrón general, encontramos que, independientemente de la identidad de las hormigas, la trayectoria ontogenética de la defensa química (HCN) es opuesta a la trayectoria del grosor foliar y azúcar en el NEF de las hojas. Sin embargo, la expresión de defensas en los estadios juvenil y adulto fueron diferentes en función de las hormigas presentes. Como se predijo, en presencia de hormigas mutualistas, las plantas expresaron una alternancia entre defensas física (grosor foliar) y biótica (contenido de azúcar en el NEF). Por el contrario, en presencia de hormigas explotadoras, la defensa física se mantuvo constante, mientras la biótica se redujo.

Caracterización de las trayectorias ontogenéticas en la defensa

Una posible explicación de la alternancia en las defensas directas e indirectas en *V. hindsii* es una reasignación de recursos para lidiar con las disyuntivas energéticas que implica la expresión simultánea de distintos atributos de defensa (Coley et al., 1985). Estas disyuntivas pueden ocasionarse por la limitación de recursos, escasez de tejido fotosintético y/o restricciones durante el desarrollo de las plantas (Stamp, 2003; Boege y Marquis, 2005; Barton y Koricheva, 2010; Quintero et al., 2013; Ochoa-López et al., 2015). Por ejemplo, en sus primeros estadios ontogenéticos, las plantas mirmecófitas expresan mecanismos alternativos de defensa ante la imposibilidad de producir suficientes recompensas para atraer a hormigas mutualistas. La producción de metabolitos secundarios representa una alternativa como defensa, puesto que en esta etapa son susceptibles al ataque por herbívoros (Barton y Koricheva, 2010; Barton y Hanley, 2013; Quintero et al.,

2013). En concordancia con estudios previos, *V. hindsii* mantiene altas concentraciones de glicósidos cianogénicos en estadios tempranos, y disminuyen en estadios posteriores (Siegler y Ebinger, 1987, Siegler, 1991).

Una vez alcanzado el tamaño suficiente para el establecimiento de hormigas, las plantas pueden disminuir su defensa química con la finalidad de invertir en la producción de recompensas para la obtención de la defensa biótica, que podría conferir una defensa más efectiva contra herbívoros (Barton y Koricheva, 2010; Barton y Hanley, 2013; Quintero et al., 2013). Se ha sugerido que la expresión de trayectorias ontogenéticas alternas en la defensa de especies mirmecófitas con más de un atributo defensivo, pueden reducir el daño foliar por ataques de herbívoro (Quintero et al., 2013; Villamil et al., 2013; Ochoa-López et al., 2015). Lo cual, resulta congruente con nuestros resultados en el daño constante por herbívoros a lo largo de la ontogenia.

Cambios en la defensa en función de la identidad de las hormigas

Nuestros resultados muestran que las plantas de *V. hindsii* tienen la capacidad de modificar facultativamente la expresión de sus defensas directas e indirectas en función de las hormigas que las colonizan. En presencia de especies explotadoras, la cantidad de recompensas producidas en los nectarios extraflorales (defensa indirecta) disminuyó, mientras que las defensas directas, como el grosor foliar aumentó. Este podría ser un mecanismo para evitar la pérdida energética causada por el consumo de recompensas por parte de hormigas explotadoras (Bronstein, 2001) y asegurar la defensa de los tejidos con mecanismos de defensa alternativos (defensas directas).

El incremento en defensas químicas y físicas en individuos adultos parece ser una opción cuando las plantas son colonizadas por hormigas explotadoras y, por tanto,

susceptibles al ataque por herbívoros (Bronstein, 2001). Se ha reportado que, una respuesta común al ataque por herbívoros es un incremento en la producción de metabolitos secundarios (Janzen, 1967; Agrawal, 2001; Heil, 2007; 2010) y el grosor foliar (incrementando la concentración de lignina) (Schädler et al., 2003). Estos rasgos de resistencia en la defensa de las plantas hacen menos atractivo el tejido vegetal para sus consumidores (Hanley et al., 2007; Clissold et al., 2009). De esta forma, las plantas de *V. hindsii*, podrían evitar pérdidas de área foliar ante escenarios de explotación.

Por otra parte, encontramos que las plantas juveniles colonizadas por hormigas mutualistas producen una mayor cantidad de azúcar en el NEF. Caso contrario, en plantas colonizadas por hormigas explotadoras, la cantidad de azúcar en el NEF disminuyó. Este mecanismo de ajuste en la expresión de rasgos defensivos podría desencadenarse ante el posible reconocimiento de especies oportunistas (Razo-Bellman y Heil, sin publicar). Diversos estudios han reportado que la calidad y cantidad de NEF depende de la presencia y la efectividad de sus hormigas defensoras (Agrawal y Rutter, 1998; Heil et al., 2005; González-Teuber et al. 2012; Heil, 2013; Quintero et al., 2013; Villamil et al., 2013). En este contexto, en presencia de organismos defensores efectivos, las plantas producen NEF con altos contenidos de azúcar y aminoácidos (Heil, 2005; 2011; 2013; González-Teuber et al., 2012). Caso contrario, cuando no existe una defensa efectiva, la cantidad de recompensas disminuye para así evitar pérdidas energéticas, como consecuencia del consumo por antagonistas (Agrawal y Rutter, 1998; Heil et al., 2005; 2009b).

Debido a que la producción de NEF implica costos energéticos para las plantas, su valor adaptativo se basa en los beneficios obtenidos por el patrullaje efectivo de hormigas (Agrawal y Rutter, 1998; Heil, et al., 2005; González-Teuber, Bueno, Heil, y Boland,

2012; Heil, 2013; Quintero et al., 2013; Heil, 2015; Hernández-Zepeda et al., 2018). Por lo que el alto contenido de azúcar en el NEF observado en el estadio juvenil podría estar relacionado con la disminución de la pérdida foliar en presencia de mutualistas. Esto sugiere una defensa biótica efectiva contra ataques de herbívoros ante la producción de recompensas de buena calidad, como se ha demostrado en otros sistemas (O'Dowd, 1982; Beattie, 1985; Koptur, 1992; Heil et al., 2001; Heil y McKey, 2003). Estos resultados son congruentes con un estudio previo de explotación en una planta mirmecófita del mismo género (Sánchez-Galván y Rico-Gray, 2011), así como en ausencia de hormigas defensoras en *V. hindsii* (González-Teuber et al., 2012). Sin embargo, hacen falta estudios para determinar los mecanismos por los cuales las plantas reconocen y responden a la presencia de explotadores.

Conclusiones

El estudio de la intervención de organismos oportunistas en sistemas mutualistas es de importancia en la teoría de la defensa de las plantas, ya que es necesario incluir tanto las restricciones internas, como el contexto ecológico en que se involucra el sistema, que determinará la expresión de los rasgos defensivos durante la ontogenia de las plantas.

Independientemente de la hormiga colonizadora, las plantas de *Vachellia hindsii* expresaron trayectorias ontogenéticas en las defensas directas opuestas, como una posible reasignación de recursos involucrados en distintos procesos, como defensa y crecimiento.

La identidad de la hormiga colonizadora determina los cambios en la expresión de defensas. En presencia de hormigas mutualistas, las plantas expresaron una alternancia entre defensas física y biótica. Por el contrario, en presencia de hormigas explotadoras, las defensas directas se mantuvieron constantes, mientras la biótica se redujo. Esta alternancia

es un posible mecanismo para evitar pérdidas energéticas debido al consumo de recompensas, pero sin un beneficio a cambio.

Tanto la alternancia en las defensas directas e indirectas, el incremento en el contenido de azúcar, como recompensa ante una defensa efectiva, parecen reducir efectivamente el ataque de herbívoros.

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