INÁCIO JOSÉ DE MELO TELES E GOMES

BENEFITS FROM ANTS TO PLANTS WITH A SPECIAL SCRUTINY ON PROTECTION AGAINST HERBIVORY: A REVISIT ON THE *Cecropia-Azteca* SYSTEM

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ecologia, para obtenção do título de *Doctor Scientiae*.

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"Se procurar bem você acaba encontrando

não a explicação (duvidosa) da vida

mas a poesia (inexplicável) da vida"

Carlos Drummond de Andrade

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SUMÁRIO

RESUMO

GOMES, Inácio José de Melo Teles e, D.Sc., Universidade Federal de Viçosa, abril de 2019. **Efeitos benéficos de formigas para plantas com um escrutínio sobre a proteção contra herbivoria: revisitando o sistema *Cecropia-Azteca*.** Orientador: Ricardo Ildefonso de Campos. Coorientadores: José Henrique Schoereder e Ricardo Ribeiro de Castro Solar.

Mutualismo e herbivoria são relações ecológicas com características distintas. Enquanto o mutualismo é uma interação que gera benefícios mútuos para as espécies envolvidas, a herbivoria é uma relação de predação, em que uma espécie herbívora é beneficiada se alimentando da planta. Essas relações são evidentes nos sistemas de plantas mirmecófitas. Nesses sistemas mutualísticos, as plantas oferecem abrigo e alimento para formigas, que, por sua vez, oferecem diversos benefícios às suas hospedeiras, sendo a proteção contra herbivoria a mais evidente. Para se proteger, as plantas ainda possuem outras estratégias, como a produção de compostos químicos e estruturas morfológicas especializadas em proteção. Além disso, as respostas das plantas à herbivoria em suas características físicas e fisiológicas podem mudar ao longo do seu desenvolvimento. Neste trabalho, nosso objetivo foi investigar todos os potenciais benefícios conhecidos das formigas *Azteca muelleri* para suas plantas hospedeiras, *Cecropia glaziovii*. Além disso, investigamos os efeitos da herbivoria sobre o crescimento das plantas ao longo do seu desenvolvimento ontogenético. Por meio de um estudo experimental de longo prazo, nós acompanhamos o desenvolvimento de plantas de *C. glaziovii* por 54 meses. Com coletas mensais, acessamos dados sobre crescimento, herbivoria, nutrição, investimento em defesas químicas e físicas e a colonização das formigas. Demonstramos que as formigas *A. muelleri* favorecem o crescimento de suas plantas hospedeiras por meio de proteção contra herbívoros e patógenos. As formigas ainda nutre a planta e reduzem gastos energéticos com produção de tricomas. Ademais, a herbivoria só se torna prejudicial para a planta depois que esta é colonizada por formigas. Apresentamos aqui uma definição conclusiva sobre os efeitos benéficos das formigas para as plantas, que vão além da clássica defesa contra herbivoria. Além disso, mostramos que a fase ontogenética da planta parece ser determinante para seu tipo de resposta à herbivoria.

ABSTRACT

GOMES, Inácio José de Melo Teles e, D.Sc., Universidade Federal de Viçosa, April, 2019. **Benefits from ants to plants with a special scrutiny on protection against herbivory: a revisit on the *Cecropia-Azteca* system.** Advisor: Ricardo Ildefonso de Campos. Co-advisors: José Henrique Schoereder and Ricardo Ribeiro de Castro Solar.

Mutualism and herbivory are antagonic ecologic relations. Mutualism is an interaction with mutual benefits for the involved species. Herbivory, on the other hand, is a predation interaction, in which a herbivore species benefits feeding on a usually impaired plant. Both interactions are found in myrmecophytic systems. In such mutualistic systems, plants offer shelter and food to ants, that, in exchange, benefit plants through many mechanisms, being herbivory protection the most evident. In parallel, plants can also use other strategies, like the production of chemical compounds and morphological structures. Moreover, plant physical and physiological responses to herbivory can change throughout its development. Our aim was to investigate all the known potential benefits from ants to plants. In addition, we addressed the effects of herbivory on plant growth along its ontogenetic development. Through an long-term experiment, we monitored *Cecropia glaziovii* individuals during 54 months. We collected data monthly on plant growth, herbivory, nutrition, investiment in chemical and physical defenses and colonization by ants. We showed here that *Azteca muelleri* ants benefit their host plants growth via protection against herbivores and pathogens, nutrition and energy saving from other defensive strategies. Moreover, herbivory only impairs plants in the phase after the ant colonization. Here, we conclusively demonstrate the beneficial effects of ants to plants, beyond the classic herbivory protection. In addition, we showed that plant ontogenetic stage is determinant to its response to herbivory.

INTRODUÇÃO GERAL

As interações entre os seres vivos podem ser classificadas quanto ao tipo de efeito causado em cada uma das espécies envolvidas. Quando a interação entre duas espécies é benéfica para ambas as partes, classificamo-la como mutualismo, uma das interações biológicas mais conhecidas e estudadas (Bronstein, 2009). Por outro lado, quando somente uma das espécies é beneficiada em detrimento da outra, podemos ter um caso de predação. A predação de plantas por herbívoros é conhecida como herbivoria, uma das mais importantes interações ecológicas, pois é a partir dela que toda a energia produzida pelas plantas se torna disponível para os demais níveis tróficos (Agrawal, 2007; Schmitz, 2008). É em um sistema de interface entre essas duas importantes interações ecológicas, mutualismo e herbivoria, que se desenvolve a presente tese.

Mutualismos são interações amplamente encontradas na natureza e desempenham importantes papeis na estruturação e funcionamento dos ecossistemas (Bronstein, 2009; Bronstein, Alarcón, Geber, et al., 2006; Herre, Knowlton, Mueller, & Rehner, 1999). As relações mutualísticas podem ser desde ocasionais, passando por relações estáveis, e podem chegar a uma relação mutualística obrigatória, quando um parceiro depende exclusivamente do outro para sua sobrevivência. Nesses casos, a relação mutualística pode ter origens distintas. Segundo Drown *et al.* (2013), os casos de mutualismo obrigatório podem ser divididos em:

i)   *Mutualismo de transmissão vertical*, em que o hospedeiro recebe seu parceiro diretamente dos seus pais, o que gera fidelidade entre os genes dos hospedeiros e seus parceiros;

ii)   *Mutualismo de transmissão horizontal*, em que o hospedeiro obtém seu parceiro diretamente do ambiente ou tendo contato infeccioso com outros hospedeiros da mesma geração.

Os estudos empíricos sobre a ecologia e evolução desses dois tipos de mutualismo não possuem ainda grandes consensos (Borges, 2015). Um clássico exemplo de mutualismo de transmissão horizontal com considerável estabilidade é o realizado pelas plantas e formigas. As interações entre plantas e formigas são encontradas no mundo todo, e podem ser desde relações ocasionais e facultativas até complexas relações obrigatórias (Martin, Heil; Doyle, 2003). Nesse último caso, as plantas produzem estruturas especializadas para abrigar (domáceas) e, muitas vezes, alimentar as formigas (e.g. trichilia, nectários extra-florais), sendo então chamadas de mirmecófitas. Uma vez morando na planta, as formigas patrulham e defendem sua planta hospedeira contra herbívoros, patógenos e outras plantas parasitas (Martin, Heil; Doyle, 2003; Mayer, Frederickson, & Mckey, 2014).

Uma das vantagens mais notórias experimentadas pelas plantas mirmecófitas é a proteção das formigas contra herbívoros. Herbivoria é uma interação geralmente negativa para as plantas, que perdem tecido fotossintético e reservas estocadas (Marquis, 1984). Na maioria dos casos, a herbivoria é prejudicial para as plantas, podendo levá-las à redução da sua performance e fitness e até mesmo à morte (Züst & Agrawal, 2017). A longa disputa evolutiva entre plantas e seus herbívoros selecionou para as primeiras um vasto leque de estratégias defensivas. Diante de tantas formas distintas de defesa já identificadas, os pesquisadores criaram diferentes classificações. Dentre elas, podemos destacar três tipos sendo o mais geral deles divididos em dois grandes grupos: *a) defesas constitutivas*, quando estão sempre presentes na planta,

independentemente da ação de herbívoros, e *b) defesas induzidas*, que são defesas desencadeadas como resposta a herbívoros, podendo ser defesas de alta especificidade (Mithöfer & Boland, 2012). Outra forma de classificação divide as defesas em: *a) defesas diretas*, quando a defesa é produzida e executada pela própria planta sob ataque; e *b) defesas indiretas*, identificadas quando uma planta atrai, nutre ou abriga organismos de outro nível trófico para diminuir a pressão da herbivoria (Heil, 2008).

Na presente tese, nós utilizaremos uma terceira classificação que divide as defesas de plantas em três grupos:

i) *Defesas químicas*: são compostos químicos produzidos pelas plantas. Além dos compostos primários, usados pelas plantas essencialmente para crescimento, elas também produzem os compostos secundários, que são utilizados para defesa. Esses compostos inibem a ação de herbívoros tornando a planta impalatável e tóxica. Os compostos secundários podem ser classificados em três grandes grupos químicos: terpenoides (e.g. piretroides e cucurbitacinas), compostos fenólicos (e.g. lignina, flavonoides e taninos) e compostos ricos em N (e.g. glicosídeos cianogênicos e alcaloides) e S (e.g. fitoalexinas e glicosinolatos) (Chaudhary, Bala, Thakur, Kamboj, & Dumra, 2018; Mithöfer & Boland, 2012).

ii) *Defesas físicas*: são estruturas de defesas morfológicas ou anatômicas das plantas. Geralmente são a primeira barreira de defesa contra os herbívoros, uma vez que são capazes de detê-los mecanicamente ou mesmo desencorajá-los ao ataque. Em muitos casos, as estruturas de defesas físicas podem agir conjuntamente com estratégias químicas, como no caso dos tricomas glandulares e das resinas das coníferas. Os

principais exemplos de defesas físicas são os espinhos, acúleos, tricomas, a dureza ou espessura foliar e as resinas (Chaudhary et al., 2018; Hanley, Lamont, Fairbanks, & Rafferty, 2007).

iii) *Defesas bióticas*: são defesas conferidas por algum parceiro que, associado em algum grau com a planta hospedeira, a protege contra herbívoros. Normalmente, esses parceiros são insetos que são atraídos e recompensados por estruturas que oferecem alimento, como néctar ou corpúsculos; ou moram nas plantas em buracos ou abrigos naturais ou mesmo em estruturas especializadas, as domáceas. Vários animais estão envolvidos nesse tipo de defesa, como ácaros, aranhas e joaninhas. Entretanto, os mais comuns, como já citado anteriormente, são as formigas (Martin, Heil; Doyle, 2003). Alguns estudos sugerem que as relações planta-formiga obrigatórias (mirmecófitas) são a mais eficiente estratégia de defesa de plantas, superando até as defesas químicas e físicas (Massad, Fincher, Smilanich, & Dyer, 2011; Zhang, Zhang, & Ma, 2015).

As plantas normalmente não utilizam apenas um tipo de defesa. As estratégias de defesa das plantas são dinâmicas e muitas vezes atuam conjuntamente. Acredita-se que o efeito sinérgico desses diferentes tipos de defesa seja mais eficaz contra uma maior variedade de herbívoros (Agrawal, 2007). Por outro lado, estudos sugerem a presença de um balanço energético entre a produção de diferentes estratégias de defesa (Coley, 1986; Koricheva & Romero, 2012). Além disso, essas estratégias de defesa variam ao longo do desenvolvimento ontogenético das plantas, sendo influenciado por aspectos fisiológicos, mas também por fatores ambientais, como disponibilidade de

recursos e a própria pressão de herbivoria (Barton & Koricheva, 2010; Boege & Marquis, 2005). É neste escopo teórico, envolvendo as relações possivelmente benéficas entre plantas mirmecófitas e suas formigas influenciando as estratégias de defesa das primeiras contra a herbivoria, que se encontra o tema central desta tese.

As plantas mirmecófitas são encontradas em todas as regiões tropicais. Seu sucesso evolutivo fez com que surgissem várias vezes independentemente em pelo menos 100 gêneros neotropicais (Martin, Heil; Doyle, 2003). Em uma escala global, os gêneros mais conhecidos são *Vachellia* (*Acacia*), *Macaranga* e *Cecropia*. As plantas do gênero *Cecropia* (Rosales: Cecropiaceae) são encontradas em toda região neotropical e têm mais de 180 espécies conhecidas, sendo a maioria mirmecófita. As formigas associadas a essas plantas são as formigas do gênero *Azteca* (Formicidae: Dolichoderinae), com raras exceções. A associação *Cecropia-Azteca* é conhecida desde o século XIX, mas somente a partir dos estudos sistemáticos de Janzen (1969), as características específicas desse sistema foram pormenorizadas.

Nesse sistema, a associação começa quando a rainha de *Azteca* perfura uma região do caule especialmente fina (prostoma) e penetra no tronco oco da *Cecropia*. O interior do tronco é composto por várias câmaras (internós) que servirão como local para a construção de ninhos para as formigas (Janzen, 1969). Além disso, a planta oferece alimento para suas formigas associadas, os corpúsculos müllerianos. A produção desses corpúsculos acontece em uma estrutura especializada chamada de *trichilium* que se localiza na base do pecíolo de todas as folhas (Yu & Davidson, 1997) (Figura 1). Nosso modelo de estudo na presente tese é a espécie *Cecropia glaziovii* Snethl, que, na região onde o estudo foi realizado, é sempre encontrada associada à formiga *Azteca muelleri* (Emery 1893).

Como mostrado no nosso modelo, os benefícios oferecidos pelas plantas para as formigas são intuitivos, uma vez que as formigas moram e se alimentam de recursos oferecidos pela própria planta. Por outro lado, os benefícios das formigas para as plantas carecem de um olhar mais analítico. Alguns estudos falharam em encontrar algum benefício das formigas para as plantas (e.g. Fáveri e Vasconcelos, 2004; Janzen, 1975; Wheeler, 1942), enquanto muitos outros atestam pelo benefício advindo das formigas (e.g. Oliveira e Pie, 1998; Pringle, Dirzo e Gordon, 2011; Schupp, 1986; Zhang, Zhang e Ma, 2015). Entretanto, o fato de que formigas podem identificar e escolher suas plantas hospedeiras baseadas em pistas sobre sua saúde (Razo-belman, Molina-Torres, Martínez, & Heil, 2018) mantém aberta a questão: as formigas de fato beneficiam as plantas, ou a melhor performance encontrada em plantas com formigas se deve ao fato de que as formigas escolheram plantas mais saudáveis para colonizar (K. N. Oliveira et al., 2015)?

Dentre os benefícios encontrados para indivíduos de *Cecropia*, o mais evidente é a proteção que as formigas conferem contra herbívoros. Entretanto, um estudo desenvolvido pelo nosso grupo de pesquisa no mesmo sistema interativo, concluiu que existem outras importantes vantagens trazidas pelas formigas para suas plantas hospedeiras: i) proteção contra patógenos que causam doenças; ii) nutrição ocasionada da absorção pela planta de resíduos das formigas; e iii) economia de energia pela possibilidade de reduzir as defesas físicas e químicas (Oliveira *et al.*, 2015 e referências). Dessa forma, investigar todos esses possíveis mecanismos de beneficiamento pelas formigas pode nos ajudar a compreender melhor como essa relação se estabiliza e evolui. A melhor compreensão dos mecanismos que geram e mantém esses mutualismos também pode contribuir para um melhor entendimento das estratégias de defesa e alocação de energia em plantas.

Figura 1. Imagens com foco nas estruturas mirmecófitas de *Cecropia glaziovii*. **A.** estrutura de uma planta já colonizada, mas ainda juvenil. **B.** internó da planta, que é oco por dentro, formando a domácea. **C.** *Trichilium* em destaque, encontrado na base do pecíolo foliar; os pontos brancos são os corpúsculos Müllerianos, usados pelas formigas para alimentação. **D.** Prostoma, local do tronco mais fino, presente nos internós, onde as formigas perfuram para ter acesso ao interior da planta.

Dentre os possíveis benefícios das formigas para as plantas, a proteção contra herbivoria é recorrentemente apontada como o principal deles. Entretanto, pouco ou quase nada se sabe sobre os efeitos da herbivoria em plantas mirmecófitas ou como elas podem mudar de acordo com o desenvolvimento ontogenético das plantas. Dessa forma, acreditamos que para a obtenção de resultados mais conclusivos sobre esse

tema, estudos experimentais e principalmente de longa duração são a melhor estratégia. Assim, a proposta desta tese é oferecer esse tipo de dado. Para isso, mudas de *C. glaziovii* foram plantadas e acompanhadas por 54 meses. Essas plantas foram divididas em três grupos experimentais: plantas protegidas contra a colonização de formigas, plantas sem formiga protegidas contra herbívoros, e plantas controle. Dispomos de dados mensais durante 54 meses acerca do crescimento de *C. glaziovii*, taxas de herbivoria, presença ou ausência de formigas, momento exato da colonização, presença de patógenos, além de dados anuais sobre defesas químicas e físicas e isótopos estáveis.

Nosso objetivo geral é investigar os reais benefícios das formigas *A. muelleri* para suas plantas mirmecófitas hospedeiras com uma abordagem múltipla e integrativa, com especial atenção e detalhamento para os efeitos da herbivoria. Para isso, esta tese está dividida em dois capítulos em formato de artigo científico.

Capítulo I: An integrative approach on mechanisms underlying benefits that ants provide to plants: a long-term manipulative experiment on the *Cecropia-Azteca* system

(Uma abordagem integrada sobre diferentes mecanismos que explicam os benefícios das formigas para as plantas mirmecófitas: um experimento manipulativo de longo prazo no sistema *Cecropia-Azteca*)

Este capítulo está formatado em formato de manuscrito para ser submetido à revista *Journal of Ecology*.

Neste capítulo, nosso objetivo principal foi testar a hipótese de que as formigas aceleram o crescimento de suas plantas hospedeiras logo após a sua colonização e a

ação dos seus mecanismos de proteção. Além disso, nós analisamos os diferentes mecanismos potenciais pelos quais as formigas poderiam acelerar o crescimento da planta, sendo eles:

i. Redução da herbivoria colonização das formigas;

ii. Aumento de fungos patogênicos em plantas não colonizadas;

iii. Maior absorção de nutrientes das formigas para as plantas colonizadas;

iv. Redução do investimento em defesas químicas e físicas após a colonização.

Capítulo II: Herbivory and plant growth: contrasting effects along ant-plant ontogeny

(Herbivoria e crescimento de plantas: efeitos contrastantes ao longo da ontogenia de uma planta mirmecófita)

Este capítulo está formatado em formato de manuscrito para ser submetido à revista *Oikos*.

Neste capítulo, nós focamos nos efeitos da herbivoria sobre o crescimento de plantas mirmecófitas. Especificamente, nós testamos as seguintes hipóteses:

i. A herbivoria reduz o crescimento das plantas;

ii. As respostas das plantas à herbivoria variam ao longo do seu desenvolvimento ontogenético, sendo que plantas mais velhas devem ser mais suscetíveis do que plantas mais jovens;

iii. A pressão da herbivoria e as mudanças ontogenéticas das plantas influenciam suas estratégias defensivas.

An integrative approach on mechanisms underlying benefits that ants provide to plants: a long-term manipulative experiment on the *Cecropia-Azteca* system

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ABSTRACT

1. In myrmecophytic systems, the benefits provided by plants to ants are easily recognizable, but reverse benefits can be less obvious, conditional and complex. Environmental variation, ontogenetic changes and the lack of long-term experimental approaches may cause contradictory outcomes. Furthermore, other seldom studied mechanisms besides anti-herbivore protection may better explain how plants benefit from ants' presence.

2. We planted individuals of *Cecropia glaziovii*, from which 36 were able to be divided in two groups (colonized by *Azteca muelleri* ants and experimentally uncolonized individuals). We monitored tree development, colonization, defensive and nutritional features from the onset of the experiment to 54 months. We quantified effects of ant colonies on plants, including plant growth, herbivory levels, fungal infection on trichilia, fertilization via ant debris and changes in physical and chemical defenses. We compared plant features in trees before and after ant colonization to understand whether the colonization preferences are random or biased by plant traits.

3. The presence of ants significantly increased tree height by 125% and leaf number by 123% after colonization compared to uncolonized individuals. This benefit is

associated with multiple, complementary positive effects of ants, including a decrease in herbivory by 83%, and protection against fungi, reduced physical (trichomes) investment, and 65% increase in leaf nitrogen and altered stable isotope composition, suggesting an effect of fertilization by ants.

4. *Synthesis*. Our results experimentally confirm and quantify the ants´ benefits for myrmecophytes, and we can exclude a possible alternative explanation that ant colonization is biased towards healthier plants, as their size and other traits were similar to uncolonized plants. Moreover, we differentiated among ant effects (i.e., ant fertilization and energy savings by reducing physical defenses) in a single experimental study, which give us a more complete picture of complex mechanisms besides anti-herbivore defenses.

INTRODUCTION

Most species on earth are, to some extent, involved in mutualisms and this mutual beneficial relationship between organisms is central for the structure and functioning of populations, communities and ecosystems (Bronstein, 2009; Herre et al., 1999; Toby Kiers, Palmer, Ives, Bruno, & Bronstein, 2010). Unfortunately, most studies involving mutualism are unilateral, focusing on only one of the interacting species (Bronstein, 1994). Therefore, the precise identification of mutual benefits between species can be considered among the greatest knowledge gaps in mutualism theory. Associations between ants and plants are among the most widespread

mutualistic interactions in nature (Bronstein, Alarcón, & Geber, 2006; Zhang et al., 2015) and so this system is an excellent model for the study of mutual benefits.

For myrmecophytes, defined as plants presenting specialized structures to house (domatia) and feed ants (Martin, Heil; Doyle, 2003; Mayer, Frederickson, Mckey, & Blatrix, 2014), the benefits provided by plants to the ants are straightforward due to two main reasons: i) ants live and feed on their host plants and ii) most of ant species colonizing myrmecophytes are exclusively found associated to their host plants (i.e., obligatory symbiosis). Conversely, ants' benefits for plants are not always clear and commonly reported in more case-specific situations (Martin, Heil; Doyle, 2003). In fact, while some studies have failed to find defensive traits in ants inhabiting myrmecophytes (Fáveri & Vasconcelos, 2004; Janzen, 1975; Wheeler, 1942), others showed an effective ant protection against plants' natural enemies (P. S. Oliveira & Pie, 1998; Pringle et al., 2011; Schupp, 1986; Zhang et al., 2015). Great part of this contradictory results is possibly influenced by two main methodological issues: lack of experiments and lack of long-term studies. Firstly, non-manipulative studies are insufficient to distinguish whether the plants' potential benefits are really provided by ants or whether such colonized plants were already healthier prior to ant colonization (K. N. Oliveira et al., 2015; Razo-belman et al., 2018). Second, even short-term ant-exclusion experiments have generated very contradictory outcomes regarding the ant benefits for plants (Trager et al., 2010). Here, we present a long-term ant-exclusion experiment where we intend to tackle both issues at the same time.

The most commonly reported benefit provided by ants to myrmecophytes is an increase in growth rate (Martin, Heil; Doyle, 2003; K. N. Oliveira et al., 2015; Schupp, 1986). Enhanced growth rates have been found to be triggered exclusively by lower herbivory levels experienced by colonized plants (e.g. Chamberlain & Nathaniel

Holland, 2009; Rosumek et al., 2009; Zhang et al., 2015). Although the causal relationship between herbivory and plant fitness is sound (but see Tiffin, 2000), herbivory and growth measures alone may represent weak proxies for the overall positive effects of ants on plant performance (see revision in Chamberlain & Nathaniel Holland, 2009; Trager et al., 2010). Unfortunately, studies on other important plant beneficial mechanisms such as ant protection against pathogens, ant-plant nutrition and plant energy saving by defensive trade-offs are scarce (but see K. N. Oliveira et al., 2015).

A key service provided by mutualistic ants to plants is defense against pathogens (Aljbory & Chen, 2018; Martin, Heil; Doyle, 2003). Ants can produce antifungal chemicals in their glands and repeal visitant fungal vectors, which has been described in some ant-plant systems, such as *Piper* (Letourneau, 1998), *Macaranga* and *Cecropia* (Roux, Céréghino, Solano, & Dejean, 2011). It has been also suggested that protection against herbivores often translates into a protection against pathogens, as the main entrance for pathogens and bacteria is via wounded tissue (Martin, Heil; Doyle, 2003). However, in spite of such potential importance, the effect of ant protection against pathogens is still poorly understood.

Other mechanism that may directly benefits host plants is the top-down ant fertilization, which is known as myrmecotrophy (Beattie, 1989). Host plants can absorb nutrients from ant's debris, such as prey remains, feces or dead ants which are deposited inside domatia (Martin, Heil; Doyle, 2003; Mayer, Frederickson, Mckey, et al., 2014; Sagers, Ginger, & Evans, 2000). Is has been recently suggested that such nutrient uptake can come from the ants' fecal droplets on leaves as well (Pinkalski, Christian, & Offenberg, 2018). Myrmecotrophy has been described in many nutrient-limited epiphytes but has also been found in terrestrial myrmecophytic plants such as

Maieta (Dejean et al., 2013) and *Cecropia* (Dejean, Petitclerc, Roux, Orivel, & Leroy, 2012; Sagers et al., 2000). Nevertheless, relevance and underlying processes of nutrient uptake are still largely unknown, and studies on myrmecotrophy are scarce.

Finally, ants may benefit plant development through indirect pathways. According to plant defense theory, there is a classical trade-off between growth or defense (Herms & Mattson, 1992). Therefore, it is reasonable to believe that if ants can provide an efficient herbivory protection, it would reduce host plant demand to invest in chemical and physical defenses, thereby saving energy for growth (Coley, 1986; Koricheva & Romero, 2012). However, the existence of resource allocation trade-offs between different defensive strategies and growth investment is still an open question for ant-plant relationships. While some studies have found higher levels of chemical investment in ant-free plants (e.g. Dyer, Dodson, Beihoffer, & Letourneau, 2001; Latteman, Mead, Duvall, Bunting, & Bevington, 2014; Rehr, Feeny, & Janzen, 1973), other studies demonstrated the lack of such trade-offs in ant-plant defensive systems (e.g. Del Val et al., 2003; Letourneau, Barbosa, & Letourneau, 1999).

Here we are presenting the first long-term manipulative experiment, where we investigate the effects of a symbiotic ant on their host plant growth also presenting a myriad of potential mechanisms behind this pattern. We monthly record plants since their seedling stage (during 54 months) and experimentally prevent part of those plants from being colonized by ants. This approach allows us to investigate the long-term consequences of the continuous absence of ants in uncolonized plants and also compare the same plant individuals before and after ant colonization. To avoid confounding factors, we used genetically related individuals of one single myrmecophytic plant species, growing under same natural conditions and colonized by a single ant species.

Our aim was to answer the following question: Do plants grow faster as a result of benefits brought by ant colonization, allowing them to be larger than their uncolonized conspecifics? In order to go further and assess why ants could possibly enhance plant growth we tested four non-exclusive hypotheses: (1) leaf herbivory rates decrease after ant colonization; (2) fungi infection is less frequent in colonized than in non-colonized plants; (3) there is an increase in plant top-down nutrient content after ant colonization, and (4) plants decrease their investment in physical and chemical defenses following ant colonization.

METHODS

Study site and biological model

Our experiment was performed at the Research, Training and Environmental Education Station "*Mata do Paraíso*" (20º48'07"S, 42º51'31"W). This reserve is a 195-ha area of Atlantic rainforest biome dominated by a montane semideciduous forest (Meira-Neto & Martins, 2002). The climate according to Köppen, is Cwa subtropical hot-summer climates, with rainy and hot summers and dry and cold winters (Alvares, Stape, Sentelhas, De Moraes Gonçalves, & Sparovek, 2013).

Our biological model was the tree species *Cecropia glaziovii* Snethl (Cecropiaceae) and the ant *Azteca muelleri* (Emery 1893) (Dolichoderine) in the Brazilian Atlantic rainforest biome. Our choice was mainly influenced by three reasons. Firstly, *Cecropia-Azteca* interaction is considered an iconic biological symbiosis as it is one of the most conspicuous ant-plant interactions in the neotropics (Janzen, 1969; Longino, 1991). Second, although *Cecropia-Azteca* interaction presents normally a low degree of species-specificity (Yu & Davidson, 1997), in our

experimental station *C. glaziovii* occurs is exclusively associated with *A. muelleri.* Lastly, most studies regarding this interaction were conducted with species from Amazonia and Central America (Davidson, 2005) and so far, there are few studies in other biomes, such as the Atlantic Forest of Brazil (but see K. N. Oliveira et al., 2015). *Cecropia-Azteca* interaction begins when a founding queen of *Azteca* pierces a *Cecropia* stem region, which is especially thin (called prostoma), and penetrates in tree trunk. Inside the trunk, there are many chambers (internodes) which will be used as ant nests (domatia) (Janzen, 1969). In addition to shelter, the plant also provides food bodies for the associated ants through a specialized structure, located under the leaf petiole, called trichilia (Yu & Davidson, 1997).

Experimental design

Seedlings of *C. glaziovii* were obtained from the same parental plant (Flora Londrina®) and vegetative and reproductive material of progenitor plant was identified and deposited at VIC herbarium under the code VIC nº 37.610 at Federal University of Viçosa, Viçosa, MG, Brazil. We planted 100, six months-old *C. glaziovii* individual seedlings in July 2012 and other 100 in July 2013. Seedlings were planted in a line transect (10 m apart from each other) in two lake borders providing *C. glaziovii* seedlings the same forest fragment surroundings. From July 2012 to December 2016 (54 months), we collected data on those seedlings monthly (see details below). From those 200 plants, we were able to use a total of 36 plants which survived until December 2016.

Plants were separated into two long-term experimental groups: (i) a control group, unmanipulated plants and thus available to ant colonization (colonized group CO) and (ii) a treatment group, plants which were prevented to be colonized by ants

(uncolonized group UN). In UN group, we used 3M Scotch Silver Tape®, passed over prostomas of all plants. In every new node the plant produced, we covered the prostoma with the tape every month. If we found any ant queen inside a UN plant group, we exterminated them injecting water in their domatia. To control a possible effect of Silver Tape® and water, plants of CO group had also tapes passed over their trunks, but not over the prostomas and water injected inside their domatia. We collected data from plants belonging to these two experimental groups for 54 months, measuring the following plant traits: plant height, herbivory level, pathogens frequency, isotope signature, physical and chemical defenses.

Plant growth

To evaluate plant growth, we used plant height and total number of leaves measured monthly. Plant height was measured from the ground to the apex of the terminal internode. Total leaves were obtained by counting the total number of leaves of each plant in each monthly visit.

Herbivory and pathogens

We obtained herbivory data monthly by measuring leaf area loss of the three most apical leaves of each plant. We took pictures in the field of each leaf (without removing it) against a white board with 1-cm marks as a reference scale. We then estimated the leaf damage using the software ImageJ (Rasband, 2006) and calculated the average foliar herbivory [(removed area/total area) x 100]. Finally, we calculated average plant herbivory as the sum of average foliar herbivory of each leaf divided by three. In our visits to each plant, we sought for fungus in the trichilia and/or other signal of ill caused by pathogens and recorded when it occurred.

Isotope signature

To evaluate nitrogen and carbon content and ^{15}N and ^{13}C signatures (see Sagers et al., 2000), we collected three leaves randomly of each plant in four distinct times (October 2013, July 2014, May 2015 and April 2016). Leaves were taken to the laboratory and stored in paper bags in boxes with silica gel for at least six months for complete drying. Afterward, dried leaves were ground to a fine powder and stored.

We used 2 to 3 mg of leaf powder for isotope ratio and elemental analyses by Continuous Flow Isotope Ratio Mass Spectrometry, employing a Thermo Scientific Delta Plus mass spectrometer coupled to a Carlo Erba CHN 1110 elemental analyzer (Isotope Ecology Laboratory of the Center for Nuclear Energy in Agriculture, CENA, University of São Paulo). The sample isotope ratios (^{15}N/^{14}N and ^{13}C/^{12}C) were compared to an international standard, atmospheric N_2 and PeeDee Belemnite carbonate, respectively. Precision, estimated from the reproducibility of the international standards IAEA-N1 and IAEA-N2 (Barrie & Prosser, 1996), was better than ±0.5°/oo (2σ). Results are expressed relative to the standards in "delta" notation: δ = [(R_{sample}/$R_{standard}$) − 1] * 1000, where R is the absolute isotopic ratio.

Physical and chemical defenses

We used the same leaves collected for isotopes analyses, but physical parameters were obtained before the grind process. To evaluate physical defenses, we obtained leaf specific mass (LSM), the used index for sclerophylly, by the ratio of the dry weigh of the leaf and leaf area. After this, we calculated average LSM per plant in g/cm^2. We estimated leaf area using the software ImageJ and obtained dry weight with a precision balance Quimis®, model Q500L210C. We counted the number of

trichomes using a stereomicroscope in two randomly selected 0.25 mm^2 plots of upper surface of each leaf, only avoiding the midvein. We then averaged total number of trichomes per leaf and per plant. Then, we divided number of leaf trichomes per plant by average plant foliar area to access trichome density.

Our focus on chemical defenses was in total phenolics and condensed tannins because these categories are widely known as defensive metabolites and are present in *Cecropia* plants (Latteman et al., 2014). For their extraction, we used 1g samples of the leaf powder and put them in 50 ml conical, screw-cap centrifuges tubes. Tubes received 20 ml of distilled water and were vortexed. Tubes were then taken to an ultrasonic cleaner Bransonic® model 2510R-DTH at 60º C for 30 minutes. After this, tubes were centrifuged for 10 min (2500 xg). Pellets were washed twice with 20 ml of distilled water. Supernatants and washes for each sample were combined, brought to 100 ml in volumetric flasks, and aliquots were used for assay. Samples were stored in amber pots at 4º C.

Total phenolics were assessed following the Folin-Ciocalteau Assay (adapted from Swain & Hillis, 1959), using tannic acid as standard and read in a spectrometer at 725 nm against the blank. The average total phenolics was expressed as milligrams of total phenolics per gram of plant dry mass. Condensed tannins were estimated using the Vanillin Assay modified by Hagerman (2002). We used catechin as standard and read the samples at 500 nm against the blank. The average condensed tannins were expressed as milligrams of tannins per gram of plant dry mass. We performed three replicates for each sample for each of the described assays.

Data analysis

To test plant healthy prior to colonization, we considered traits of colonized plants six months before ant colonization, when we could assure the absence of ant effects. For uncolonized plants, we considered data from six months before the average age of plant colonization (29 months). Then, we performed general linear models (GLM) using plant height and total number of leaves as response variable and group (colonized or uncolonized) as explicative variable. We specified a Poisson distribution of errors for total number of leaves.

To assess the effect of ant colonization in plant size and herbivory along time, we performed linear mixed effect models (LMER) using plant height, total number of leaves or herbivory rate as response variables and time (in months), colonization status (colonized or non-colonized) and the interaction between both as fixed effects. Individual plant identity was defined as random effect to account for repeated measures. Herbivory rate was log transformed to comply with test assumptions of normality of residuals and homoscedasticity.

To test whether plants from different groups (colonized or non-colonized) differ in nutrient top-down intake and investment in physical and chemical defenses, we conducted another LMER considering $\delta^{15}N$ signature, total nitrogen content, $\delta^{13}C$ signature, total carbon content, LSM, trichome density, concentrations of total phenolics and condensed tannins as response variables and colonization status (colonized or non-colonized) as fixed effect; plant identity was used as random effect too. Trichome density and total phenolics were log transformed to achieve normality of residuals and homoscedasticity.

To assess whether time since colonization affects the same above-mentioned isotopic and defensive variables, we performed a third LMER using $\delta^{15}N$ signature, total nitrogen content, $\delta^{13}C$ signature, total carbon content, LSM, trichome density,

total phenolics and condensed tannins as response variables and time since colonization (in months) as response variable. Once again, we used plant identity as random effect. Trichome density was log transformed to achieve normality of residuals and homoscedasticity.

In order to evaluate and compare the development of plant features after colonization of CO group by ants, we defined the moment when plants of UN group were able to receive queens and thus could have been colonized. We used the average height of CO plants in the moment of colonization (91 cm) and UN plants >= 91 cm were considered able to be colonized. We thus performed the same LMER model described above. To test an alternative model that might explain the trends in uncolonized plant features, we considered plant height as an explanatory variable for ontogenetic change. Given that plant height and time since colonization were strongly correlated ($p<0.001$), we could not include both in the same model. We thus performed additional LMER using $\delta^{15}N$ signature, total nitrogen content, $\delta^{13}C$ signature, total carbon content, LSM, trichome density, total phenolics and condensed tannins of UN plants as response variables, plant height as fixed effect and plant identity as random effect. Trichome density was log transformed and total phenolics was square transformed to achieve normality of residuals and homoscedasticity.

To estimate the effect sizes (i.e., the comparative percentage of the influence ants have on each plant trait analyzed) of plant growth and herbivory, we compared the mean trait value at the final month of the colonized plants (μ_1) with the mean of the uncolonized plants (μ_2) in the same moment as follows: ($\mu_1 - \mu_2$) / μ_2). For estimation of ant effect sizes on plant nutritional and defensive traits ($\delta^{15}N$ signature, total nitrogen content, $\delta^{13}C$ signature, total carbon content, LSM, trichomes, total phenolics and condensed tannins) along time, we used the mean trait value at the final

month of colonized plants (μ_f) with the mean of the first month of colonization (μ_i): ($\mu_f - \mu_i$) / μ_i). Means from transformed values were re-transformed for this calculation.

We checked for spatial autocorrelation in plant distribution by using Mantel tests (9999 permutations) of pairwise spatial distances and pairwise differences in any of the parameters (growth rate, herbivory, δ^{15}N signature, total nitrogen content, δ^{13}C signature, total carbon content, LSM, trichomes, total phenolics and condensed tannins). Because we have data available from different moments, we used the last measure available, because it was when the effect of ant's presence or absence should be at its maximum. Finally, we found no significant spatial autocorrelation between growth, herbivory, plant isotopic and defensive features and plants (Spearman r <= 0.2, p >= 0.05).

We used software R 3.4.1 (R Core Team 2017) for all analyses. We performed residual analyses for all models and checked for the distribution of errors. We performed LMER tests using the package lme4 (Bates, Mächler, Bolker, & Walker, 2015) and Mantel tests using the package ade4 (Dray & Dufour, 2015).

RESULTS

A total of 26 months passed since the first tree was colonized by ants and the average age of plant colonization by ants was 28.9 ± 2.5 (SE). We found no differences in plant height ($F_{1,25} = 3.03$; $p = 0.09$) and total number of leaves (χ^2 (1, N = 27) = 12.44; $p = 0.18$) between colonized and uncolonized groups of plants previously to ant colonization.

Plant growth

Plants colonized by ants increased their height in average 125% more than uncolonized plants after 26 months since the first colonization and 54 months of observation. Plant height increased along with time (χ^2 (1, N = 36) = 3215.95; $p <$ 0.001) and colonization status (χ^2 (1, N = 36) = 718.92; $p < 0.001$), but colonized plants grew faster than uncolonized ones (χ^2 (1, N = 36) = 631.01; $p < 0.001$) (Figure 1a). In addition, the number of leaves was 123% higher in colonized than uncolonized plants, also with a positive effect of time (χ^2 (1, N = 36) = 123.78; $p < 0.001$) and colonization status (χ^2 (1, N = 36) = 219.87; $p < 0.001$), with colonized plants developing more new leaves than uncolonized ones (χ^2 (1, N = 36) = 42.31; $p < 0.001$) (Figure 1b).

Herbivory defense

Herbivory was 83% lower in colonized than uncolonized plants after 26 months since the first colonization. Herbivory did not decrease along with time (χ^2 (1, N = 36) = 0.47; p = 0.45), except for colonized plants that showed a strong decrease in herbivory rates after ant colonization (χ^2 (1, N = 36) = 8.44; p = 0.004) (Figure 2).

Pathogen protection

We were able to find 31 occurrences of fungi and mites' (Acari: Eryophidae) colonies. From those, 30 (96.77% of observations) were found in uncolonized plants. In addition, the unique occurrence of such pathogen in a colonized plant was in a month that ant colony activity was abnormally low, leading to colony death in the next month.
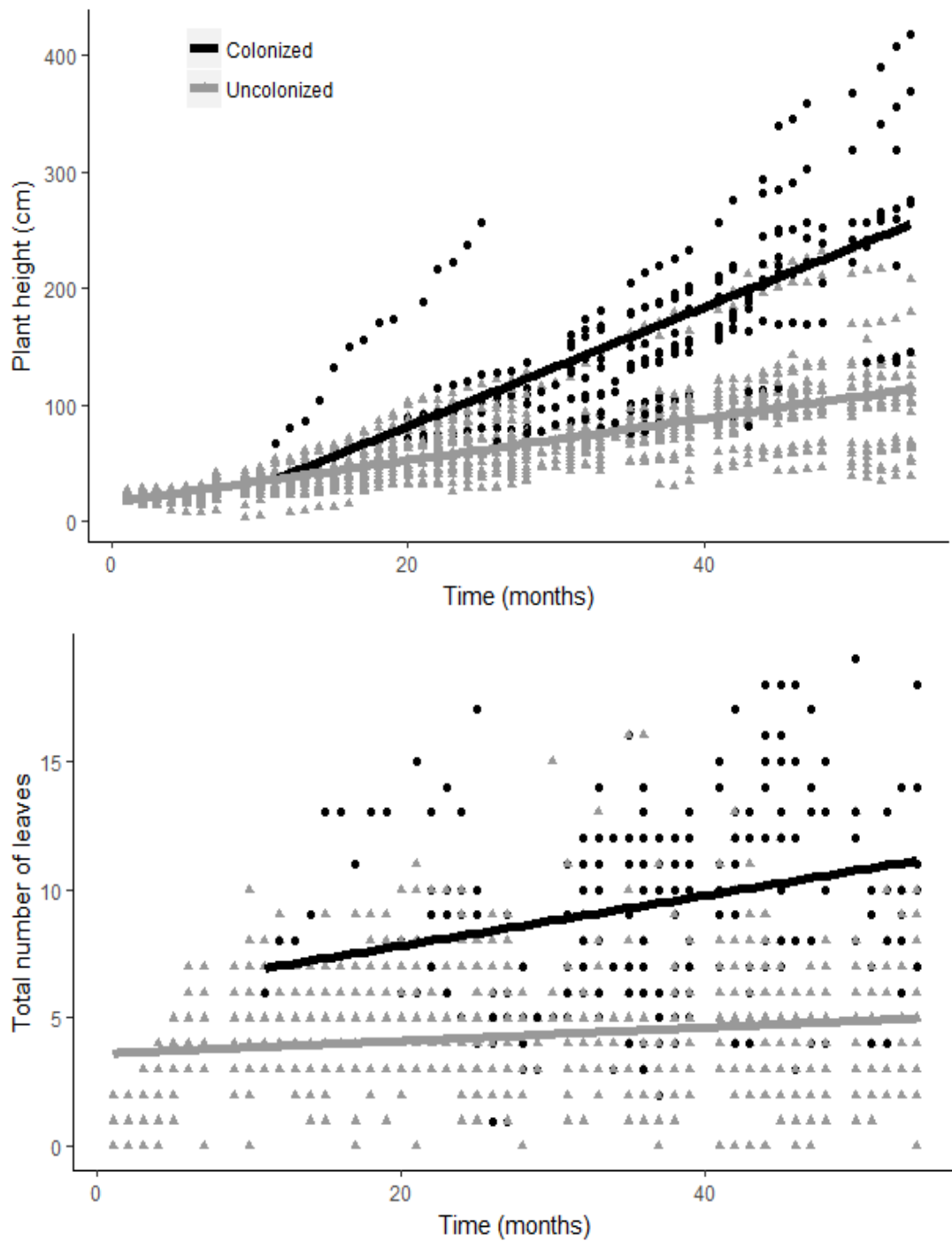
Figure 1. Relationship between Plant height (A) and Total number of leaves (B) and time according to groups of colonized or uncolonized plants.

Top-down fertilization

We found no differences in plant nutritional traits (δ^{15}N signature, total nitrogen content, δ^{13}C signature, total carbon content) between colonized and

uncolonized plants. However, we found that total carbon content was higher in colonized than uncolonized plants (Table 1). The effect of time after colonization on plant features showed an increase in $\delta^{15}N$ signature (Fig. 3a) (Effect size = 146%) and total nitrogen content (Effect size = 65%). On the other hand, we found no effects of time in uncolonized plants after they were considered prone to be colonized (p >= 0.15) (Table 1). Total C content increased in uncolonized plants after being able to be colonized (Table 1). We found no relationship between plant height and uncolonized plant nutritional features for any of the variables, but only a positive relation with $\delta^{13}C$ signature (S1).
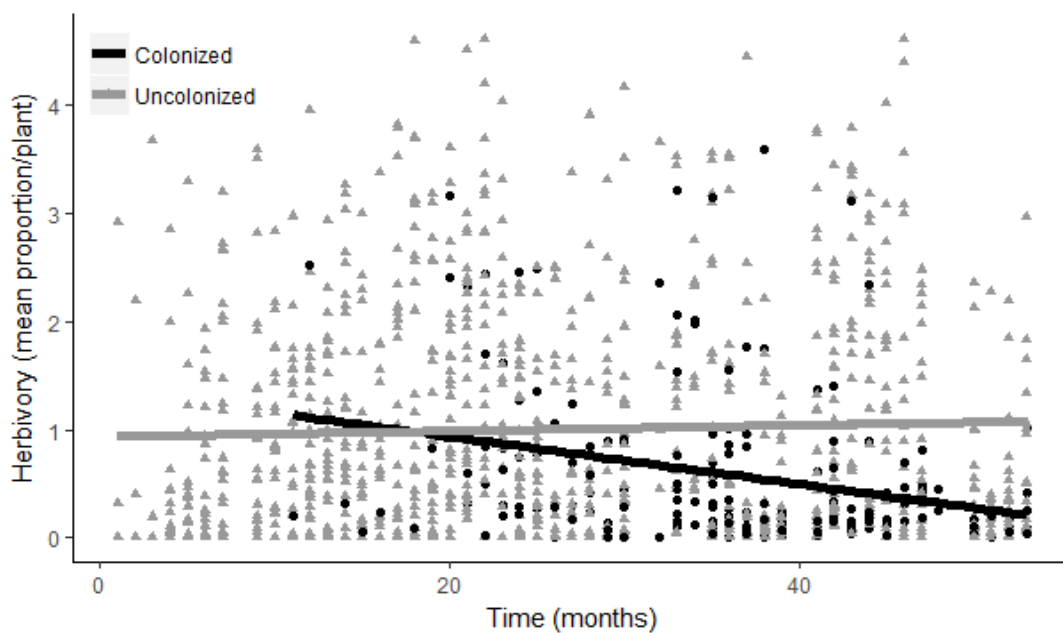
Figure 2. Relationship between herbivory rates and time according to groups of colonized or uncolonized plants. Herbivory values are in Log_{1p}.

Energy saving in physical and chemical defenses

The comparison of chemical and physical defensive features between colonized and uncolonized groups showed that only trichome density was lower in colonized than uncolonized plants (Table 1). The effects of time since colonization on

plant features showed a decrease in trichome density (Effect size = -93%) (Fig. 3b), while time since plants became able to be colonized did not affect these features in uncolonized plants ($p >= 0.2$) (Table 1). On the other hand, LSM increased in uncolonized plants after being able to be colonized (Table 1). We found no relationship between plant height and uncolonized plant features for any of the variables (S1).

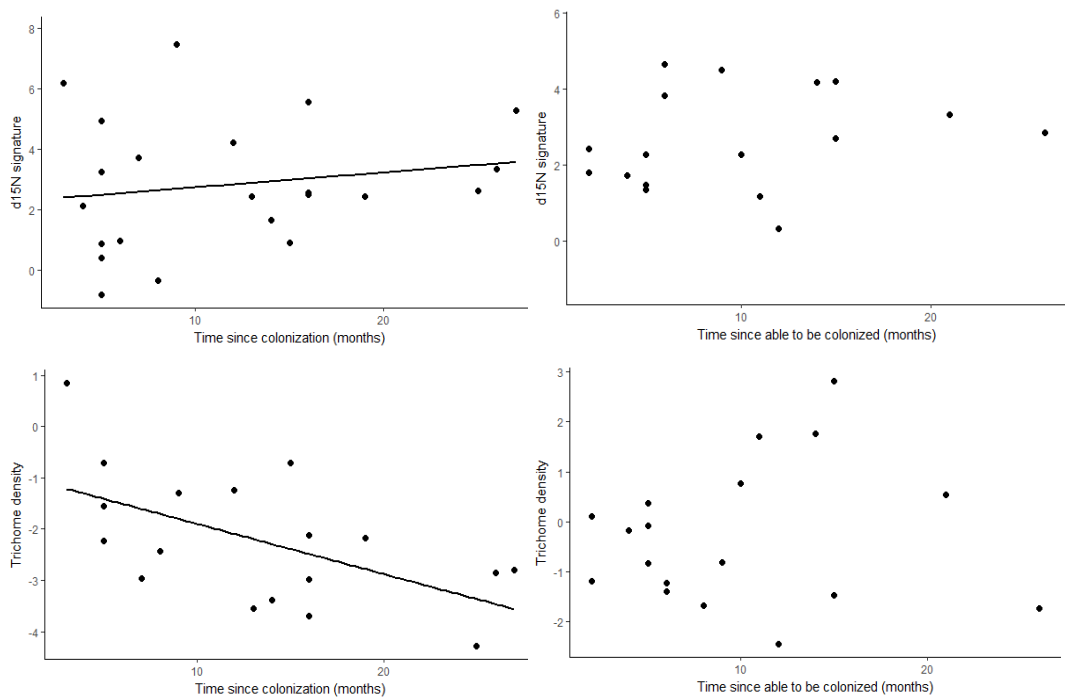**Figure 3.** Variations of $\delta^{15}N$ signature and trichome density since colonization by ants begun in plants (26 months since first colonization) and since plants of the experimentally uncolonized group were prone to be colonized (91cm height).

Table 1. Relationship between plant isotopic and defensive features and plant group (colonized of uncolonized), time since colonization and plant height as a proxy for ontogenetic phase. Statistically significant results are presented in bold (p<0.05).

Variable	Plant group			Time since colonization				Time since able to be colonized			
	df	χ^2	p	df	χ^2	p	Effect	df	χ^2	p	Effect
^{15}N	, N=57	0.16	0.69	1, N=12	7.63	**0.005**	+	1, N=15	0.94	0.33	
N %	, N=57	1.03	0.31	1, N=12	22.26	**<0.001**	+	1, N=15	2.05	0.15	
^{13}C	, N=57	1.7	0.19	1, N=12	0.65	0.42		1, N=15	0.55	0.46	
C %	, N=57	8.19	**0.004**	1, N=12	1.32	0.25		1, N=15	6.71	**0.009**	-
Trichomes	, N=42	4.65	**0.03**	1, N=12	12.11	**<0.001**	-	1, N=15	1.63	0.2	
LSM	, N=58	2.56	0.11	1, N=12	2.21	0.14		1, N=15	6.38	**0.01**	+
Phenolics	1, N=38	1.72	0.19	1, N=12	2.97	0.08		1, N=12	0.12	0.88	
Tannins	1, N=38	0.01	0.91	1, N=12	1.29	0.26		1, N=12	0.21	0.65	

S1 Fig. Linear mixed effect models between plant traits and plant height as a proxy for ontogenetic development.

Variable	Plant height			
	df	χ^2	p	Effect
Trichomes	1, N=34	2.18	0.14	
LSM	1, N=52	0.97	0.33	
Phenolics	1, N=31	0.0005	0.98	
Tannins	1, N=31	0.12	0.72	
^{15}N	1, N=52	0.17	0.68	
N %	1, N=52	0.15	0.7	
^{13}C	1, N=52	7.91	**0.005**	+
C %	1, N=52	1.43	0.23	

DISCUSSION

In our long-term manipulative experiment, we showed that symbiotic *Azteca* ants have a strong and positive effect on *Cecropia* plants growth. We demonstrated that after 54 months, plants colonized by *Azteca* ants experienced a significant increase in growth rate which makes more than twice as large as their uncolonized conspecifics. We also show that ant defense against herbivores, top-down nutrient uptake from ants to plants and plant energy saving in physical defenses are the likely the promoting

mechanisms promoting an increased growth. Since the pioneer studies by Janzen (1966) on myrmecophytes, many studies have addressed the presumably positive effects of mutualistic ants on ant-plants (K. N. Oliveira et al., 2015; P. S. Oliveira & Pie, 1998; Pringle et al., 2011; Schupp, 1986). However, the effect of ant colonization along plant ontogeny in a long-term manipulative experiment is unique, and demonstrates how the presence of *Azteca muelleri* enhances *Cecropia glaziovii* growth over time. A critical question in the observational studies of ant-plant interactions has been whether an increase in plant growth is promoted by the ant's presence, or whether colonized plants were already healthier prior to colonization (K. N. Oliveira et al., 2015; Razo-belman et al., 2018). Because we compared traits of individual plants before and after ant colonization, our experiment supports the evidence that ant colonization itself is responsible to enhance plant growth.

Herbivory avoidance is usually reported as the main mechanism explaining ant effects in myrmecophytes (e.g. Del Val et al., 2003; Martin, Heil; Doyle, 2003; K. N. Oliveira et al., 2015; Schupp, 1986; Zhang et al., 2015). Our data support such findings considering that the presence of *A. muelleri* decreased herbivory rates by 83% at 54-month-old trees comparing colonized and uncolonized plants. It is well known that herbivory can be a severe injury for plants and impair their growth, survivorship and fitness (Coley & Barone, 2002; Marquis, 1984; Warner & Cushman, 2002) and that plants that suffer less herbivore attacks are those who offers nest and food for ants (Zhang et al., 2015). Protection against herbivory is presumably related to the predatory and aggressive behavior of *A. muelleri* which recruit rapidly and can effectively avoid chewing and big insects from attacking their host plant (Yu & Davidson, 1997; Gomes, pers. obs). *Azteca muelleri* ants have actually been reported to prey on one of the *Cecropia* specialist herbivore, the beetle *Coelomera* sp.

(Coleoptera: Chrysomelidae) (Rocha & Bergallo, 1992; Silveira, dos Anjos, & Zanúncio, 2002). In addition to *Coelomera* beetles, *Colobura dirce* (Lepidoptera: Nymphalidae), another *Cecropia* herbivore specialist (Willmott, Constantino, & Hall, 2001), were the most common in our study site.

A meta-analysis using data from plants of the whole world pointed out that the category of plants that suffer less herbivore attacks are those who offers nest and food for ants and its herbivory rate is about 3% (Zhang et al., 2015). They also pointed out that ant-excluded plants suffer more than 10% of herbivory. In contrast, the average herbivory level for in our study was only 1.8% for colonized and 5.3% for uncolonized plants. However, Zhang et al. (2015) conducted experimental exclusion of ants, whereas we controlled for prevented colonization. In such studies using experimental exclusion, plants already had allocated more resources to ant rewards and may thus appear more fragile to herbivore attacks. Uncolonized plants in our study never received ants, thus their constitutive defensive traits could have been more efficient.

We also demonstrated that fungi infection occurs almost exclusively on *Cecropia* trichilia from uncolonized plants. This pattern suggests that the symbiotic ants might be evolved in plant protection against such pathogens. In fact, the fungus *Fusarium moniliforme* can infect *Cecropia* trichilia in uncolonized plants (Roux et al., 2011). This fungus produces toxins that causes necrosis in plants (Cole, Kirdsey, Cutler, Doupnik, & Peckham, 1973) and the protection of plant trichilia by physical or chemical means might improve plant growth (Roux et al., 2011). In our system, we neither identify fungi nor mite species found in trichilia, but it was obvious that fungal colonies disabled trichilia function, which is presumably harmful for at least the plants (K. N. Oliveira et al., 2015). It's important to notice that fungi prevalence was very low in our study and we only found fungus infection in ten plant individuals. The

effects of time and colonization status on plant height did not change after excluding these samples from our analysis. Therefore, despite the evidence of ant protection against fungi found here, we cannot consider fungus infection as a determinant factor influencing plant growth in our study.

After ant colonization, plant $\delta^{15}N$ isotope signature increased by 146% and at the same time, we did not find any significant variation in these variables for uncolonized plants. The increase in $\delta^{15}N$ signature indicates that plants are somehow absorbing this nutrient from their mutualistic ant-partners (Sagers et al., 2000). *Azteca* ants, by feeding on exogenic food sources such as herbivore insects usually deposit their debris inside the *Cecropia* steams (Trimble & Sagers, 2004). The absorption of these nitrogen-rich nutrients is what we call myrmecotrophy (Beattie, 1989), one of the most novel and unrevealed mechanism underlying ant effects on ant-plants (Mayer, Frederickson, & Mckey, 2014). Our results support a strong top-down uptake of nutrients by ants in this system, which can lead to increased benefits for plants (Roux & Dejean, 2012). Oliveira et al. (2015) found higher N content in leaves of colonized individuals of *C. glaziovii* but could not attest for ant nutrition, once $\delta^{15}N$ signature did not change between colonized and uncolonized plants. Here, we also could not find any difference between colonized and uncolonized plants, but we did find a sharp increase in $\delta^{15}N$ and N after colonization by ants, which did not occur in the uncolonized plants after prone to be colonized. It is likely that the effect of ants on nurturing plants only became perceptible as plants become taller and ant colonies bigger. Future studies on *Cecropia-Azteca* system should focus on the mechanisms underlying this top-down nutrient transference from ant nests to plants.

Regarding physical plant defenses, we found that trichomes are denser in leaves of uncolonized than in colonized plants. We also show that while *C. glaziovii*

individuals reduce their trichome density by 93% after being colonized by ants, uncolonized plants in the same ontogenetic stage do not present any change in their trichome density. Our results support the hypothesis that a biological defense provided by ants decreases plant dependency on physical defenses, such as trichomes (Coley, 1986; Koricheva & Romero, 2012; but see Del Val et al., 2003). It is reasonable to hypothesize that higher trichome density in uncolonized than colonized plants found here might be promoted as plant response to leaf herbivory. It was already shown that higher trichome density can be induced by herbivory in *Alnus* and *Endospermum* trees (Baur, Binder, & Benz, 1991; Letourneau et al., 1999).

Despite some evidences of different energy allocation for trichomes, other plant features such as chemical secondary metabolites and leaf toughness did not differ between treatment groups and did not change after ant colonization. In fact, the revision performed by Heil and Mackey (2003) stated that defensive trade-offs in ant-plant systems seems to be weaker than they were claimed before (e.g. Eck, Fiala, Linsenmair, Hashim, & Proksch, 2001; Rehr et al., 1973; Seigler & Ebinger, 1987). Absolute presence or absence of trade-offs are rare because multiple defenses are useful against dynamic attackers and if plants present the energy potential to invest on then at the same time they will do (Agrawal & Rutter, 1998). Moreover, it is important to consider that phenolics, tannins and sclerophylly are responsible for different plant physiological roles other than defense (Agrawal & Fishbein, 2006).

Regarding the relative importance of our three significant mechanisms, we demonstrated that the strongest beneficial effects of *Azteca* ants on *Cecropia* plants were observed on $\delta^{15}N$ signature followed by trichomes and herbivory (Fig. 4). Thus, protection against herbivory might play a minor role in plant development when compared to top-down ant fertilization, at least in the studied ontogenetic stage (Boege

& Marquis, 2005). This result sheds light to the fact that the classical ant-plant protective mutualism might be actually less important than a reciprocal nutritional mutualism (Mayer, Frederickson, & Mckey, 2014). However, these findings might be interpreted with caution as we did not measure how energy costly such traits were to plants prior colonization. Furthermore, it is possible that the synergistic effect of those mentioned mechanisms is more relevant than each mechanism individually. Finally, the relative importance of each mechanism can be only achieved through specific manipulative experiments (Stanton & Palmer, 2011).
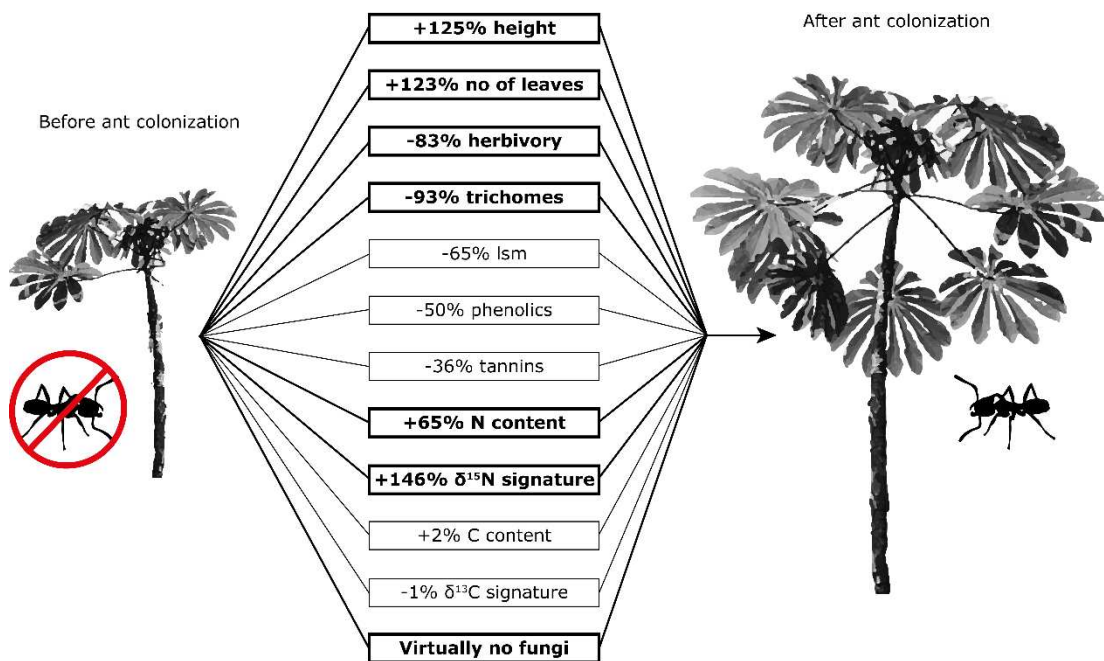
Figure 4. Effect size of ants on plant growth and herbivory (estimated by the mean trait value at the end of the 54º month of the colonized versus the mean of the uncolonized plants); and effect of ants on defensive and isotopic traits (estimated by the mean of the mean trait value at the final month of colonized plants versus the mean of the first month of colonization). Statistically significant traits are presented in bold.

Our study is pioneer to experimentally conclude that ants actually benefit plants, independently from plants prior conditions. In addition, because we used an integrative approach, we also present that plants are benefited by ants directly, via

herbivory control and nutrient uptake, and indirectly, via reducing defensive energy costs with trichomes. We educe such clear conclusions from a long-term manipulative experiment, which demonstrates the importance of such approach for avoiding inconsistences present in studies focusing on the evolutionary outcomes of ecological interactions (Agrawal & Fishbein, 2006). We suggest that future studies on ant-plant interactions should focus on whether these benefits shown in plant growth might be translated into an increase in plant reproductive success. Moreover, as we showed that other ant-plant mutualistic mechanisms (e.g. top-down fertilization) rather than the classical herbivory defense effect have also important roles, we suggest that the investigation on the individual importance of each mechanism should constitute an important focus for future studies. Finally, the study of obligatory species interactions, as presented here, has a great conservation importance because they are particularly sensitive to global change (Mayer, Frederickson, & Mckey, 2014) and the fully comprehension of species mutual benefits and its mechanisms can contribute to prevent and mitigate human impacts in a current changing world.

AUTHORS' CONTRIBUTIONS

I.J.M.T.G., K.N.O., R.I.C. and R.S. conceived the ideas and designed different parts of the methodology; I.J.M.T.G., K.N.O and L.R.B. collected the data; I.J.M.T.G., R.S., M.Z.M. and N.B. analyzed the data; and I.J.M.T.G. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Herbivory and plant growth: contrasting effects along ant-plant ontogeny

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ABSTRACT

Herbivory can represent a risk to plant development. To resist, plants can use different strategies such as chemical, physical or biological defenses, which usually decreases the negative effects of herbivory. Conversely, plants can reduce the negative effects of herbivore damage on fitness via compensatory physiological mechanisms (tolerance). Plants show different responses to herbivory along their ontogeny. In addition, ant-plants development has particular characteristics such as the colonization by ants. We aimed to test the effects of herbivory on an ant-plant growth, defense traits and how such responses change along ontogeny. We found that herbivory has a negative effects of plant growth after ant colonization, but not in pre-colonized by ants' ontogenetic stages. It is possible that plants tolerate herbivory during the first stages, but they lose this trait after ant colonization, possibly due to trade-off decisions or nutrients availability. Moreover, trichome density decreases after ant colonization, but we suggest it may happen because this trait is induced by high levels of herbivory. The classical detrimental effects of herbivory have different effects on ant-plants ontogenetic stages and shifts triggered by ants is determinant to such contrast.

Keywords: herbivory effects, mutualism, myrmecophytes, trichomes

INTRODUCTION

Herbivory is the starting process governing energy flow in ecological food webs (Agrawal, 2007; Schmitz, 2008). Such a key importance is probably what makes plant-herbivore interactions one of the most studied topics in ecology. A remarkable effect of herbivory is to impair plant growth, as it causes loss of photosynthetic tissues (Marquis, 1984; Züst & Agrawal, 2017). On the other hand, by means of compensatory physiological mechanisms, herbivory may trigger neutral or positive effects on plant development (Barton, 2013; Fornoni, 2011; Hawkes & Sullivan, 2001). Such contradictory effects evolving herbivory and plant growth has been responsible for a rich theoretical debate evolving two plant evolutionary strategies: resistance and tolerance (Agrawal, 2004).

The classic resistance strategies to avoid herbivore´s impacts can be summarized in three principal defense strategies: i) the production of chemical compounds toxic to herbivores, ii) physical structures which impairs herbivore consumption and/or iii) mutualistic associations with other species which defend plants against herbivory in exchange for food and/or shelter (Chaudhary et al., 2018; Hanley et al., 2007; Massad et al., 2011; Zhang et al., 2015). Conversely, herbivory tolerance is generally defined as the capacity of plants to reduce the negative effects of herbivore damage on fitness (Stowe, Marquis, Hochwender, & Simms, 2000; Strauss & Agrawal, 1999). In response to herbivory, plants can show increased levels of photosynthesis, phenological changes, compensatory growth and activation of dormant meristems (review in Tiffin 2000). Finally, the plant "evolutionary decision" or trade-off between to resist or tolerate herbivory is highly context-dependent and it seems to be derived from the interaction between plant genotype and the environment

(e.g. herbivory pressure and resource availability) (Fornoni, 2011; Fornoni, Valverde, Núñez-farfán, & Nunez-Farfan, 2003).

Plant responses to herbivory such as tolerance and resistance may also change along individual development (i.e. ontogeny), even for related species with similar life stories (Barton, 2013; Barton & Koricheva, 2010). Ontogenetic changes in plant responses to herbivory might be promoted mainly by herbivore pressure fluctuation during plant development or to resource allocation trade-offs among life history traits along plant ontogeny (e.g. defense, growth and reproduction) (Barton & Koricheva, 2010; Boege & Marquis, 2005). For instance, if a young plant has its physical and chemical defense production limited by the acquisition and allocation of resources, one might expect an ontogenetic increase in defense (Herms & Mattson, 1992), which makes juveniles more susceptible to herbivore attacks. However, from the few studies focusing on the ontogenetic changes in plant defense traits, many theoretical predictions found no support on empirical data and their general patterns and mechanisms remain unclear (Barton & Koricheva, 2010; Boege & Marquis, 2005).

Most studies investigating the effects of herbivory on plant responses were focused on chemical or physical defense traits and much less attention was given to biological-mutualistic defenses (Barton & Koricheva, 2010). Interactions between ants and plants constitute some of the most outstanding cases of mutualism in nature. Such interactions can range from opportunistic, facultative relationships to highly specialized associations in which plants produce hollow structures for nesting ants and can feed them too, being denominated myrmecophytes or ant-plants (Martin, Heil; Doyle, 2003; Mayer, Frederickson, Mckey, et al., 2014). Whereas ants benefit from nesting space and food rewards, plants can benefit from protection against herbivores and pathogens, nutrition from ants and energy savings due to the decrease of other

defensive strategies (Sagers et al. 2000, Martin, Heil; Doyle 2003, Koricheva and Romero 2012, Oliveira et al. 2015, first chapter of this thesis). As defense against herbivores is considered one of the major benefits provided by ants to myrmecophytes (Martin, Heil; Doyle, 2003; K. N. Oliveira et al., 2015) it is reasonable to believe that ant-plants will be highly and negatively affected by herbivory. Finally, despite the lack of studies, the nature of ant-plant mutualistic systems makes them a perfect model for studying the effects of herbivory on plant ontogenetic development.

Drastic ontogenetic changes are expected to happen because myrmecophytes experience "the ant colonization" which might be considered a major shift during juvenile plant development (Martin, Heil; Doyle, 2003). When ant-plants are young, they do not have any myrmecophytic structure, therefore they must face herbivores with no biological defense (Del Val & Dirzo, 2003). Before ant colonization, plants should first develop structures to host (e.g. domatia) and feed (e.g. extrafloral nectaries or Müllerian bodies) the ant colony (Del Val & Dirzo, 2003; Janzen, 1969; Yu & Davidson, 1997). Such energetic requirements for ant´s reception might impose some changes in plant physiology even before ant colonization. In addition, herbivory levels are much higher before ant colonization (Oliveira et al. 2015, first chapter of this thesis). Consequently, along plant development, it is reasonable to expect two main shifts in plant responses: i) a decrease in plant tolerance to herbivory (Del Val & Dirzo, 2003) and ii) a decrease in chemical and physical defenses (Koricheva & Romero, 2012). Moreover, it is possible that such changes begin even earlier, when plants start to produce myrmecophytic structures and are, thus, prone to be colonized. Despite this fascinating ecological scenario, very few studies have documented the effects of herbivory and plant responses along ant-plants ontogeny (but see Del Val and Dirzo 2003 for ontogenetic changes in chemical and physical defenses).

Here, we aim to experimentally test the effect of herbivory on plant growth and defensive traits of a neotropical myrmecophytic plant species along 54 months by testing the following hypotheses: (1) overall herbivory pressure impairs ant-plants and decrease their growth; (2) there is a shift in individual plant responses to herbivory along plant ontogeny, as older and ant-colonized plants should be more affected by herbivory than younger and antless plants; (3) the ontogenetic shifts affect plant defensive strategies, promoting a decrease in chemical and physical defenses as the plant age.

METHODS

Study site and biological model

We collected data at the Research, Training and Environmental Education Station "*Mata do Paraíso*" (20º48'07"S, 42º51'31"W). This reserve is a 195-ha area of Atlantic rainforest biome dominated by a montane semideciduous forest (Meira-Neto & Martins, 2002). The climate according to Köppen, is Cwa subtropical hot-summer climates, with rainy and hot summers and dry and cold winters (Alvares et al., 2013).

Our plant model was *Cecropia glaziovii* Snethl (family Cecropiaceae) which is a pioneer tree distributed throughout the Brazilian Atlantic Forest, naturally occurring in our experimental station. Some species of genus *Cecropia* present the more conspicuous symbiotic relationship between ants and plants in the neotropics (Janzen, 1969; Longino, 1991), and this association begins when a founding queen of *Azteca* pierces a stem region which is especially thin (called prostoma) and penetrates in its hollow trunk. Inside the trunk, there are many chambers (internodes) which will be used as ant nests (domatia) (Janzen, 1969). In addition to shelter, the plant also

provides food bodies for the associated ants through a specialized structure, located under the leaf petiole, called trichilium (Yu & Davidson, 1997). In our study site, the tree species *C. glaziovii* is exclusively associated to *Azteca muelleri* (Emery 1893).

Field experiment

We obtained 260 seedlings of *C. glaziovii* (six months-old) from Flora Londrina®. These seedlings came from the same parental plant and the vegetative and reproductive material of progenitor plant was identified and deposited at VIC herbarium under the code VIC nº 37.610 at Federal University of Viçosa, MG, Brazil. Out from those 260 *C. glaziovii* seedlings we obtained, acclimated and planted 100 in July 2012, 100 in July 2013 and 60 in December 2014 in our field area (always six months-old seedlings). Seedlings were planted in a line transect (10 m apart from each other) in two lake borders providing for our planted *C. glaziovii* seedlings the same type of habitat experienced by natural *C. glaziovii* individuals already presented in our sampling station. To test our three hypothesis those seedlings were separated into two different field experiments as follows.

1 –Effect of herbivory reduction on plant growth and defenses

We used 60 *C. glaziovii* seedlings planted in 2014 as described previously. Between October 2015 and October 2016, we randomly selected two groups with the plants that had survived until that moment. The first group had 12 plants protected from herbivores (protected group), covered by a translucent, voile fabric fixed with plastic rods. In the control group, we used nine plants non-protected from herbivores (non-protected group). From those 19 plants we recorded plant growth and herbivory levels monthly during one year. In May 2015 and April 2016, we collected three leaves

of each plant to measure the physical and chemical defenses (see below for details on plant growth, herbivory, physical and chemical measures). To control the light availability for each plant and a potencial effect of casing, we measured PAR (Photosynthetically Active Radiation) intensities three times per day and then used it as an average measure.

2 – Effect of plant ontogeny on herbivory effects

We used 100 *C. glaziovii* seedlings planted in July 2012 and 100 in July 2013 as described previously. Out from those 200 seedlings we randomly selected two long-term experimental groups: i) a control group, unmanipulated plants and thus available to ant colonization (CO) and ii) a treatment group, plants which were prevented to be colonized by ants (uncolonized group UN). In UN group, we used 3M Scotch Silver Tape®, passed over prostomas of all plants. In every new node the plant produced, we covered the prostoma with the tape every month. If we found any ant queen inside a UN plant group, we exterminated them injecting water in their domatia. To control a possible effect of Silver Tape® and water, plants of CO group had also tapes passed over their trunks, but not over the prostomas and water injected inside their domatia. From July 2012 to December 2016, we collected data on plant height and herbivory level of those seedlings monthly (see details bellow), totaling 54 months. Out from the 200 plants, we were able to use a total of 35 plants which survived until December 2016.

During these 54 months, we could identify three distinct ontogenetic stages: i) the "juvenile" stage, when plants were seedlings or saplings and had no structures to host ants yet. After juvenile stage, comes the "pre-mature" stage, when plants were prone to be colonized (i.e. started to produce trichilia and Müllerian bodies, with

average height of 91 cm). Because we have strong evidence that ants can trigger changes in plant traits (Oliveira et al. 2015, first chapter of this thesis), we separated pre-mature plants into ii) "non-colonized pre-mature"; and iii) "colonized pre-mature". We had no plants in the mature stage until December 2016.

In October 2013, July 2014, May 2015 and April 2016, we collected three leaves from each plant, brought it to the lab and stored them in paper bags in boxes with silica gel for at least six months for complete drying. Those leaves were used to assess the physical and chemical defenses along plant ontogenetic stages.

Plant growth measures

To access plant growth, we used plant growth rate measured monthly. Growth rate was obtained by the increase in height (cm/day) using the following formula: GR = $(H_{final} - H_{initial})/t$, where H is height measured from the ground to the apex of the terminal internode, and t is time in days.

Herbivory rate measures

We obtained herbivory per month by measuring leaf area loss of the three most apical leaves of each plant. We took pictures in the field of each leaf (without removing it) against a white board with 1-cm marks as a reference scale. We then estimated the leaf damage using the software ImageJ (Rasband, 2006) and calculated the average foliar herbivory [(removed area/total area) x 100]. Finally, we calculated average plant herbivory as the sum of average foliar herbivory of each leaf divided by total number of leaves.

Physical and chemical defenses

To evaluate physical defenses, we obtained specific leaf mass (SLM), the used index for sclerophylly, by the ratio of the dry weigh of the leaf and leaf area. After this, we calculated an average per plant in g/cm^2. We estimated leaf area using the software ImageJ and obtained dry weight with a precision balance Quimis®, model Q500L210C. We counted the number of trichomes using a stereomicroscope in two randomly selected 0.25 mm^2 plots of upper surface of each leaf, only avoiding the midvein. We then averaged total number of trichomes per leaf and per plant. Then, we divided number of leaf trichomes per plant by average plant foliar area to access trichome density. Hereafter, leaves were ground to a fine powder.

Our focus on chemical defenses was in total phenolics and condensed tannins. Those categories are widely known as defensive metabolites and are present in *Cecropia* plants (Latteman et al., 2014). For their extraction, we used 1g samples of the leaf powder and put them in 50 ml conical, screw-cap centrifuges tubes. Tubes received 20 ml of distilled water and were vortexed. Tubes were then taken to an ultrasonic cleaner Bransonic® model 2510R-DTH at 60º C for 30 minutes. After this, tubes were centrifuged for 10 min (2500 xg). Pellets were washed twice with 20 ml of distilled water. Supernatants and washes for each sample were combined, brought to 100 ml in volumetric flasks, and aliquots were used for assay. Samples were stored in amber pots at 4º C.

Total phenolics were assessed following the Folin-Ciocalteau Assay (adapted from Swain and Hillis 1959), using tannic acid as standard and read in a spectrometer at 725 nm against the blank. The average total phenolics was expressed as milligrams of total phenolics per gram of plant dry mass. Condensed tannins were estimated using the Vanillin Assay modified by Hagerman (2002). We used catechin as standard and read the samples at 500 nm against the blank. The average condensed tannins were

expressed as milligrams of tannins per gram of plant dry mass. We performed three replicates for each sample for each of the described assays.

Data analysis

In the herbivory reduction experiment, we performed LMER using herbivory level as response variable, group (protected or non-protected) as fixed effect and plant identity as random effect. To assess the effects of herbivory on plant growth, we used the leaf area lost rate of the previous month. We used it because plant increment in growth in a given month should mirror the effects of past herbivory (previous month). To test the effects of herbivory on plant traits, we performed LMER analyses using plant growth rate, SLM, trichome density, total phenolics and condensed tannins as response variable, group (protected or non-protected) as fixed effect and plant identity as random effect.

To test the effects of herbivory on plant growth of the 54-months monitoring experiment, we performed linear mixed effect models (LMER) using plant growth rate as response variable and previous month herbivory and ontogenetic stage (juvenile, uncolonized pre-mature or colonized pre-mature) as fixed effects. Individual plant identity was used as random effect. To verify the effects of the ontogenetic stage on plant defense traits, we used SLM, trichome density, total phenolics and condensed tannins as response variable, ontogenetic stage as fixed effect and plant identity as random effect. Trichome density was log transformed to achieve normality of residuals and homoscedasticity.

For the 12 months of herbivory protection experiment and the 54 months monitoring experiment, we checked for spatial autocorrelation by using distinct Mantel tests (9999 permutations) of pairwise spatial distances and pairwise

differences in any of the parameters (growth rate, herbivory, SLM, trichome density, total phenolics and condensed tannins). Because we have data available from different moments, we standardized to use the last measure available. We compared PAR intensities performing a Generalized Linear Model (GLM) using PAR measurements as response variable and group as explanatory variable.

We used software R 3.4.1 (R-Core-Team, 2016) for all analyses. We performed residual analyses for all models and checked for the distribution of errors. We performed LMER tests using the package lme4 (Bates et al., 2015) and Mantel tests using the package ade4 (Dray & Dufour, 2015).

RESULTS

Effect of herbivory reduction on plant growth and defenses

Herbivory reduction was successfully performed as plants non-protected from herbivores had 2.89 times more leaf area lost than plants protected against herbivores ($\chi2$ (1, N = 21) = 5.13; p = 0.02). Contrary to expected, we found that daily growth rate was not different between protected and non-protected plants ($\chi2$ (1, N = 21) = 1.84; p = 0.17). On the other hand, as expected, non-protected group presented higher SLM ($\chi2$ (1, N = 18) = 13.76; p < 0.001; Figure 1a) and trichome density ($\chi2$ (1, N = 18) = 4.25; p = 0.04; Figure 1b) than protected group. Finally, we found no differences in total phenolics ($\chi2$ (1, N = 12) = 2.15; p = 0.14) and condensed tannins ($\chi2$ (1, N = 12) = 0.94; p = 0.33) between protected and non-protected plants.

We found no differences in light availability between groups. Photosynthetic Active Radiation (PAR) was not different between protected and non-protected plants ($F_{20,1}$ = 1.14, p = 0.27). We also found no spatial correlation between growth, herbivory, plant chemical traits, trichome density and plants (Spearman r <= 0.15, p

$>=0.1$). However, we found a significative spatial correlation for SLM and plants (Spearman r = 0.34, p = 0.006).
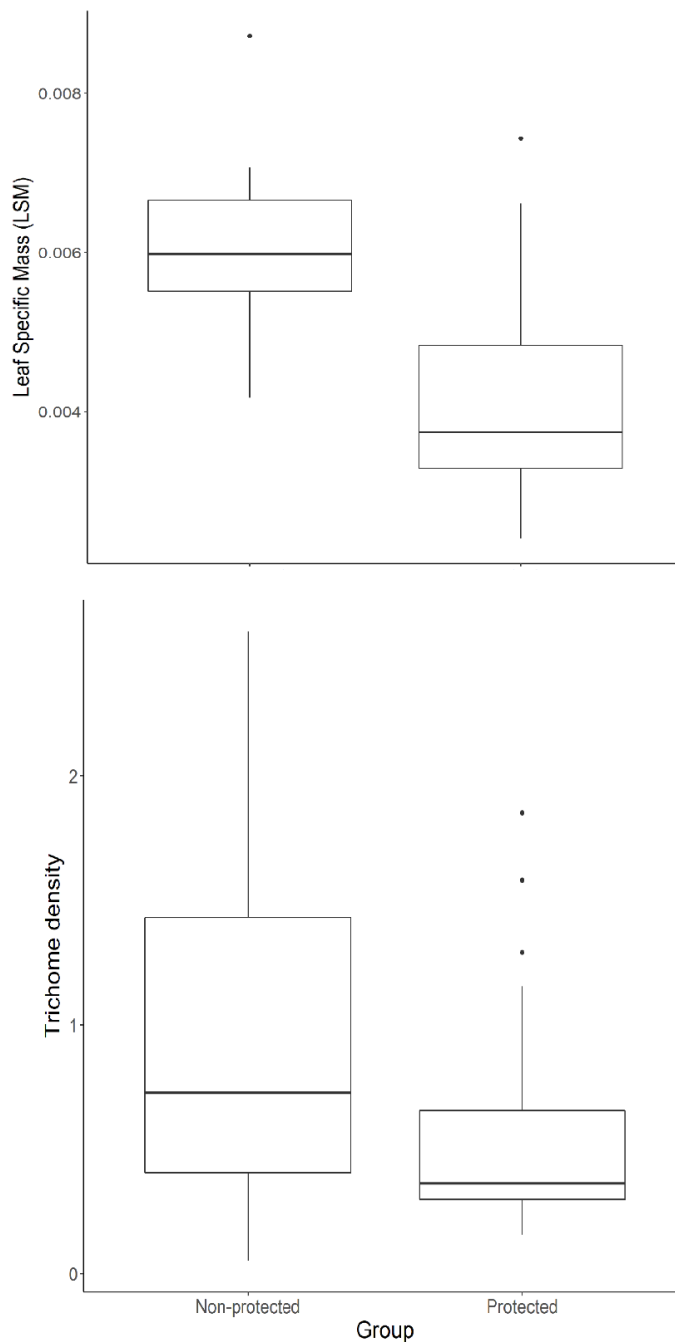
Figure 1. Effects of the experimental reduction of herbivory (protected and non-protected against herbivores groups) on physical defense traits: **A)** specific leaf mass (g/cm^2) and **B)** trichome density (no. trichomes x (1/4 mm^2)$^{-1}$). The horizontal line in each box indicates the median, box limits are first and third distributional quartiles, whiskers extend to most extreme data point, and dots indicate outlying data points.

Effect of plant ontogeny on plant responses to herbivory

Based on our overall model (considering the 54 months), we found that the herbivory suffered in the previous month had no effects on present plant growth (χ2 (1, N = 35) =1.8; p = 0.18). On the other hand, as expected, when we categorize plants according to their ontogenetic stage, we found that plant growth from older and ant-colonized plants were negatively affected by herbivory, while herbivory had no effect on plant growth on younger and ant-less plants (χ^2 (1, N = 35) = 13.35; *p* = 0.001; Figure 2).
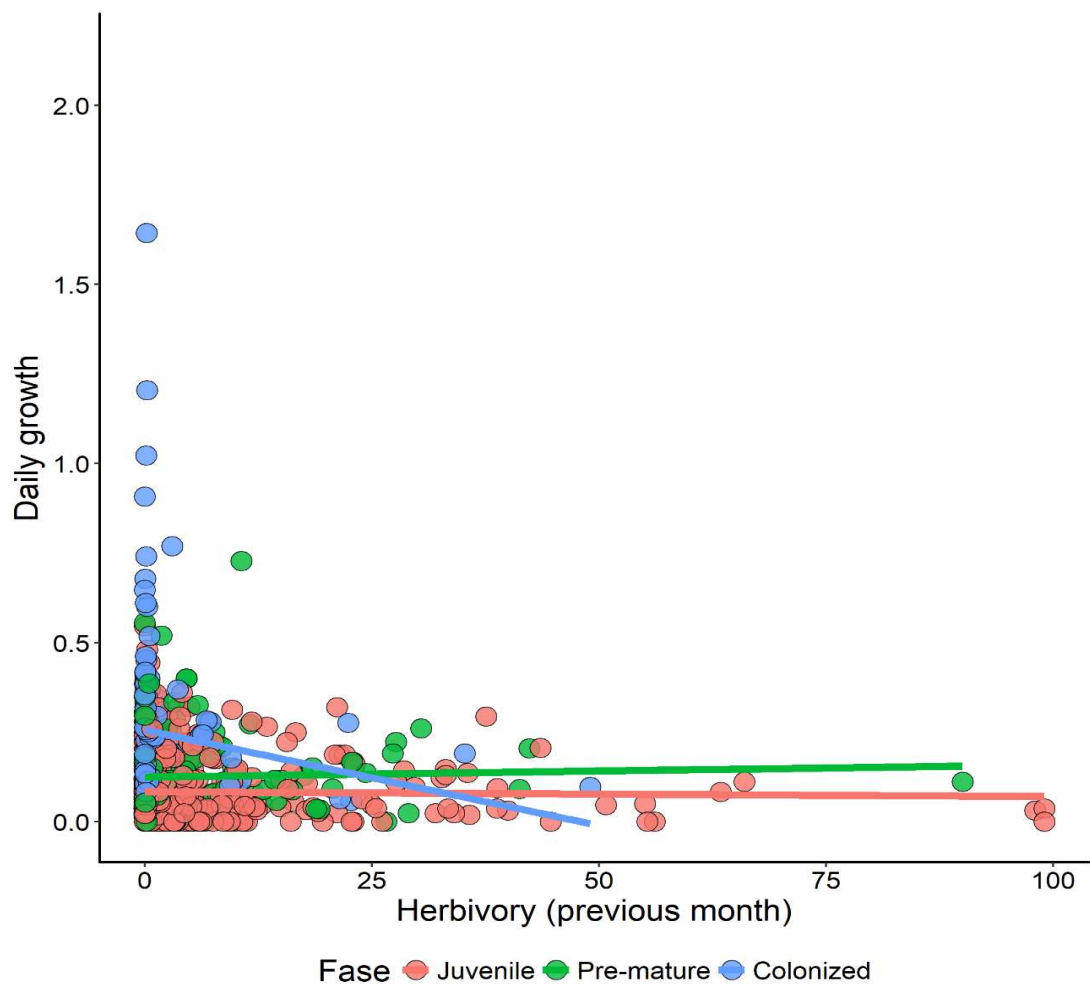

Figure 2. Relationship between daily growth rate (cm/day) and the percentage of leaf are lost in the previous month (number of observations: 917; number of groups: 35). Different circles colors represent different ontogenetic stages.

Conversely to our expectations, most chemical and physical defense traits did not change among ontogenetic stages (SLM: χ^2 (1, N = 36) =0.96; p = 0.62; total phenolics: χ^2 (1, N = 31) =1.34; p = 0.51; and condensed tannins: χ^2 (1, N = 31) =1.7; p = 0.43). However, after colonization by ants, there was a reduction on trichome density of plants (χ^2 (1, N = 34) =22.13; p < 0.001; Figure 3). Finally, we found no spatial correlation between growth traits, herbivory and plants (Spearman r <= -0.05, p >= 0.54).
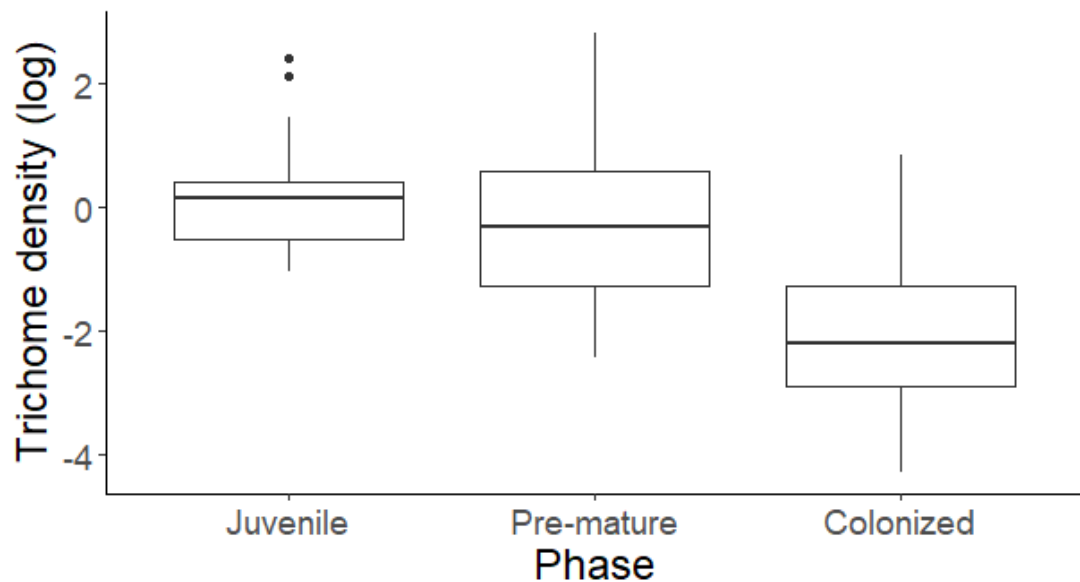
Figure 3. Effects of ontogenetic development on density of leaf trichomes (no. trichomes x (1/4 mm^2)$^{-1}$). The horizontal line in each box indicates the median, box limits are first and third distributional quartiles, whiskers extend to most extreme data point, and dots indicate outlying data points.

DISCUSSION

Contrary to our expectations, we found no overall effects of herbivory on plant growth neither in our 54-months-old monitoring nor in our herbivory reduction experiment. However, considering plant ontogeny, our data reveals contrasting effects of herbivory on plant growth along its development. During the first stages of

development, when plants are juvenile, or pre-mature with myrmecophytic structures but without ants yet, herbivory does not impair plant growth. Such finding is especially interesting, because the commonly found pattern of reduced herbivory and increased growth in colonized plants has been always linked as a causal relationship (Martin, Heil; Doyle, 2003; K. N. Oliveira et al., 2015; Schupp, 1986; Zhang et al., 2015). Here, we present direct evidence that herbivory only impairs *C. glaziovii* growth after ant colonization. By a long-term monitoring on plant herbivory and growth rate, we can for the first time confirm that the reduced herbivory (Oliveira et al. 2015, first chapter of this thesis) results in an increment in plant height, but only after the ant arrival.

Counterintuitively, our findings suggest that herbivores have limited effects on plant growth during the pre-colonization stages, as we could not recognize any trend in plant responses to enhanced herbivory. In addition to our long-term monitoring, our experimental reduction of herbivory in pre-colonized plants did not cause an increase in plant growth rates. Interestingly, such pattern is observed exactly when *Cecropia* plants are more attacked by herbivores (Del Val and Dirzo 2003, Oliveira et al. 2015, first chapter of this thesis). Such results diverge from classical studies which suggest that plants are more susceptible to the negative effects of herbivory during the first ontogenetic stages of plant development (Crawley, 1989; Dirzo, 1984; Marquis, 1984). At some degree, it is possible that antless plants are tolerating herbivory. Non-colonized *C. glaziovii* plants have higher herbivory rates (Oliveira et al. 2015, first chapter of this thesis) and have only trichomes as additional defense compared to their older sympatric relates. Trichomes are efficient defense traits but with a limited range of protection (Coley, 1983; Levin, 1973; Valverde, Fornoni, & Núñez-Farfán, 2001). Although these young plants present higher herbivory and humble defense strategies, their growth is not impaired. Therefore, we suggest that juveniles and non-colonized

pre-mature individuals of *C. glaziovii* might be tolerant to herbivory in these stages. *Cecropia peltate* seems to present the same tolerance pattern during its pre-colonized stages (Del Val & Dirzo, 2003).

Nevertheless, after ant colonization, during the colonized pre-mature ontogenetic stage, herbivory clearly impairs growth of *C. glaziovii* individuals. Indeed, plants face many changes in their lives at this stage: as the colony start to increase, its demand for space and food increases too. Then, plants are required to invest more in the production of larger hollow trunks and Müllerian bodies (Folgarait, Johnson, & Davidson, 1994; Yu & Davidson, 1997). The production of trichomes decreases but there is no evidence of any change in other physical or chemical defense trait. Moreover, because of the presence of ants, herbivory rates drastically decrease in more than 80% (first chapter of this thesis), which support the assumption that the protection by myrmecophytic ants is the most efficient among physical, chemical or biological defenses (Massad et al., 2011; Zhang et al., 2015). We suggest that, once colonized by ants and therefore better protected against herbivores, plants lose their previous abilities to handle harmoniously with herbivory, i.e. tolerate herbivory. In obligate mutualisms, partners are not expected to survive in the absence of each other. Although young myrmecophytes begin their lives without ants (Martin, Heil; Doyle, 2003; Mayer, Frederickson, Mckey, et al., 2014), it is possible that, as plants start to be benefitted by ants (and vice-versa), the mutualistic relationship "officially begins" and thus come the obligate interdependency. During our observations, we could monitor three colonized pre-mature individuals that for unknown reasons lost their ant colonies, which led those plants to permanently lose all leaves and be considered as dead.

An alternative explanation for the fact that herbivory only begins to be harmful after ant colonization comes from the nutritional uptake from ants to plants. Despite

controversial debate in literature, inorganic nutrient level has been found to be negatively associated with tolerance in natural and agricultural systems (Strauss and Agrawal 1999, Wise and Abrahamson 2007 and references therein). Because colonized plants increase their isotopic N in 146% (first chapter of this thesis) compared to non-colonized plants, it is possible that this uptake of nutrient availability causes the decrease of tolerance level of plants. Unfortunately, despite growing knowledge on tolerance, we still have a poor understanding on this anti-herbivore strategy (Fornoni, 2011; Tiffin, 2000) and its interpretation is difficult. To conclusively assess if tolerance is the strategy used by our plants, future studies on photosynthetic rates, shifts on biomass and nutrient allocation, utilization of stores reserves and phenological changes are needed (Barton, 2013; Fornoni, 2011; Tiffin, 2000).

In our experiment of reduction of herbivory, young plants exposed to herbivores had higher sclerophylly and trichome density than protected ones. It suggests that such increment in physical defenses is an induced response to herbivory. Induced responses are "immune-like" responses to injuries that reduce the performance or preference of herbivores and is widely found in plants (Karban & Baldwin, 1997). Sclerophylly of as not yet colonized plants could be higher than colonized ones as a response to higher herbivory levels. Sclerophylly is a trait that may have evolved primarily as a protection against dehydration, but it is also a good strategy to deter herbivores because as more tough a leaf is, more unpalatable it is (Agrawal & Fishbein, 2006). Thus, as many other physical and chemical defenses, one might expect that increased sclerophylly is a response to increased herbivory intensity. However, such results should be interpreted with caution, as we found a spatial correlation analysing this parameter.

We also have evidence from both the 54 months monitoring and the experiment of herbivory reduction that trichome density is also an induce response to herbivory. For instance, it is known that *Alnus* and *Endospermum* trees can increase their trichome production in response to increased levels of herbivory (Baur et al., 1991; Letourneau et al., 1999). The first chapter of this thesis suggest that lower trichome densities found in colonized *C. glaziovii* individuals may be related to energy saving. Once plants are protected by ants, they could diminish their investment in other traits, such as trichomes. However, our conjugated results from both experiments suggest that it is not the presence of ants (and thereby their protection) that directly trigger the reduction on trichome production. In truth, what seems to regulate trichome production is the herbivory intensity. In other words, as higher the herbivory intensity is, higher is the trichome production.

In short, we provide here the contrasting responses of an ant-plant to herbivory and their defenses during three distinct ontogenetic stages (Figure 4). Juvenile and non-colonized pre-mature stages showed that herbivory has no effects on plant growth and defense traits remained stable among both stages. On the other hand, after being colonized, plants showed a reduction on leaf trichome production and a negative effect of herbivory on their growth. Our 54 months of monitoring since the seedlings´ plantation were not enough to reach plants reproduction (mature stage). However, growth measures are a good proxy for plant fitness, especially in long-lived woody species (Züst & Agrawal, 2017). Thus, we present here strong evidence of herbivore impacts on plant performance. Our results diverge from the most found patterns in woody plants, as tolerance was expected to remain stable and resistance to increase or maintain along plant ontogeny (Barton & Koricheva, 2010; Boege & Marquis, 2005). Nevertheless, the lack of this kind of approach on myrmecophytes (Barton &

Koricheva, 2010) may have contributed to the disagreement between our study and theoretical expectations. We show here that only the colonization by ants, and not the beginning of plant´s preparation of myrmecophytic structures, promotes significant changes in plant responses to herbivory. To definitely address this issue, further studies that check for the reproductive and senescence stages and the success of plants offspring are required.
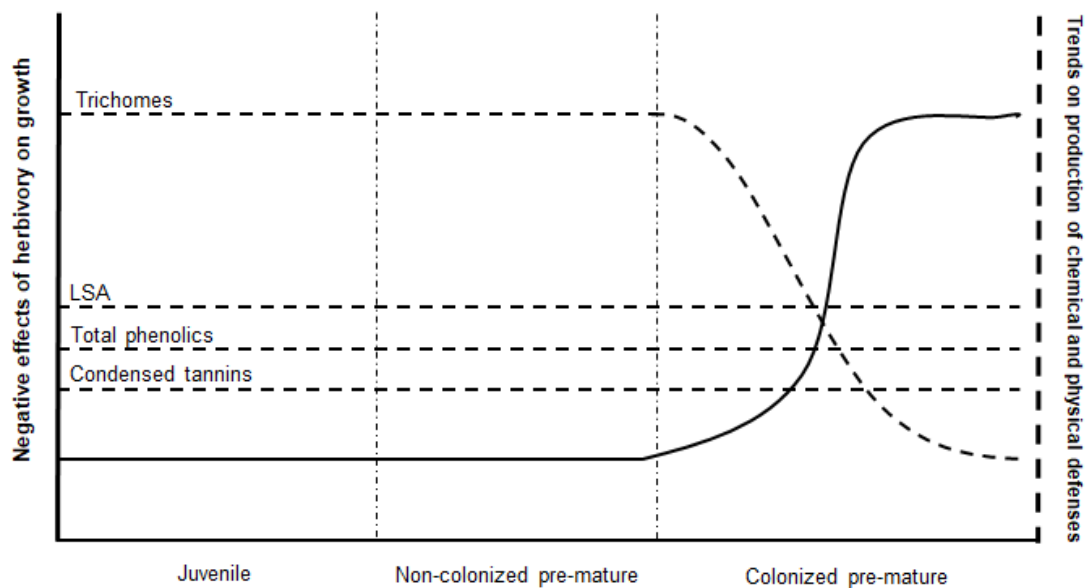
Figure 4. Summarized ontogenetic changes in responses of plants to herbivory pressure and trends in the production of chemical and physical defenses in individuals of *C. glaziovii*. During the juvenile and non-colonized pre-mature stages, herbivory causes non-detectable injury to individuals and physical and chemical defenses remain stable. In the colonized pre-mature stage, negative effects of herbivory on plant growth increase while trichomes production decreases and LSA, phenolics and tannins remain stable.

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CONCLUSÕES GERAIS

Nosso trabalho corrobora a ideia de que indivíduos da planta *Cecropia glaziovii* são beneficiadas pelas suas formigas mutualistas *Azteca muelleri*. Mais do que isso, dentro desse sistema mutualístico conspícuo e ainda assim pouco estudado na Mata Atlântica, ele contribui para eliminar totalmente os possíveis vieses e dúvidas sobre a influência da qualidade das plantas previamente à sua colonização pelas formigas. Por meio de nosso experimento de longa duração, nós podemos atestar que, independentemente da qualidade e condição prévia da planta, todos os indivíduos de planta colonizados por formigas são beneficiados e crescem mais do que os não colonizados. Nosso trabalho ainda demonstra que, além da defesa contra a herbivoria, existem outros importantes mecanismos pelos quais as formigas beneficiam as plantas. Demonstramos aqui que as formigas ainda protegem as plantas contra patógenos, fornecem nutrientes para as plantas e finalmente diminuem os gastos energéticos da planta com algumas defesas (principalmente tricomas glandulares).

Além disso, também mostramos como a planta responde à herbivoria ao longo do seu desenvolvimento ontogenético. A herbivoria parece ser prejudicial às plantas somente a partir do momento em que essas são colonizadas por formigas. O estágio ontogenético da planta parece ser determinante para o tipo de resposta que as plantas têm para os ataques de herbívoros.

Dessa forma, os resultados dessa tese contribuem para uma melhor compreensão das associações mutualísticas na natureza, que são tão importantes sob o ponto de vista de suas populações e comunidades. Também fazemos importantes acréscimos à teoria de defesa de plantas, e sobre como todas essas interações, mutualismo e herbivoria, interagem e se manifestam de acordo com o estágio

ontogenético da planta. Sob uma ótica conservacionista, nossos dados podem trazer informações que aumentem a nossa compreensão sobre os fatores e mecanismos que afetam o estabelecimento e sucesso de plantas em ambientes tropicais.

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