ENGGEL BEATRIZ SILVA DO CARMO

EFFECTS OF MULTIPLE HERBIVORE INFESTATION ON CHEMICALLY MEDIATED INTERACTIONS: FROM META-ANALYSIS TO CASE STUDY

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Entomologia, área de concentração em Entomologia, para a obtenção do título de Doutora em Ciências.

Profa. Dra. Maria Fernanda Gomes Villalba Peñaflor

Orientadora

Dr. Renato Chaves de Macedo Rego

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EFEITOS DA INFESTAÇÃO MULTIPLA DE HERBÍVOROS NAS INTERAÇÕES MEDIADAS QUIMICAMENTE: DE META-ANÁLISE A ESTUDO DE CASO

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"*Um passo à frente e você não está mais no mesmo lugar*."

Chico Science

RESUMO

As respostas das plantas à herbivoria são moduladas tanto pelo nível de infestação quanto pela identidade dos herbívoros, influenciando as defesas da planta, o desempenho dos herbívoros e suas interações com inimigos naturais (INs). Este estudo investiga como infestações múltiplas por artrópodes afetam a indução de defesas de planta e a emissão de voláteis induzidos pela herbivoria (ou herbivore-induced plant volatiles, HIPVs), com impactos nas interações tritróficas. Em uma análise mais ampla dessas interações, através de uma meta-análise, constatamos que, embora as co-infestações alterem a composição dos voláteis, elas não necessariamente prejudicam a localização dos hospedeiros pelos INs. INs especialistas não diferenciaram HIPVs emitidos por plantas infestadas pelo seu hospedeiro sozinho ou em co-infestação com herbívoros não hospedeiros, sugerindo que a comunicação química se mantém estável, apesar da complexidade imposta pela herbivoria. Contrariando as expectativas, INs generalistas não demonstraram preferência por voláteis emitidos por plantas que abrigavam uma maior diversidade de hospedeiros, embora tenha sido observada uma tendência de preferência por plantas infestadas por herbívoros de diferentes guildas alimentares (sugador e mastigador) em relação a plantas infestadas por apenas um hospedeiro. Em um caso de estudo específico com plantas de café (*Coffea arabica*) infestadas pelas cochonilha-verde (*Coccus viridis*) e cochonilha-farinhenta (*Planococcus citri*), analisamos as vias de defesa da planta, o desempenho das cochonilhas e a resposta do predador coccidófago *Cryptolaemus montrouzieri*. Nossos resultados indicaram que *P. citri* induzem um aumento dos níveis de ácido jasmônico (JA) e ácido salicílico (SA), ao passo que a herbivoria por *Co. viridis* desencadeou o acúmulo de SA sem alteração da via do JA, um padrão conhecido como *crosstalk* negativo entre SA-JA comumente observado em plantas sob infestação de insetos sugadores de floema. Essa modulação hormonal resultou em um perfil de metabólitos secundários (alcaloides e compostos fenólicos) nas folhas distinto das plantas sob infestação de *Co. viridis* e *P. citri*. A infestação por uma das cochonilhas não influenciou a escolha subsequente da outra espécie de cochonilha, mas a co-infestação impactou o desempenho de *P. citri*. A joaninha *Cr. montrouzieri* foi capaz de reconhecer plantas infestadas por cochonilhas ou cochonilhas-farinhentas pelos HIPVs, preferindo voláteis emitidos por plantas infestadas que plantas não infestadas. Porém a infestação múltipla modificou este perfil de modo que não foi reconhecido pela joaninha como um sinal da presença de presas. Esses resultados ressaltam a complexidade das interações multitróficas mediadas por defesas de plantas, destacando a indução de defesas espécie-específicas e a resiliência da sinalização tritrófica. A compreensão dessas dinâmicas fornece insights valiosos sobre estratégias de resistência de plantas, controle biológico e suas implicações no manejo de pragas em sistemas agrícolas.

PALAVRAS-CHAVE: interação tritrófica; metabólitos secundários; defesa induzida; inimigo natural; ataque duplo; sinergia hormonal; controle biológico.

ABSTRACT

The response of plants to herbivory are modulated by the level of infestation and identity of the herbivores, influencing plant defenses, herbivore performance, and their interactions with natural enemies (NEs). This study explores how multiple infestations by arthropods affect plant defense induction and herbivore-induced plant volatiles (HIPVs), with consequences for tritrophic interactions. Through a broader analysis of these interactions via meta-analysis, we found that while co-infestations alter volatiles composition, they do not necessarily impair the ability of NE to locate their host. Specialist NEs did not differentiate between HIPVs from their host alone or in co-infestation with non-host herbivores. This result suggests that chemical communication remains stable despite the complexity imposed by herbivory. Contrary to expectations, generalist NEs exhibit a preference for volatiles emitted by plants hosting a greater diversity of herbivores. However, a tendency was observed for generalists to prefer plants infested by herbivores from distinct feeding guilds (piercing sucking and chewing) over those infested by a single host species. In a specific case study with coffee plants (*Coffea arabica*) infested by *Coccus viridis* (green scales) and *Planococcus citri* (citrus mealybugs), we examined plant defense pathways, herbivore performance, and the response of the coccidophagous predator *Cryptolaemus montrouzieri*. Our results indicated that mealybugs induced an increase in jasmonic acid (JA) and salicylic acid (SA) levels, whereas herbivory by green scales triggered SA accumulation without altering the JA pathway, a pattern known as SA-JA negative crosstalk, commonly observed in phloem-feeding insect infestation. This hormonal modulation resulted in a secondary metabolite profile (alkaloids and phenolic compounds) in the leaves that differed from plants infested by *Co. viridis* and *P. citri*. Infestation by one scale species did not influence the subsequent choice of the other species, but the co-infestation impacted *P. citri* performance. The ladybird beetle *Cr. montrouzieri* was able to recognize plants infested by green scales or mealybugs through HIPVs, showing a preference for volatiles emitted by infested plants over non-infested ones. However, multiple infestations altered the volatile profile in a way that was not recognized by the ladybird as a cue for prey presence. These findings highlight the complexity of multitrophic interactions mediated by plant defenses, emphasizing species-specific defense induction and the resilience of tritrophic signaling. Understanding these dynamics provides valuable insights into plant resistance strategies, biological control, and their implications for pest management in agricultural systems.

KEYWORDS: tritrophic interaction; secondary metabolites; induced defense; natural enemy; dual attack; hormonal synergy; biological control.

INDICADORES DE IMPACTO

Os resultados desta pesquisa evidenciam a complexidade das interações ecológicas quando a planta é atacada por múltiplos herbívoros. A meta-análise conduzida revelou que a herbivoria múltipla por insetos hospedeiros e não-hospedeiros não impacta a atratividade dos voláteis induzidos por herbivoria (HIPVs) para inimigos naturais (INs). Apesar de estudos anteriores indicarem efeitos variados da infestação múltipla na eficiência dos INs, os resultados obtidos sugerem que esses impactos são específicos para determinados sistemas ecológicos. A estabilidade da comunicação química nos sistemas tritróficos permite que os INs localizem seus hospedeiros mesmo na presença de herbívoros não-hospedeiros, demonstrando a resiliência desse mecanismo ecológico. Estes achados oferecem subsídios para estratégias de manejo integrado de pragas (MIP) em sistemas agrícolas a partir do controle. No estudo de caso envolvendo as cochonilhas *Coccus viridis* e *Planococcus citri* em plantas de café, observou-se que a infestação simples por uma das espécies alterou os perfis hormonais e metabólicos da planta, afetando diretamente o desempenho dos herbívoros e a atração de inimigos naturais. Embora a infestação múltipla não tenha influenciado a seleção de hospedeiros pelos herbívoros, afetou negativamente o desempenho de uma das espécies em co-infestação, sugerindo que a competição intra-guilda mediada por alterações nas defesas da planta pode ser um fator determinante na dinâmica populacional desses insetos. A análise do papel de metabólitos secundários, como cafeína e catequina, na defesa das plantas e na dinâmica tritrófica, pode abrir novas perspectivas para o manejo sustentável de pragas, fortalecendo a resiliência dos sistemas agrícolas frente aos desafios ambientais e econômicos. Os resultados também revelaram que a joaninha predadora *Cryptolaemus montrouzieri* reconhece plantas infestadas pelas cochonilhas, mas sem preferência por plantas co-infestadas, indicando efeito neutro da infestação múltipla na atração de predadores, mas que a presença de determinados compostos no perfil de voláteis é determinante para essa atratividade. A pesquisa reforça práticas sustentáveis e à redução do uso de inseticidas, especialmente na cafeicultura, ao caracterizar o sistema de defesa da planta e sua interação com INs. Seu impacto tecnológico e econômico se reflete no setor agropecuário, ao fornecer bases para o controle biológico e manejo sustentável de pragas, beneficiando agricultores e setores do agronegócio que adotam práticas ambientalmente responsáveis. Os impactos se inserem nas áreas temáticas da Política Nacional de Extensão em "Tecnologia e Produção", "Meio Ambiente" e "Trabalho", favorecendo inovação tecnológica e preservação ecológica. A pesquisa também se alinha aos Objetivos de Desenvolvimento Sustentável (ODS) da ONU, como ODS 2 (Fome Zero e Agricultura Sustentável), ODS 12 (Consumo e Produção Responsáveis) e ODS 15 (Vida Terrestre), ao contribuir para o desenvolvimento de práticas agrícolas mais sustentáveis e minimizar impactos ambientais. As discussões destacam a importância de abordagens integradas nas interações planta-herbívoro e herbívoro-inimigo natural, abrindo novas perspectivas para o manejo ecológico de pragas e fortalecendo a resiliência dos sistemas agrícolas.

IMPACT INDICATORS

The results of this study highlight the complexity of ecological interactions when plants are attacked by multiple herbivores. The conducted meta-analysis revealed that multiple herbivory by host and non-host insects does not impact the attractiveness of herbivore-induced plant volatiles (HIPVs) to natural enemies (NEs). While previous studies have indicated varying effects of multiple infestations on NE efficiency, our findings suggest that these impacts are specific to particular ecological systems. The stability of chemical communication in tritrophic systems enables NEs to locate their hosts even in the presence of non-host herbivores, demonstrating the resilience of this ecological mechanism. These findings provide insights into integrated pest management (IPM) strategies in agricultural systems through biological control. In the case study involving the phloem feeders' insects, *Coccus viridis* (green scale) and *Planococcus citri* (citrus mealybug), in coffee plants, single-species infestation altered the plant's hormonal and metabolic profiles, directly affecting herbivore performance and NE attraction. While multiple infestations did not influence herbivore host selection, they negatively impacted the performance of one species in co-infestation, suggesting that intra-guild competition mediated by plant defenses may be a key factor in insect population dynamics. The predatory lady beetle *Cryptolaemus montrouzieri* recognizes plants infested with scale insects but shows no preference for co-infested plants, indicating a neutral effect of multiple herbivory on predator attraction. This research reinforces sustainable practices and the reduction of pesticide use, particularly in coffee farming, by characterizing the plant's defense system and its interactions with NEs. Its technological and economic impact extends to the agricultural sector, providing a foundation for biological control and sustainable pest management, benefiting farmers and agribusiness sectors that adopt environmentally responsible practices. The study's impacts align with the thematic areas of the National Extension Policy, specifically in "Technology and Production," "Environment," and "Work," fostering technological innovation and ecological preservation. Additionally, the research aligns with the United Nations Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land), by promoting sustainable agricultural practices and minimizing environmental impacts. These findings underscore the importance of integrated approaches to plant-herbivore and herbivore-NE interactions, opening new perspectives for ecological pest management and strengthening the resilience of agricultural systems.

SUMÁRIO

PRIMEIRA PARTE

INTRODUÇÃO GERAL

As plantas são fundamentais para a vida na Terra, servindo como fonte de alimento para diversos organismos que se nutrem de seus tecidos e subprodutos, como seiva, néctar e pólen. Frente à ação de herbívoros e patógenos, plantas desenvolveram diversos mecanismos de proteção (LUNDGREN; DES MARAIS, 2020). As defesas das plantas incluem barreiras físicas, como lignificação, cera, espinhos, tricomas e espessamento da parede celular, além da produção de compostos orgânicos classificados em metabólitos primários e secundários (WAR et al., 2012). Enquanto os metabólitos primários, como proteínas, ácidos graxos, polissacarídeos e clorofila, têm funções essenciais para o crescimento e desenvolvimento das plantas (ERB; KLIEBENSTEIN, 2020), os metabólitos secundários desempenham papéis cruciais na defesa e comunicação das plantas com outros organismos. Sua composição varia conforme a espécie e as interações com o ambiente (ERB; KLIEBENSTEIN, 2020; KESSLER; KALSKE, 2018; WINK, 2018).

Os metabólitos secundários podem atuar como defesas constitutivas, presentes durante todo o ciclo fenológico da planta, ou como defesas induzidas, ativadas em resposta a sinais de herbivoria (MITCHELL et al., 2016; MITHÖFER; BOLAND, 2012). Ao reconhecer compostos presentes nas secreções orais e os fluídos de oviposição dos herbívoros, as plantas produzem respostas induzidas específicas em relação à identidade do herbívoro (DICKE; BALDWIN, 2010; KANT et al., 2015; KARBAN, 2020). A produção dessas respostas é regulada por redes de fitohormônios, como ácido jasmônico (JA), ácido salicílico (SA), ácido abscísico (ABA) e etileno, que coordenam as defesas e influenciam o crescimento e desenvolvimento das plantas (DICKE; VAN LOON; SOLER, 2009; THALER; HUMPHREY; WHITEMAN, 2012). Esses hormônios podem interagir de forma sinérgica, como no caso do JA e do etileno; ou antagônica, como entre o JA e o SA, em que a ativação de uma via pode inibir a outra (*cross-talk* negativo), dependendo do tipo de dano causado (DICKE; HILKER, 2003; THALER; HUMPHREY; WHITEMAN, 2012). Essas respostas específicas resultam em perfis de defesa que variam conforme a guilda alimentar do herbívoro e outros fatores ambientais (DICKE; VAN LOON; SOLER, 2009; TURLINGS; ERB, 2018). Por exemplo, herbívoros mastigadores ativam vias de JA, enquanto herbívoros sugadores e patógenos tendem a ativar vias de SA (KANT et al., 2015; LUO et al., 2023).

Para evitar ou mitigar o efeito dessas defesas, muitos herbívoros desenvolveram estratégias, como redução de danos mecânicos às células, enzimas de detoxificação e associações simbióticas para manipular e metabolizar as substâncias produzidas pela planta (ALI; AGRAWAL, 2012; CHUNG et al., 2013; ERB; ROBERT, 2016; WALLING, 2008; WINK, 2018). Além disso, as defesas induzidas podem ser diretas, afetando a ação dos herbívoros (KANT et al., 2015; MITCHELL et al., 2016; STENBERG; MUOLA, 2017); ou indiretas, atraindo inimigos naturais (ALJBORY; CHEN, 2018; TURLINGS; ERB, 2018).

Entre as estratégias de defesa indireta, destaca-se a emissão de voláteis de plantas induzidos pela herbivoria (ou herbivore-induced plant volatiles, HIPVs), que desempenham um papel central na comunicação das plantas com inimigo naturais (TURLINGS; ERB, 2018). Além de atraírem predadores e parasitoides, esses compostos podem repelir os herbívoros e mediar a comunicação entre as plantas, induzindo defesas antecipadas nas plantas vizinhas (KARBAN, 2020; NINKOVIC; MARKOVIC; RENSING, 2021).

A composição dos HIPVs varia amplamente de acordo com fatores como espécie de planta e herbívoro, idade e fase fenológica da planta, a guilda alimentar do herbívoro e sua densidade populacional (DICKE; HILKER, 2003; ERB; KLIEBENSTEIN, 2020; RUSSAVAGE et al., 2024; TURLINGS; ERB, 2018). Quando múltiplos herbívoros infestam a mesma planta (herbivoria múltipla), cenário comum em ambientes naturais, a composição dos voláteis emitidos frequentemente difere daquela produzida sob infestação simples (DICKE; VAN LOON; SOLER, 2009; STAM et al., 2014). A sequência e modo de infestação (simultânea ou sequencial) (FERNÁNDEZ DE BOBADILLA et al., 2021, 2022) e a identidade taxonômica dos herbívoros também afetam a intensidade e a especificidade das defesas induzidas (GÓMEZ; ORIANS; PREISSER, 2012; KUNDU et al., 2023). Além disso, a herbivoria por artrópodes da mesma guilda alimentar pode potencializar defesas induzidas (FERNÁNDEZ DE BOBADILLA et al., 2021; PEÑAFLOR et al., 2017), enquanto a herbivoria por artrópodes de guildas distintas pode suprimir uma das vias de defesa (FERNÁNDEZ DE BOBADILLA et al., 2022; LIU et al., 2024; STAM et al., 2014). Essas diferenças impactam diretamente as interações tritróficas mediadas quimicamente, como a eficiência de forrageamento de inimigos naturais.

Os inimigos naturais, como predadores e parasitoides, desempenham um papel crucial na regulação de populações de herbívoros. Seu sucesso depende da capacidade de identificar pistas químicas específicas que indicam a localização e a identidade de suas presas ou hospedeiros (BUKOVINSZKY et al., 2012; DICKE; BALDWIN, 2010; STRAND; OBRYCKI, 1996). Em geral, os parasitoides tendem a ser mais especializados em relação aos

seus hospedeiros do que os predadores em relação às suas presas (SNYDER; IVES, 2003). Enquanto os parasitoides parasitam estágios específicos de vida do hospedeiro e superam suas defesas imunes, os predadores geralmente consomem múltiplas presas ao longo da vida, dependendo da disponibilidade (STRAND; OBRYCKI, 1996). No entanto, inimigos naturais especialistas (predadores e parasitoides) são mais aptos a localizar, reconhecer e parasitar ou se alimentar de um táxon específico (STILMANT et al., 2008). Estudos recentes sugerem que esses organismos utilizam subconjuntos específicos de compostos voláteis, conhecido como *search templates* (modelos de busca), para navegar na alta variabilidade das composições químicas dos voláteis de plantas (AARTSMA et al., 2019). Entretanto, a presença de herbívoros não-hospedeiros em plantas co-infestadas pode confundir os inimigos naturais, aumentando o tempo necessário para localizar suas presas e comprometendo seu desempenho (BUKOVINSZKY et al., 2012; LI et al., 2017; PEÑAFLOR et al., 2017; VOSTEEN; VAN DEN MEIRACKER; POELMAN, 2019).

Embora a interação tritrófica envolvendo os voláteis de plantas seja amplamente estudada, ainda há lacunas no entendimento sobre como a herbivoria múltipla afeta essas interações. Diversos sistemas foram estudados e apresentaram diferentes respostas dos inimigos naturais. Por exemplo, alguns estudos mostram que os inimigos naturais preferem os voláteis emitidos exclusivamente pela herbivoria de suas presas (MOAYERI et al., 2007; PONZIO et al., 2014; SHIOJIRI et al., 2001), enquanto outros indicam que a atratividade dos voláteis de plantas é aumentada sob a herbivoria múltipla (BUKOVINSZKY et al., 2012; MOAYERI et al., 2007; OLIVEIRA; PAREJA, 2014). Essa diferença destaca a necessidade de uma análise mais abrangente sobre o tema.

Esta tese tem como objetivo geral investigar os efeitos da herbivoria múltipla nas defesas das plantas e nas interações tritróficas. O primeiro capítulo é composto de uma meta-análise que sintetiza o conhecimento de pesquisas publicadas ao longo de duas décadas, as quais avaliaram a resposta olfativa dos inimigos naturais a plantas infestadas por múltiplos herbívoros. Os dados foram coletados em estudos disponíveis nas bases de dados *Web of Science* e *Scopus*, com foco em ácaros e insetos como inimigos naturais. Avaliamos padrões gerais na resposta destes organismos aos voláteis induzidos por herbivoria, testando hipóteses relacionadas à preferência de predadores e parasitoides, considerando fatores como especialização alimentar e guilda alimentar dos herbívoros.

No segundo capítulo, focamos em um sistema específico, estudando a interação entre cochonilhas, joaninhas e plantas de café. O café (*Coffea arabica* L.) (Rubiaceae) é uma cultura de grande importância econômica, frequentemente atacada por uma diversidade de herbívoros

(VENZON, 2021). Essa espécie produz compostos secundários, como alcaloides e ácidos fenólicos, conhecidos por seu papel na resistência contra a herbivoria (FERNANDES et al., 2011, 2012; FILHO; MAZZAFERA, 2000).

Dentre os herbívoros que atacam o café, destacam-se cochonilhas da ordem Hemiptera, como *Planococcus citri* (Maskell) (Pseudococcidae) e *Coccus viridis* (Green) (Coccidae). Essas espécies se alimentam da seiva do floema, causando danos significativos, como nanismo, descoloração, desfolhamento e redução da produtividade das plantas (FERNANDES et al., 2009, 2012; RAVUIWASA et al., 2009; RODRIGUES-SILVA et al., 2021). Pesquisas sugerem que herbívoros sugadores podem compartilhar hospedeiros explorando tecidos diferentes (LIU; TURLINGS; LI, 2023) ou que uma espécie dominante pode manipular as defesas da planta, melhorando seu desempenho, mas prejudicando o de espécies subsequentes (GÓMEZ; ORIANS; PREISSER, 2012; LIN et al., 2019; ZHAO et al., 2015). Entretanto, ainda não foi relatada a interação simultânea de cochonilhas, tais como *Co. viridis* e *P. citri,* no mesmo hospedeiro, nem se há uma espécie dominante entre elas que pode manipular as defesas da planta, alterando o desempenho das espécies na interação (GÓMEZ; ORIANS; PREISSER, 2012; LIN et al., 2019; MERTENS et al., 2021).

Insetos predadores, como *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), desempenham um papel crucial no controle biológico de cochonilhas. Essa espécie utiliza sinais químicos da própria presa e da planta para localizar suas presas (ANDRADE et al. 2023, BOZORG-AMIRKALAEE et al., 2015; FINLAY-DONEY; WALTER, 2012; MERLIN; LEMAITRE; GRÉGOIRE, 1996). Ambas as espécies, *Co. viridis* e *P. citri*, são presas de *Cr. montrouzieri* (ATTIA et al., 2011; BIBI et al., 2023; KAIRO et al., 2013; MANI et al., 2008), mas não se sabe se voláteis induzidos pela herbivoria múltipla dessas espécies afetam o forrageamento e a preferência predatória dessa joaninha.

Para responder a essas perguntas, realizamos testes comportamentais de dupla escolha, avaliando a preferência das cochonilhas entre plantas co-infestadas por espécies diferentes, e seu desempenho em plantas previamente infestadas. Foram coletados e analisados metabólitos secundários voláteis (GC-MS) e não voláteis (LC-MS) de plantas induzidas. Também realizamos testes de preferência de presas, avaliando o consumo pela joaninha e a preferência por voláteis de plantas sob infestação simples e múltipla.

Esta tese busca avançar o entendimento sobre os efeitos da herbivoria múltipla nas defesas das plantas e nas interações tritróficas. Através de uma abordagem que combina meta-análise e um estudo de caso, esperamos contribuir tanto para a ciência básica quanto para o desenvolvimento de estratégias de controle biológico mais eficazes. Os resultados deste

trabalho podem colaborar para o desenvolvimento de estratégias de controle biológico mais eficazes, especialmente em culturas de importância econômica, como o café.

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SEGUNDA PARTE

ARTIGO 1

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Herbivory by multiple arthropods does not hinder the attraction of natural enemies to plant volatiles: insights from a meta-analysis

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Abstract

Plants under herbivore attack emit herbivore-induced plant volatiles (HIPVs) that recruit natural enemies (NEs) of the herbivores for defense. The composition of HIPVs is often specific to the herbivore species, and infestation by multiple herbivore species produces a distinct volatile blend compared to single infestations, potentially influencing tritrophic interactions. Although two decades of research have investigated how multiple herbivory can affect chemically-mediated tritrophic interactions, a comprehensive understanding on this topic remains elusive, as studies have shown varying results depending on the system examined. We performed a quantitative synthesis of 29 studies, extracting effect sizes from 94 experiments that assessed the olfactory preferences of NEs for HIPVs emitted from multiple-infested and single-infested plants. Our analysis revealed that multiple infestations do not affect the attractiveness of HIPVs to NEs, regardless of whether the plant is infested by nonhosts, hosts from different or the same feeding guild, the NE dietary specialization, or guild. However, specialist NEs prefer HIPVs emitted from plants with hosts even if they are infested by multiple herbivores over those infested by only a single non-host herbivore. Our meta-analysis provides valuable insights into the complexity of chemically-mediated tritrophic interactions, demonstrating that the co-infestation with nonhosts or multiple hosts do not affect attractiveness of HIPVs to NEs.

Keywords: herbivore-induced plant volatiles, insects, infochemical use, olfactometer, parasitoids, predators, foraging efficiency, tritrophic interactions.

Introduction

Arthropod predators or insect parasitoids are natural enemies (NEs) of herbivores that play a crucial role in regulating their populations. Their effectiveness depends on their ability to search for prey/hosts, a process optimized by chemical cues that convey information about the location, identity, and suitability of prey/hosts [1,2]. Compared to odors emitted by the herbivores themselves, plants represent an important source of chemical cues for NEs as they emit abundant volatile organic compounds (VOCs) [3]. When under attack, plants release a distinct blend of VOCs known as herbivore-induced plant volatiles (HIPVs), which serve as an indirect anti-herbivore defense by facilitating location of prey/hosts by NEs [4]. The production of herbivore-induced plant defenses is regulated by a network of phytohormones, such as jasmonic acid (JA), salicylic acid (SA), ethylene and abscisic acid, which orchestrate a specific induced plant response according to the herbivore's identity [5]. However, when plants are attacked by multiple herbivore species, which is the most common scenario in natural settings, the composition of HIPVs often differs from when plants are infested by herbivores separately [6,7].

Several herbivore-related factors can influence the composition of HIPVs, including herbivore feeding guilds (phloem-feeding, chewing, and cell-feeding herbivores), the presence of nonhost (i.e., unsuitable for parasitoid development) or nonprey herbivores (i.e., not accepted as food by predators), the identity of the herbivores, the order of infestation, and whether infestations occur sequentially or simultaneously [8,9]. Studies have proposed that NEs exploit this variability in HIPV composition enhancing their fitness within the framework of optimal foraging theory [10]. As our understanding of foraging behavior of NEs by chemical cues has advanced, novel theories have emerged to explain how NEs cope with this complexity. More recently, it has been proposed that to navigate the high variability of HIPV compositions, NEs may rely not on the full blend but on a subset of volatile components likely correlated with the host, referred to as the search template [11]. The range of plant volatile compounds and the ratios within the blend involved in the search templates of NEs during foraging can vary depending on their dietary breath, prior experience, and the rarity of their resources [12]. If search templates are too specific or too general, NEs may experience perception errors, reducing their efficiency in locating resources or potentially leading to attraction to non-resources [12].

Despite research on the effect of multiple herbivory on chemically-mediated tritrophic interactions dating back to the early 2000s [13], we still lack a comprehensive understanding

of how infestation by multiple herbivores impacts chemically-mediated tritrophic interactions [8,14]. Thus, we examine this question using a meta-analysis approach, considering the potential factors that may shape NEs' responses to plant volatiles emitted by single-infested and multiple-infested plants. By analyzing 29 studies from which we extracted 94 effect sizes quantifying the innate olfactory responses of NEs to plant volatiles (Figure 1), we challenge predictions from classical theories and test the hypotheses (i-v) detailed below:

- NE guilds: As specialists are fine-tuned to locate, recognize, and parasitize or feed on a specific taxon [15], the presence of nonhost/nonprey herbivores on host-infested plants can affect the foraging efficiency of NEs by increasing the time and effort required to locate their host, thereby increasing the chances of the NE leaving the patch [16–18]. Moreover, specialist NEs likely prefer volatile blends most reliably associated with the presence of their prey [3,11]—i.e., those emitted by plants hosting only the prey, without the influence of other non-target biotic stressors that could introduce variability into the composition of the volatile blend [19]. Thereby, we hypothesize that specialist NEs prefer volatiles specifically associated with their hosts, such as originating from plants infested exclusively by their host/prey (hereafter referred to as 'host') over those from plants co-infested with a host and a nonhost/nonprey ('nonhost' hereafter) (i) (Figure 1). Overall, parasitoids are typically more adapted to their hosts than predators are to their respective prey [20]. Parasitoids parasitize specific life stages and overcome host defenses, whereas predators usually consume multiple prey over lifetime, depending on availability [2]. Within the guild of specialist NEs, we hypothesize that specialist parasitoids have a stronger preference for host-infested plants over those from plants co-infested with hosts and nonhosts than specialist predators (ii) (Figure 1). Conversely, when plants are infested with multiple hosts, we expect that generalist predators and parasitoids prefer volatiles from multiple-infested plants, as these provide the advantage of exploiting a broader range of host types to feed on or parasitize, over single-infested plants (iii) (Figure 1).

- Herbivore's feeding guilds: The mechanical damage on leaf tissues by chewing herbivores often triggers the JA signaling pathway, which regulates increased levels of herbivore-induced defenses [5]. Phloem-feeding herbivores, in turn, elicit the SA signaling pathway, either by injection of effectors or microbes, which can interact antagonistically with JA [21,22], resulting in the suppression of JA-dependent defenses. When herbivores of distinct feeding guilds (e.g., chewing and phloem-feeding herbivores) coexist on a plant, herbivore-induced defenses can be suppressed [7,23]. In contrast, herbivory by two herbivores of the same feeding guild can potentiate induced defenses [24,25]. Thus, we predict that NEs prefer volatiles from plants infested with multiple hosts of the same feeding guild over those from single host-

infested plants but prefer the latter over volatiles from plants infested with herbivores of different feeding guilds (iv) (Figure 1).

- Infestation with nonhosts: The specificity of HIPVs allows NEs to discriminate between volatiles emitted by plants with their hosts [10, 17,18]. Therefore, NEs should prefer volatiles emitted from plants co-infested with host and nonhost over those from nonhost-infested plants (v) (Figure 1).

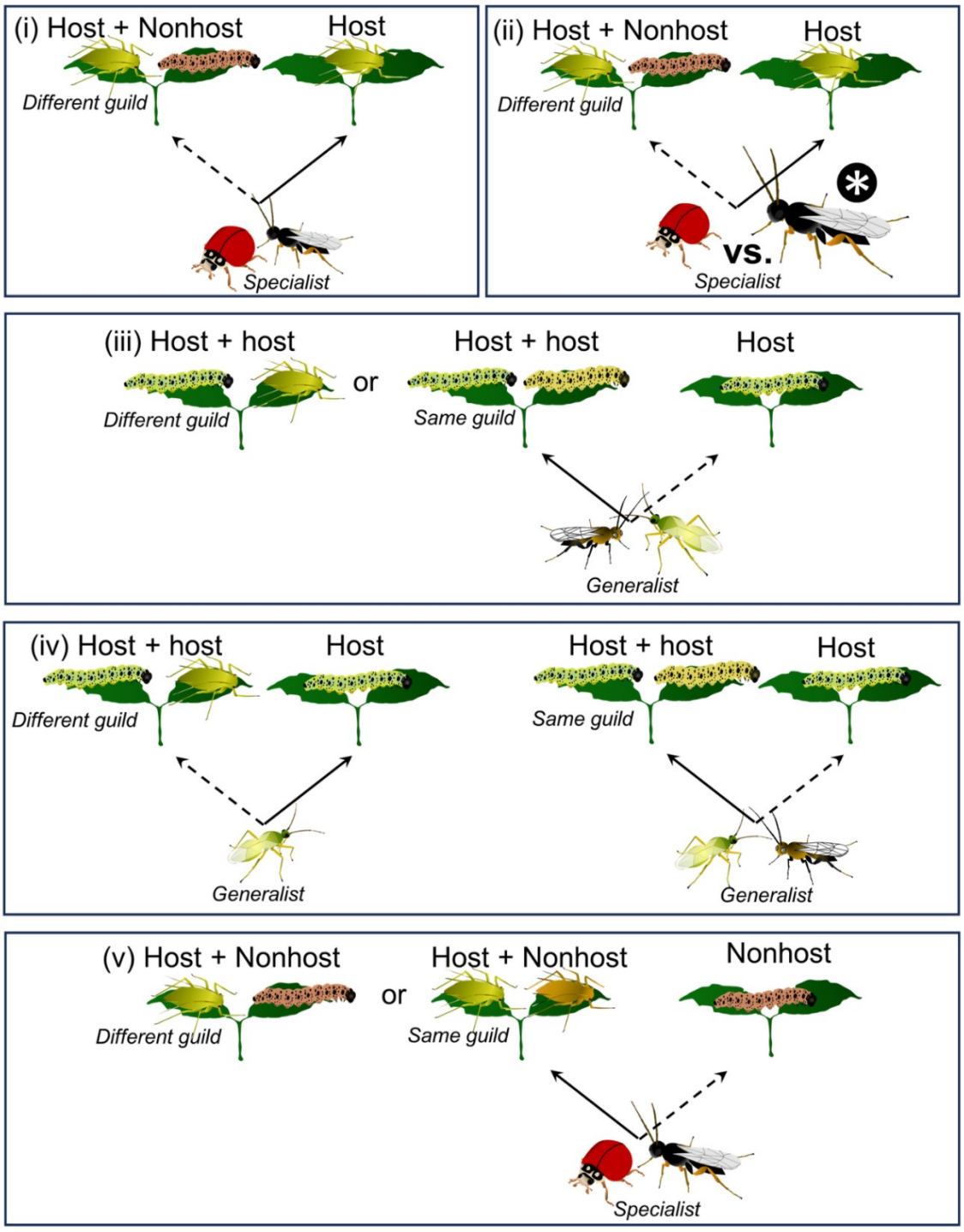

Figure 1. The dataset of the meta-analysis examining the olfactory preferences of natural enemies (NEs) for herbivore-induced plant volatiles (HIPVs) emitted from multiple-infested and single-infested plants. The meta-analysis considered NEs' dietary specialization (specialist or generalist), NEs guild (parasitoid or predator), the feeding guild of herbivores and the suitability of herbivores to NEs (host and nonhosts) as moderator variables. The numbers from (i) to (v) represent the hypotheses. Hypotheses (i) and (ii) were restricted to study systems involving herbivores from different feeding guilds due to the limited availability of studies

addressing infestations by host and nonhost herbivores within the same feeding guild. Arrows originating from predators or parasitoids indicate the potential attraction of NEs to the treatments. Solid arrows correspond to the NE preference as predicted by the hypotheses. An asterisk above and the larger size of the parasitoid indicate its greater ability to recognize plants harboring only hosts over those infested by both hosts and nonhosts, as hypothesized, in contrast to predators. The ladybug represents a specialist predator, while the hemipteran represents a generalist predator. The wasps represent the parasitoids guild, with the darker wasp being a specialist parasitoid and the lighter wasp a generalist.

Methodology

We conducted a systematic search in *Web of Science* (core collection) and *Scopus* databases for studies examining the effect of multiple-herbivory on NEs (i.e., mites and insects) response (for more on the literature search, see Supplementary Material I). Using the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) methodology [26] (Supplementary Material II, Figures S1 and S2), we screened 761 studies. Studies were excluded if they were off-topic (e.g., focused on plant nutrition, phytopathogens, herbivory by mammals or birds, hyperparasitism) or lacked sufficient details, such as the absence of NEs, single-infested plants used as plant pairs, or transgenic plant studies. To avoid potential bias influenced by prior experience of the NEs, we only included studies investigating the innate responses of NEs, focusing exclusively on systems with olfactory stimuli (excluding studies that relied on visual cues). We extracted effect sizes from 29 studies that quantified the preference of NEs when given two options: single-infested and multiple-infested plant (Supplementary Material III, Table S1). When extracting data from figures, we used the software WebPlotDigitizer 4.6 [27]. Here, the effect size (odds ratio, OR) represents the odds of choosing the single-infested plant over the multiple-infested plant, and it was calculated following [28,29]. As ORs do not follow a gaussian distribution, we log-transformed the collected effect sizes and the respective variances using the *escalc* function (*metafor* package, [30]) in R Software [31] (see [32] and Table S3). Positive effect sizes (logOR) indicate NEs preference for single-infested plants, while negative effect sizes indicate NEs preference for multiple-infested plants.

The collected data on NEs' responses to single-infested and multiple-infested plants included 8 to 37 effect sizes for testing hypotheses (i) to (v) (Figure 1). The data used to test hypotheses (i) and (ii) focused on the olfactory response of specialist parasitoids and predators

(analyzed collectively and separately) to volatiles from plants co-infested with hosts and nonhosts, compared to plants infested solely with hosts. Due to limitations in the available literature, these analyses were restricted to systems involving hosts and nonhosts from different guilds. For hypothesis (iii), the data examined the olfactory preference of generalist parasitoids and predators (analyzed collectively and separately) for volatiles from plants co-infested with two host species, regardless of whether they belonged to the same or different feeding guilds, compared to single-infested plant with hosts. For hypothesis (iv), the same data used for hypothesis (iii) were analyzed but focused on contrasting the influence of host herbivore feeding guilds (different vs. same guilds) on generalist NEs' preferences. For hypothesis (v), the data referred to the olfactory preferences of specialist NEs (parasitoids and predators analyzed collectively) for plants infested with both hosts and nonhosts, regardless of feeding guilds, compared to plants infested with nonhosts. We standardized the term 'host' to refer to herbivore species suitable for parasitoid development, or as prey for the NE. Conversely, the term 'nonhost' includes herbivores unsuitable for parasitoid development or arthropod predation. For information on the classification of specialists and generalists, see Table S2 (Supplementary Material IV).

We analyzed the responses of NEs using multilevel models [33] and the *rma.mv* function (*metafor* package, [30]). We controlled data dependency by including as random effects [34]: studies identities, the phylogeny of NEs, the phylogeny of plants, and herbivore species in single-infested plants. The phylogenetic matrices were obtained from the TimeTree.org Database [35] and created using the *vcv* function (*ape* package, [36]; for more, see Supplementary Material V). We investigate the variability between studies by estimating data heterogeneity (I^2) [37,38] with the *i2_ml* function (*orchaRd* age, [39]). We assessed the contribution of each random variable to this variation in each subgroup and checked for potential publication biases by running Egger's regressions [33].

Results

Our dataset contains 94 effect sizes derived from experiments on 22 species of NEs (Figure 2A), involving 19 herbivore species (Figure 2B) and 16 plant species (Figure 2C).

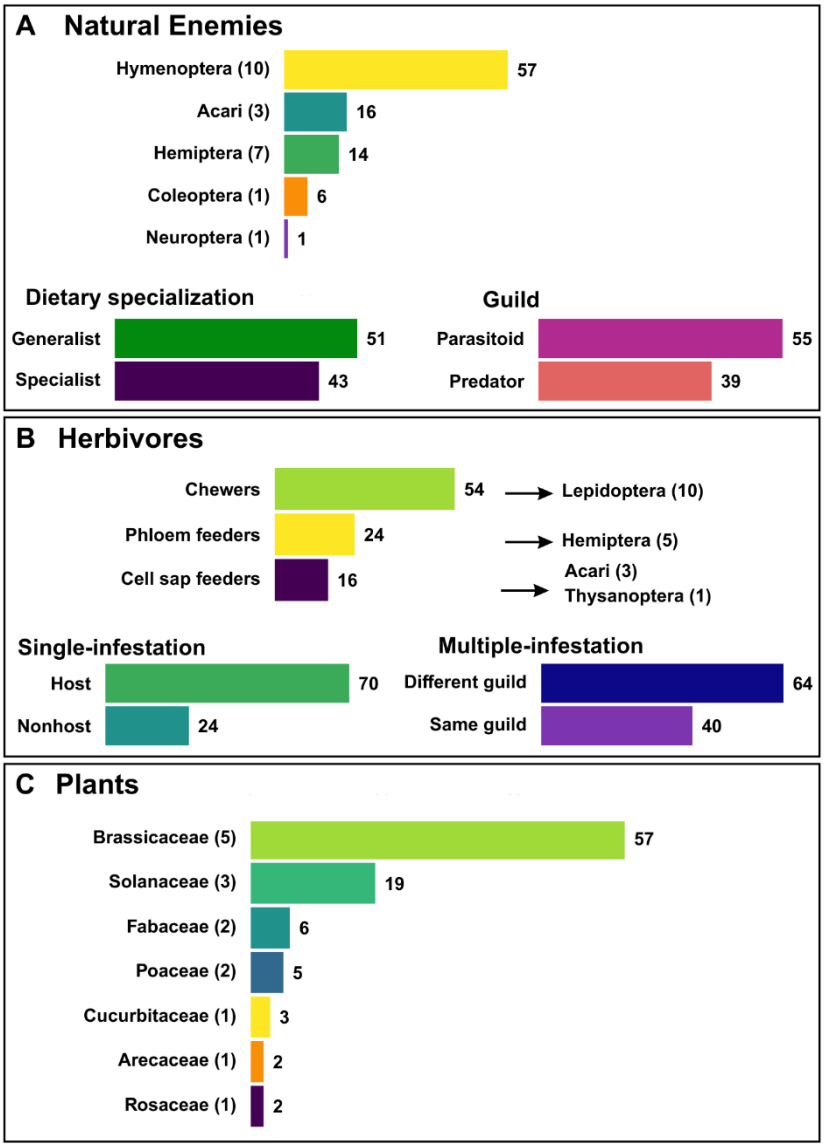

Figure 2. Characterization of the study systems involving natural enemy, herbivore, and plant species from the 29 studies examined in the meta-analysis. (A) NEs' traits: taxonomic identity, dietary specialization, and guild; (B) Herbivores' traits: taxonomic identity, feeding guild and combinations. Herbivores on single-infested plants were classified as either hosts or nonhosts, while those on the multiple-infested plants were categorized according to their feeding guilds. (C) Plants: number of species belonging to each plant family. Numbers in brackets indicate the number of species of the insect order or plant family. The numbers on the bars represent the quantity of effect sizes of each group.

The meta-analysis revealed that specialist NEs showed no preference for volatiles emitted from plants infested solely by a single host species or those from plants co-infested by a host and a nonhost species (p= 0.77) (hypothesis i, Figure 3). This pattern was consistent among both parasitoid and predator specialists, which also exhibited no preference for these

treatments (p= 0.51) (hypothesis ii, Figure 3). No publication bias was detected, and data variation was moderate (58.72%). The largest source of variation was plant phylogeny (33.38%), followed by smaller contributions from study identity (13.10%) and NE phylogeny (12.25%) (Table S4).

Similarly, generalist NEs showed no preference for volatiles emitted from plants infested exclusively by a host species compared to those emitted from plants co-infested by two host species (p= 0.22, hypothesis iii, Figure 3). Consistently, within generalists, neither parasitoids nor predators showed any preference towards these treatments (p= 0.39). Moreover, irrespective of the combination of herbivore feeding guilds, generalist NEs showed no preference between plants infested by a single host or two hosts (p= 0.13, hypothesis iv, Figure 3). No publication bias was detected, and most of the data variation was explained by herbivore species identity (31.00%), with a smaller contribution from NE phylogeny (9.80%) (Table S4).

In contrast, specialist NEs preferred volatiles emitted from plants co-infested with a host and a nonhost species over those emitted from plants infested solely by the nonhost species (p<0.0001) (hypothesis v, Figures 1 and 3). No publication bias was detected, and almost all data variation was explained by study identity (30.99%) (Table S4).

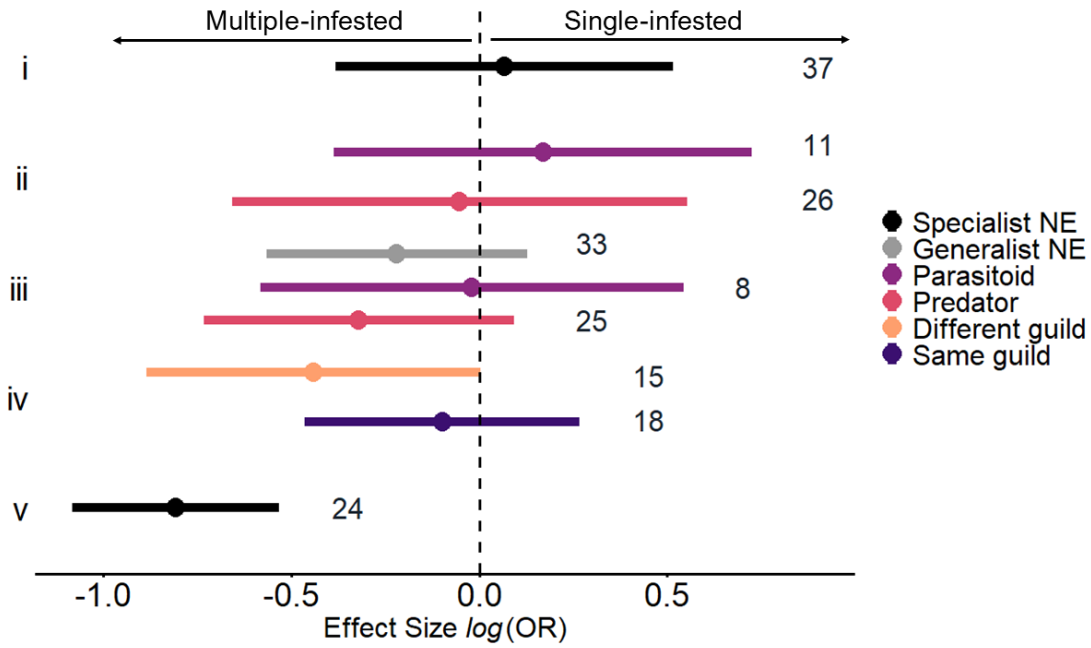

Figure 3. Results of multilevel meta-analyses on natural enemies' (NEs) choice between multiple- and single-infested plants on each of the five hypotheses. Effect sizes are the standardized mean differences *log*(OR), horizontal lines represent 95% confidence intervals (CIs), and the number of effect sizes are shown next to each estimate. CIs overlapping zero

(dashed line) indicates no difference on NE choice between volatiles from single-infested and multiple-infested plants. Negative effect sizes [mean *log*(OR) ± CI indicates preference of NEs for volatiles emitted from multiple-infested plants.

Discussion

Over the last two decades, it has been debated whether infestations by multiple herbivores affect the attractiveness of HIPVs to NEs, potentially impacting their foraging efficiency [8,9,14]. While many studies have demonstrated that co-infestations induce a distinct composition of HIPVs, both quantitatively and qualitatively, compared to those from plants infested with herbivores separately [11,40], the outcome of multiple infestation on tritrophic interactions seems to be unpredictable [8,41]. Our results revealed a consistent pattern: regardless of their guild or dietary specialization, NEs showed no preference for HIPVs emitted by multiple-infested or singly-infested plants when the host is present. These findings support the notion that chemical communication in tritrophic systems is sufficiently stable to withstand disruptions caused by the presence of a second herbivore, whether it is a host or a nonhost [42]. This pattern might emerge because (a) NEs are not able to distinguish HIPVs emitted by multiple-infested or singly-infested plants, or (b) because, despite distinguishing these chemical cues, NEs do not respond differently in each scenario.

The presence of nonhosts on the same plant can affect foraging efficiency of specialist NEs, particularly at long ranges [43]. At long range, HIPVs play a crucial role in the initial steps of foraging, guiding NEs in their host-searching behavior [44]. However, infestation by nonhost herbivores can alter the blend of volatiles released by the plant, thereby reducing the attractiveness of host patches [17,24,45,46]. This effect is more pronounced when the density of nonhosts exceeds a certain threshold, causing NEs to struggle to locate their hosts [45,47, 48] and to track specific volatile compounds [49]. We tested whether co-infestation with hosts and nonhosts (hypothesis i), without considering abundance of herbivores on plants as they are generally standardized in treatments contrasted in olfactometry assays, could reduce the attractiveness of HIPVs to specialist NEs. Given that the analysis of hypothesis i was limited to plants infested by hosts and nonhosts of different feeding guilds, with most NEs targeting chewing herbivores as hosts, the reduced attractiveness of HIPVs to NEs could be due to a negative crosstalk between JA and SA signaling pathways [17,50]. Nevertheless, we found that specialist NEs showed no preference for volatiles from plants harboring both hosts and nonhosts, compared to plants infested only with hosts. Similarly, both specialist predators and

parasitoids exhibited the same pattern, refuting our hypothesis ii that specialist parasitoids are more efficient at detecting the presence of nonhosts on the plant through HIPVs than specialist predators.

As predicted, specialist NEs clearly prefer HIPVs emitted by plants harboring hosts and nonhosts, both from the same and different feeding guilds, over those singly infested by nonhosts (hypothesis v). This finding confirms that specialist NEs effectively follow olfactory plant cues specifically induced by herbivory from their hosts, even when non-target herbivores are feeding on the plant [51,52]. However, we also detected a scarcity of data for this analysis, which was based on only seven studies using only two classical model systems of infochemicals use. This indicates that further efforts should be directed towards testing this scenario more comprehensively.

Overall, these results on the innate olfactory preferences of specialist NEs suggest that they cope with the high variability in HIPVs by employing search templates broad enough to recognize volatile profiles from both multiple-infested and singly-infested plants as indicators of host presence, yet sufficiently specific to avoid being attracted to plants infested solely by non-host. Using narrower search templates that exclude volatile blends from plants co-infested with hosts and nonhosts could be disadvantageous, potentially leading to missed opportunities for NEs in locating hosts [12]. Thereby, we expect that specialist NEs, upon arriving on a plant co-infested with hosts and nonhosts, can use various short-range cues, including not only chemicals from the hosts themselves but also visual, gustatory, and tactile cues, allowing them to locate their hosts even in the presence of a non-host [12,47]. However, it is also possible that specialist NEs may leave co-infested plants more rapidly or even parasitize unsuitable hosts [9,53].

Unlike specialists, generalists' parasitoids and predators parasitize or feed on a broader range of hosts and are attracted to more generic HIPVs [52], including blends emitted by artificially damaged plants or nonhost-infested plants [24]. In hypothesis iii, we specifically predicted that generalist NEs would prefer volatiles emitted from plants with a greater diversity of hosts over those harboring a single host species [54,55]. Nevertheless, NEs were equally attracted to HIPVs from both multiple-infested and singly-infested plants, a pattern also observed when analyzing the responses of parasitoids and predators separately. This lack of preference of generalist NEs likely reflect their broad search templates that allow them to locate host-infested plants. As specialists NEs, they may rely on other modalities of cues at short range to make foraging decision, such as parasitizing or consuming a given host.

Our results did not confirm the hypothesis that plants infested by two host species from the same guild would increase the attractiveness of HIPVs to NEs, as suggested in the literature [8,54]. Even though herbivory by insects from the same guild can amplify volatile emission [24,45]. generalist NEs were equally attracted to HIPVs emitted from single-infested and plants harboring two host species from the same feeding guild (hypothesis iv). As the quantities of insects are generally balanced across treatments in olfactometer assays, this result suggests that generalist NEs either fail to perceive quantitative and/or qualitative differences in the volatile blends emitted from plants harboring a single or two host species or ignore cues indicating greater host diversity, as it may not offer additional benefits. Interestingly, we observed a tendency for generalist NEs to prefer volatiles emitted from plants infested with hosts from distinct guilds over single-infested plants, contrasting to our hypothesis iv that multiple attack by herbivores of distinct guilds would reduce the attractiveness of HIPVs to NEs. It seems likely that the suppression of herbivore-induced defenses by herbivores from distinct feeding guilds is specific to particular systems [50,56,57], or that the reduction of some compounds in the blend does not impact the attractiveness to NEs [58]. On the other hand, the attenuated defenses of plants infested by herbivores from different guilds may lead to greater abundance [41,59], which could explain the tendency of NEs to prefer this treatment over plants harboring only hosts.

Conclusion

Given that co-infestations are the most common condition in natural settings [25,60], adaptations that allow NEs to recognize distinct compositions of HIPVs emitted by plants co-infested with hosts and nonhosts are crucial for locating their hosts and ensuring their reproductive success. Our meta-analysis showed that herbivory by nonhosts does not hamper the attractiveness of HIPVs to NEs. Although there are reports indicating that multiple herbivory can either increase or reduce attractiveness of HIPVs to NEs, our study suggests that these outcomes are specific to particular systems involving plants, herbivores and NE. For instance, Andrade et al. [41] found that the same HIPV blend from plants co-infested with arthropods of distinct guilds had varying effects on NEs, depending on the NE's identity [12]. We were unable to test whether the experience of parasitoids and predators with the plant-herbivores complexes influences their preference for volatiles from multiple-infested plants, as only a few studies have considered this variable [61,62]. As NEs may find reward on plants infested by a single hosts or co-infested with a non-host, we do not expect different outcomes

with experienced NEs, except for the hypothesis v (host+nonhost *vs.* nonhost-infested plant), in which they may fine-tune their foraging behavior to avoid plants harboring only nonhosts [12,53]. Due to a lack of replication, our meta-analysis was limited in testing some hypotheses but not others, particularly those concerning differences in olfactory preferences between specialist and generalist NEs in response to volatiles from plants co-infested with hosts and nonhosts *vs.* host-infested plants. This review also highlighted the need to diversify study systems to obtain a more comprehensive understanding of the effects of multiple herbivory on chemically-mediated tritrophic interactions by including studies involving non-lepidopteran chewing and root-feeding insects, as well as plants beyond classical model systems. For example, the analyses of hypotheses i and ii were restricted to systems in which hosts and nonhosts belong only to different feeding guilds, while hypothesis v was limited to systems involving plants from the Brassicaceae and Solanaceae families. Additionally, most studies investigating the effect of multiple herbivores on the recruitment of NEs have not considered the natural coexistence of herbivores and their order of arrival on plants when selecting the study system [25,60]. Investigating ecologically relevant combinations of herbivores on plants is important because each herbivore triggers a specific plant response, which can either facilitate or impede the colonization of a subsequent herbivore [23,60]. Future research should also investigate the density levels and diversity of non-host species that, when co-infested with hosts, disrupt the attractiveness of HIPVs to NEs [43].

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Data Availability

The data supporting the findings of this study are available at https://doi.org/10.17605/OSF.IO/9J7BE and in the Supplementary Material.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Enggel B. S. Carmo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Illustration, Project administration, Writing - Original Draft, Writing - Review & Editing. **Renato C. Macedo-Rego:** Data curation, Formal analysis, Methodology, Project administration, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding Acquisition. **Maria Fernanda G. V. Peñaflor:** Conceptualization, Project administration, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding Acquisition.

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Leaf Mining Moth *Tuta absoluta* and the Whitefly *Bemisia tabaci*, J. Chem. Ecol. 44 (2018) 29–39. https://doi.org/10.1007/s10886-017-0909-x.

56. A. Kroes, B.T. Weldegergis, F. Cappai, M. Dicke, J.J.A. van Loon, Terpenoid biosynthesis in *Arabidopsis* attacked by caterpillars and aphids: effects of aphid density on the attraction of a caterpillar parasitoid, Oecologia 185 (2017) 699–712. https://doi.org/10.1007/s00442-017-3985-2.

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Supplementary Material

Supplementary Material I

Keywords

We conducted a systematic search in *Web of Science* (core collection) and *Scopus* databases on September 29th, 2022. The first group contained terms related to **multiple herbivory** by both simultaneous and sequential infestation of herbivores, as well as whether the organisms served as prey or hosts for NEs. In the second group of words, the terms were related to **herbivore-infested plants**, including information about the different feeding guilds of herbivores (sucking or chewing) and feeding sites (roots, leaves, or phloem), as well as induced plant defenses. The third group of words contained terms related to the behavioral responses of **NEs** to the signals emitted by herbivore-infested plants.

We conducted the searches on August 13th, 2022. The search for articles was conducted in the *Web of Science* (core collection) database, where we found 97 studies, and in the *Scopus* database, which provided 107 studies. After removing duplicates, the search resulted in a total of 124 studies. We then selected studies within the topic of this meta-analysis based on their titles and abstracts, resulting in the initial selection of 47 articles. Subsequently, after a full-text review of these studies, we found that 21 of them addressed the responses of NEs to volatiles of both single and multiple-infested plants. In addition to these, six articles found in literature reviews and book chapters on the topic that met the inclusion criteria were added to our database [1–3].

A- Keywords of the initial search

Group 1 – Multiple herbivory (first brackets).

Group 2 – Herbivore-infested plants (second pair of brackets).

Group 3 – NEs (third pair of brackets).

TS = [("multipl* herbivor*" OR "multipl* infest*" OR "multi-herbivor*" OR "multipl* attack" OR "dual herbivor*" OR "simultaneous* herbivor*" OR "co-infestat*" OR "dual* infest*" OR "simultaneous* feed*" OR "belowground and aboveground" OR "Prey and non-prey" OR "Host and non-host") AND

TS = ("attack* herbivor*" OR "damage* plant*" OR "feeding guild" OR "herbivore attack" OR "herbivore specie*" OR "herbivorous insect*" OR "infested plant*" OR "insect

herbivor*" OR "insect interaction" OR "phloem feed*" OR "plant-herbivore interaction*" OR "plant-insect interaction*" OR "plant response*" OR "plant volatile*" OR "plant* damag*" OR "plant* infest*" OR "volatiles induced" OR "volatiles emitted" OR "induced plant defen*" OR "plant defen*" OR "herbivore-induced plant volatile*" OR "volatile organic compound*" OR "chew* feed*" OR "root herbiv*" OR "root damag*" OR "plant signaling" OR "induced defen*") AND

TS = ("natural enem*" OR "predator* attract*" OR "predator* response*" OR "parasitoid* response*" OR "tritrophic interaction*" OR "host-parasite interactions" OR "indirect defen*" OR "indirect plant defen*" OR "indirect plan volatile*" OR "induced volatile*" OR "parasitoid attract*" OR "trophic level" OR "olfactometer" OR "olfactory response*" OR "Cotesia" OR "parasitoid*" OR "predator*" OR "multitrophic interaction*")].

To further expand our search results and mitigate the possibility of overlooking pertinent literature in data collection, we conducted a second search using a novel combination of terms on September 29th, 2022. This search used five sets of keywords (B). The first group of terms describes **herbivore-infested plants** and contained terms that characterized the presence and action of herbivores on plants. The second group comprised terms related to **herbivore-induced plant responses**, such as induced defense and signal emission. The third group included terms related to **multiple herbivory**, describing the quantity of host/prey and herbivores simultaneously present on the plant. The fourth group involved the **NEs** in the study systems. Finally, the fifth group of terms describes **cross-trophic interactions**. This second search resulted in 551 articles in the *Web of Science* (core collection) database. After reviewing the titles and removing duplicates from the initial search, 25 articles were selected. Then, after reading the abstracts, four articles were selected. Among these, we added two articles to our database, while two were rejected due to lack of information on the proportions of responses from NEs.

B- Keywords of the complementary search

Group 1 – Herbivore-infested plants (first brackets).

Group 2 –Herbivore-induced plant responses (second pair of brackets).

Group 3 – Multiple herbivory (third pair of brackets).

Group 4 – NEs (fourth pair of brackets).

Group 5 – Cross-trophic interactions (fifth pair of brackets).

TS = [("prey and non-prey" OR "herbivor* damage" OR "plant* damag*" OR “root* damag*” OR "leaf damag*" OR "leaves damag*" OR "phytophag* damage" OR "infest* plant*" OR "infest* leaf" OR "infest* leaves" OR "infest* root*" OR "plant* infest*" OR "leaves infest*" OR "leaf infest*" OR "root* infest*" OR "damag* plant*" OR "damag* leaf" OR "damag* leaves" OR "damag* root*" OR "plant* damag*" OR "leaves damag*" OR "leaf damag*" OR "root* damag*" OR "attack* herbivor*" OR "herbivor* attack*" OR "attack* phytophag*" OR "phytophag* attack*" OR "phloem feed*" OR "feeding guild" OR "chew* feed*" OR "feed* on lea*" OR "feed* on root*" OR "feed* on plant*" OR "eat* lea*" OR "eat* root*" OR "eat* plant*" OR "herbivor* species" OR "herbivor* insect*" OR "insect herbivor*" OR "plant herbivor*" OR "phytophag* species" OR "phytophag* insect*" OR "insect phytophag*" OR "plant-phytophag*" OR "root herbivor*" OR "leaf herbivor*" OR "root phytophag*") AND

TS = ("plant* response*" OR "plant* respond*" OR "volatile*" OR "plant* signal*" OR "plant* defen*" OR "induce* defen*" OR "defen* induce*" OR "indirect defen*" OR "olfactometer" OR "olfactory response*") AND

TS = ("multipl* herbivor*" OR "multipl* infest*" OR "multi-herbivor*" OR "multipl* attack" OR "multipl* phytophag*" OR "dual herbivor*" OR "dual* infest*" OR "simultaneous* herbivor*" OR "simultaneous* feed*" OR "simultaneous* attack*" OR "simultaneous* infest*" OR "co-infestat*" OR "two herbivor*" OR "three herbivor*" OR "four herbivor*" OR "five herbivor*" OR "six herbivor*" OR "seven herbivor*" OR "eight herbivor*" OR "nine herbivor*" OR "ten herbivor*" OR "2 herbivor*" OR "3 herbivor*" OR "4 herbivor*" OR "5 herbivor*" OR "6 herbivor*" OR "7 herbivor*" OR "8 herbivor*" OR "9 herbivor*" OR "10 herbivor*" OR "species of herbivor*" OR "two phytophag*" OR "three phytophag*" OR "four phytophag*" OR "five phytophag*" OR "six phytophag*" OR "seven phytophag*" OR "eight phytophag*" OR "nine phytophag*" OR "ten phytophag*" OR "2 phytophag*" OR "3 phytophag*" OR "4 phytophag*" OR "5 phytophag*" OR "6 phytophag*" OR "7 phytophag*" OR "8 phytophag*" OR "9 phytophag*" OR "10 phytophag*" OR "species of phytophag*") OR

TS = ("natural enem*" OR "field condition*" OR "natural condition*" OR "natural predat*" OR "natural herbiv*" OR "natural phytophag*" OR Predator* OR Parasitoid* OR Tritrophic) AND

TS = ("insect interaction*" OR "*trophic interaction*" OR "host-parasite interaction*" OR "trophic level" OR "Host and non-host")]

Supplementary Material II

We considered studies that quantified the preference of arthropod NEs (*i.e.*, mites and insects) choosing between single- and multiple-infested plant. However, we noted that in some studies, the NEs' response to the same treatments was assessed before and after experience to volatiles from the host/prey-infested plant [4–6]. To prevent potential bias influenced by prior experience of the NE, we only considered the innate responses of the NEs from these studies in our analyses. We also excluded studies that use Bt plants and studies that use setups with NE visual cues as on greenhouses, cages, and petri dishes. Due to the limited number of studies, we were unable to restrict our selection to those where herbivore loads were comparable between singly- and multiple-infested plants, as we considered all treatments from the same study with varying herbivore densities.

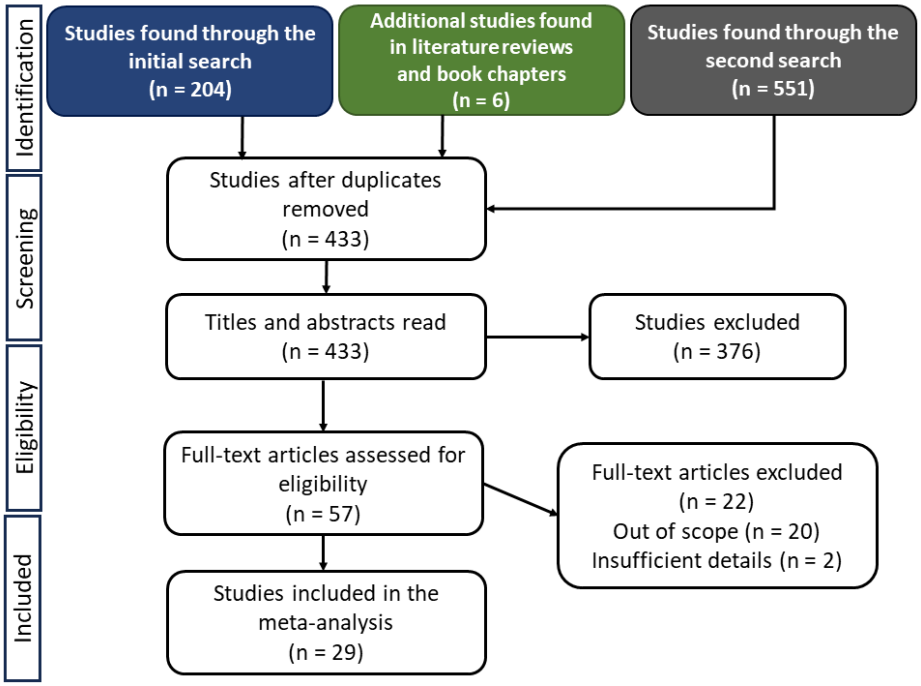


Figure S1. PRISMA diagram depicting the stages from study selection to data collection for the meta-analysis.

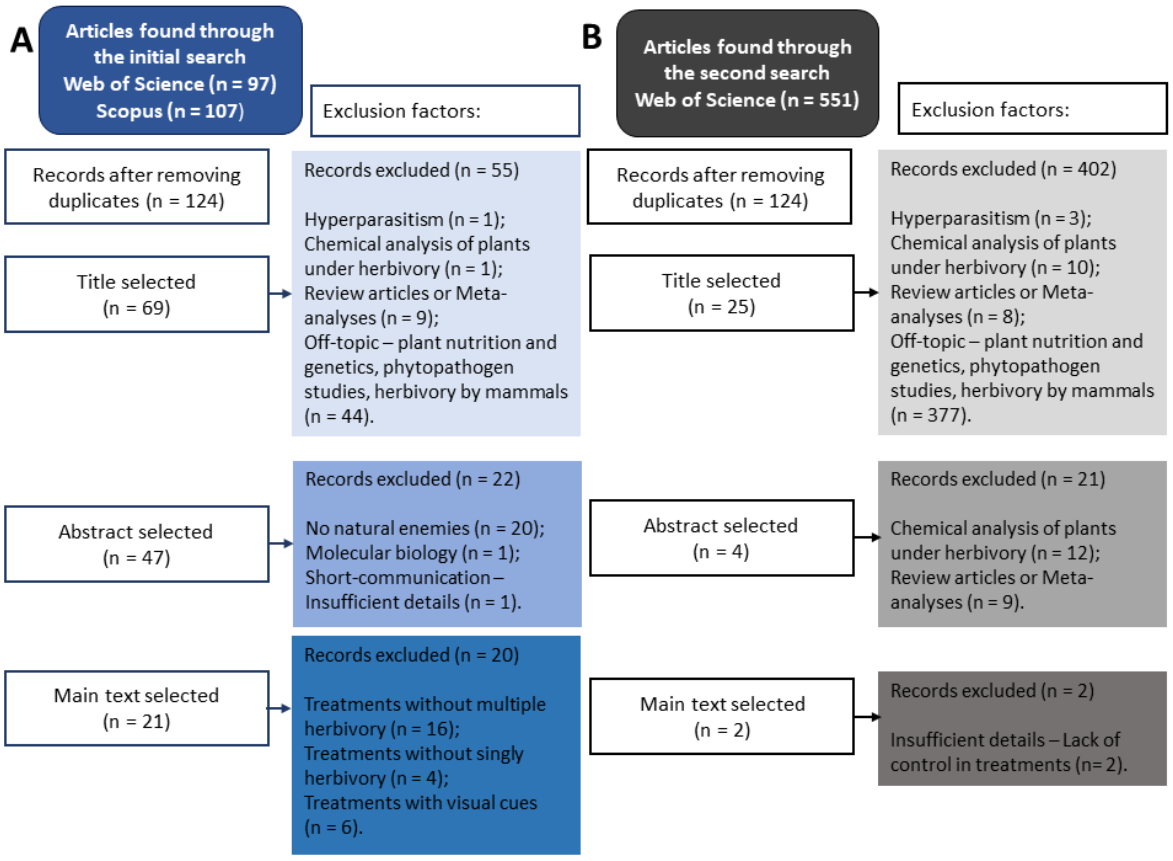


Figure S2. Diagram illustrating the reasons for excluding studies in (A) the initial search, and (B) the second search with keyword updates.

Supplementary Material III

Table S1. Studies distributed on each subgroup and respectively natural enemies' (NEs) guild (Parasitoid or predator) and herbivore guild pairs (same guild or different guild).

Subgroup	NE's guild		Herbivores guild pairs	
	Predator	Parasitoid	Same guild	Different guild
Specialist NE	[1–5]	[6–14]	-	[1–14]
Generalist NE	[7,19–25]	[15,26–29]	[15,19,22,24,26–29]	[7,20,21,23,25]
Specialist NE	[2,4]	[15–18]	[15–18]	[2,4,17]

List of studies used on Meta-analysis:

63. Botti J.M.C., Franzin M.L., Fadini M.A.M., Melo J.O.F. Preference *of Neoseiulus californicus* (Acari: Phytoseiidae) for volatiles of Bt maize induced by multiple herbivory. *Rev. Bras. Entomol*. 63 (2019) 283–289. https://doi.org/10.1016/j.rbe.2019.09.003.

64. de Boer J.G., Hordijk C.A., Posthumus M.A., Dicke M. Prey and Non-prey Arthropods Sharing a Host Plant: Effects on Induced Volatile Emission and Predator Attraction. *J. Chem. Ecol*. 34 (2008) 281–290. https://doi.org/10.1007/s10886-007-9405-z.

65. Dicke M., De Boer J.G., Höfte M., Rocha-Granados M.C. Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods. *Oikos* 101 (2003) 38–48. https://doi.org/10.1034/j.1600-0706.2003.12571.x.

66. Menzel T.R., Huang T.-Y., Weldegergis B.T., Gols R., van Loon J.J.A., Dicke M. Effect of Sequential Induction by *Mamestra brassicae* L. and *Tetranychus urticae* Koch on Lima Bean Plant Indirect Defense. *J. Chem. Ecol.* 40 (2014) 977–985. https://doi.org/10.1007/s10886-014-0499-9.

67. Zhang P.-J., Zheng S.-J., van Loon J.J.A., Boland W., David A., Mumm R., Dicke M. Whiteflies interfere with indirect plant defense against spider mites in Lima bean. *Proc. Natl. Acad. Sci. U.S.A*. 106 (2009) 21202–21207. https://doi.org/10.1073/pnas.0907890106.

68. Agbogba B.C., Powell W. Effect of the Presence of a Nonhost Herbivore on the Response of the Aphid Parasitoid *Diaeretiella rapae* to Host-infested Cabbage Plants. *J. Chem. Ecol*. 33 (2007) 2229–2235. https://doi.org/10.1007/s10886-007-9379-x.

69. Silva R., Walter G.H., Wilson L.J., Furlong M.J. Effects of single and dual species herbivory on the behavioral responses of three thrips species to cotton seedlings: Thrips responses to herbivory. *Insect Sci.* 24 (2017) 684–698. https://doi.org/10.1111/1744-7917.12340.

70. Kroes A., Stam J.M., David A., Boland W., van Loon J.J.A., Dicke M., Poelman E.H. Plant-mediated interactions between two herbivores differentially affect a subsequently arriving third herbivore in populations of wild cabbage. *Plant Biol. J.* 18 (2016) 981–991. https://doi.org/10.1111/plb.12490.

71. Li Y., Weldegergis B.T., Chamontri S., Dicke M., Gols R. Does Aphid Infestation Interfere with Indirect Plant Defense against Lepidopteran Caterpillars in Wild Cabbage? *J. Chem. Ecol*. 43 (2017) 493–505. https://doi.org/10.1007/s10886-017-0842-z.

72. Ponzio C., Cascone P., Cusumano A., Weldegergis B.T., Fatouros N.E., Guerrieri E., Dicke M., Gols R. Volatile-mediated foraging behaviour of three parasitoid species under conditions of dual insect herbivore attack. *Anim. Behav.* 111 (2016) 197–206. https://doi.org/10.1016/j.anbehav.2015.10.024.

73. Ponzio C., Gols R., Weldegergis B.T., Dicke M. Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore: Plant volatiles and parasitoid foraging behaviour. *Plant Cell Environ.* 37 (2014) 1924–1935. https://doi.org/10.1111/pce.12301.

74. Rasmann S., Turlings T.C.J. Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecol. Lett.* 10 (2007) 926–936. https://doi.org/10.1111/j.1461-0248.2007.01084.x.

75. Zhang P., Broekgaarden C., Zheng S., Snoeren T.A.L., Loon J.J.A., Gols R., Dicke M. Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. *New Phytol*. 197 (2013) 1291–1299. https://doi.org/10.1111/nph.12106.

76. Erb M., Foresti N., Turlings T.C. A tritrophic signal that attracts parasitoids to host-damaged plants withstands disruption by non-host herbivores. BMC Plant Biol. 10 (2010) 247. https://doi.org/10.1186/1471-2229-10-247.

77. Bukovinszky T., Poelman E.H., Kamp A., Hemerik L., Prekatsakis G., Dicke M. Plants under multiple herbivory: consequences for parasitoid search behaviour and foraging efficiency. *Anim. Behav*. 83 (2012) 501–509. https://doi.org/10.1016/j.anbehav.2011.11.027.

78. de Rijk M., Zhang X., van der Loo J.A.H., Engel B., Dicke M., Poelman E.H. Density-mediated indirect interactions alter host foraging behaviour of parasitoids without altering foraging efficiency. *Ecol. Entomol*. 41 (2016) 562–571. https://doi.org/10.1111/een.12325.

79. de Rijk M., Yang D., Engel B., Dicke M., Poelman E.H. Feeding guild of non-host community members affects host-foraging efficiency of a parasitic wasp. *Ecology* 97 (2016) 1388–1399. https://doi.org/10.1890/15-1300.1.

80. de Rijk M., Wang Q., Papagiannaki E., Dicke M., Poelman E.H. Herbivore species identity rather than diversity of the non-host community determines foraging behaviour of the parasitoid wasp *Cotesia glomerata. Entomol. Exp. Appl*. 161 (2016) 20–30. https://doi.org/10.1111/eea.12493.

81. Lima D.B., Oliveira H.K.V., Melo J.W.S., Gondim M.G.C., Sabelis M., Pallini A., Janssen A. Predator performance is impaired by the presence of a second prey species. *Bull. Entomol. Res.* 107 (2017) 313–321. https://doi.org/10.1017/S0007485316000900.

82. Lins J.C., van Loon J.J.A., Bueno V.H.P., Lucas-Barbosa D., Dicke M., van Lenteren J.C. Response of the zoophytophagous predators Macrolophus pygmaeus and *Nesidiocoris tenuis* to volatiles of uninfested plants and to plants infested by prey or conspecifics. *BioControl* 59 (2014) 707–718. https://doi.org/10.1007/s10526-014-9602-y.

83. Moayeri H.R.S., Ashouri A., Poll L., Enkegaard A. Olfactory response of a predatory mirid to herbivore induced plant volatiles: multiple herbivory vs. single herbivory. *J. Appl. Entomol*. 131 (2007) 326–332. https://doi.org/10.1111/j.1439-0418.2007.01177.x.

84. Oliveira M.S., Pareja M. Attraction of a ladybird to sweet pepper damaged by two aphid species simultaneously or sequentially. Arthropod-Plant Interact. 8 (2014) 547–555. https://doi.org/10.1007/s11829-014-9336-x.

85. Silva D.B., Bueno V.H.P., Van Loon J.J.A., Peñaflor M.F.G.V., Bento J.M.S., Van Lenteren J.C. Attraction of Three Mirid Predators to Tomato Infested by Both the Tomato Leaf Mining Moth *Tuta absoluta* and the Whitefly *Bemisia tabaci*. J. *Chem. Ecol*. 44 (2018) 29–39. https://doi.org/10.1007/s10886-017-0909-x.

86. Sousa A.L.V., Silva D.B., Silva G.G., Bento J.M.S., Peñaflor M.F.G.V., Souza B. Behavioral response of the generalist predator *Orius insidiosus* to single and

multiple herbivory by two cell content-feeding herbivores on rose plants. *Arthropod-Plant Interact.* 14 (2020) 227–236. https://doi.org/10.1007/s11829-019-09729-5.

87. Schettino M., Grasso D.A., Weldegergis B.T., Castracani C., Mori A., Dicke M., Van Lenteren J.C., Van Loon J.J.A. Response of a Predatory ant to Volatiles Emitted by Aphid- and Caterpillar-Infested Cucumber and Potato Plants. J. Chem. Ecol. 43 (2017) 1007–1022. https://doi.org/10.1007/s10886-017-0887-z.
88. Chabaane Y., Laplanche D., Turlings T.C.J., Desurmont G.A. Impact of exotic insect herbivores on native tritrophic interactions: a case study of the African cotton leafworm, *Spodoptera littoralis* and insects associated with the field mustard *Brassica rapa*. *J. Ecol.* 103 (2015) 109–117. https://doi.org/10.1111/1365-2745.12304.
89. Desurmont G.A., Guiguet A., Turlings T.C.J. Invasive insect herbivores as disrupters of chemically-mediated tritrophic interactions: effects of herbivore density and parasitoid learning. *Biol. Invasions* 20 (2018) 195–206. https://doi.org/10.1007/s10530-017-1526-x.
90. Martorana L., Foti M.C., Rondoni G., Conti E., Colazza S., Peri E. An invasive insect herbivore disrupts plant volatile-mediated tritrophic signalling. *J. Pest Sci*. 90 (2017) 1079–1085. https://doi.org/10.1007/s10340-017-0877-5.
91. Peñaflor M.F.G.V., Gonçalves F.G., Colepicolo C., Sanches P.A., Bento J.M.S. Effects of single and multiple herbivory by host and non-host caterpillars on the attractiveness of herbivore-induced volatiles of sugarcane to the generalist parasitoid *Cotesia flavipes*. *Entomol. Exp. Appl.* 165 (2017) 83–93. https://doi.org/10.1111/eea.12623.

Supplementary Material IV

Table S2. Classification of NEs based on their dietary specialization: References were added in cases where (1) study systems used hosts from the same guild, or (2) papers did not specify the dietary range of the NEs used in the study.

Natural enemies	Number of effect sizes	Dietary specialization	References
Aphidius colemani	1	Specialist	[1]
Cotesia glomerata	7	Specialist	[2–5]
Cotesia marginiventris	2	Specialist	[6,7]
Diadegma semiclausum	10	Specialist	[8–10]
Diaeretiella rapae	4	Specialist	[5,11]
Microplitis mediator	3	Specialist	[12–14]
Neoseiulus californicus	2	Specialist	[15,16]
Phytoseiulus persimilis	12	Specialist	[17–21]
Trichogramma brassicae	2	Specialist	[22]
Campyloneuropsis infumatus	2	Generalist	[23–25]
Chrysoperla externa	1	Generalist	[1,26]
Cotesia flavipes	1	Generalist	[27,28]
Cotesia glomerata	24	Generalist	[2,3,29–31]
Cycloneda sanguinea	6	Generalist	[32]

Engytatus varians	2	Generalist	[25]
Formica pratensis	2	Generalist	[33]
Macrolophus basicornis	2	Generalist	[25]
Macrolophus caliginosus	2	Generalist	[34]
Macrolophus pygmaeus	2	Generalist	[35]
Neoseiulus baraki	2	Generalist	[36,37]
Nesidiocoris tenuis	2	Generalist	[35]
Orius insidiosus	2	Generalist	[38,39]
Trissolcus basalis	1	Generalist	[40–42]

Supplementary Material V

Phylogenies of plants and NEs

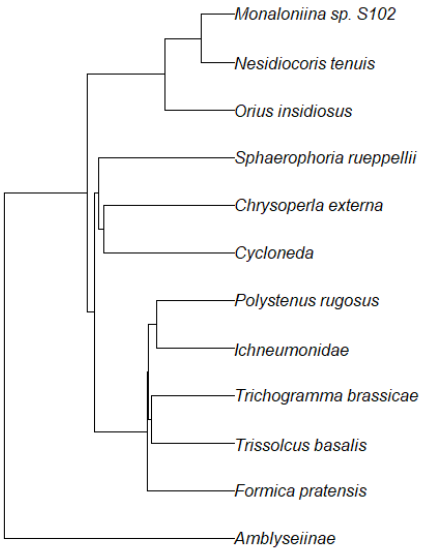
To account for the non-independence of data due to the phylogenetic relationship between taxa of natural enemies and between taxa of plants, we included the phylogenies of both groups in the models as random variables [23–25].

Our dataset included 25 species of arthropod NEs, comprising insects and mites characterized according to their guild, i.e., predator or parasitoid., as well as 15 plant species. We created correlation matrices using the *ape* package, utilizing the *vcv* function [26] in R Software 4.3.0[27].

The plants phylogeny and the majority of the phylogenetic matrix for the NEs (including divergence times between species) was obtained from the TimeTree.org Database [28,29]. For the species absent in the TimeTree Database, we expanded the phylogeny and calculated the length of the unknown branches through a literature search, using the Phylocom software to address this issue [30] (Supplementary Data III). To determine the lengths of the new undated branches, the principle of parsimony was employed. The Phylocon program stipulated that branches with unknown lengths would have half the value of the maximum possible length.

First phylogenetic tree of NE species, with approximate species for the nearest taxon available in Time.tree

(Amblyseiinae:563.30898000,(((Formica_pratensis:214.53714000,((Trissolcus_basalis:203.99635000,Trichogramma_brassicae:203.99635000)'14':8.12836000,(Ichneumonidae:190.04625000,Polystenus_rugosus:190.04625000)'13':22.07846000)'25':2.41243000)'37':129.46286000,((Cycloneda:321.22010000,Chrysoperla_externa:321.22010000)'36':11.44747000,Sphaerophoria_rueppellii:332.66757000)'35':11.33243000)'34':17.53224000,(Orius_insidiosus:170.54237000,(Nesidiocoris_tenuis:81.66410000,Monaloniina_sp._S102:81.66410000)'43':88.87827000)'33':190.98987000)'51':201.77674000);

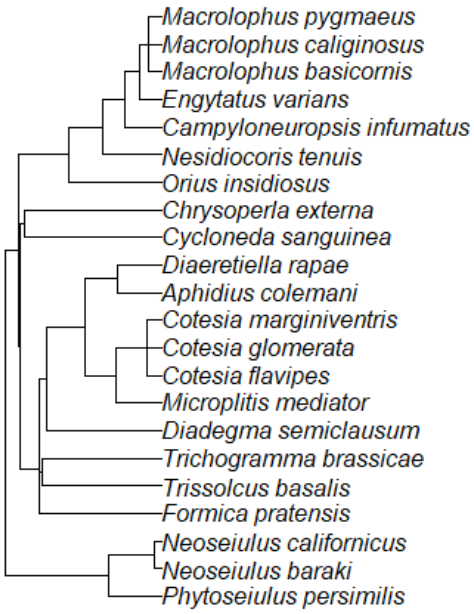


To construct the phylogeny using the Time.tree framework, editing was necessary to introduce the missing species. The Amblyseiinae subfamily of mites had to be subdivided into three distinct groups: *Phytoseiulus persimilis*, *Neoseiulus baraki*, and *Neoseiulus californicus*. The divergence time between *Phytoseiulus persimilis* and the genus *Neoseiulus* was determined according to Zhang et al. (2019). The Braconidae family was fragmented into various distinct species (*Microplitis mediator*, *Cotesia flavipes*, *Cotesia glomerata*, *Cotesia marginiventris*, *Cotesia plutellae*, *Cotesia rubecula*, *Aphidius colemani*, and *Diaeretiella rapae*). Importantly, *Aphidius colemani* and *Diaeretiella rapae*, belonging to the Aphidiinae subfamily, were distinctly separated from the others, which are part of the Microgastrinae subfamily, which comprised the *Cotesia* genus, except *Microplitis mediator*, facilitating the differentiation of genera within this subfamily. The Dicyphini tribe, represented by the species *Monaloniina* sp., was divided into its various species based on the phylogeny presented by Namyatova et al. (2016) (*Campyloneuropsis infumatus*, *Engytatus varians*, *Acrolophus basicornis*, *Macrolophus caliginosus*, and *Macrolophus pygmaeus*).

Phylogeny tree of NE species

((Phytoseiulus_persimilis:334.17737590,(Neoseiulus_baraki:041.7536710,Neoseiulus_californicus:041.7536710)Node3:292.42370490)Node2:665.82262410,(((Formica_pratensis:781.25490780,((Trissolcus_basalis:759.88968320,Trichogramma_brassicae:759.88968320)Node8:018.604192140,(Diadegma_semiclausum:737.36617360,((Microplitis_mediator:292.46696750,(Cotesia_flavipes:087.674642950,Cotesia_glomerata:087.674642950,Cotesia_marginiventris:087.674642950)Node12:204.79232460)Node11:194.23869160,(Aphidius_colemani:284.086

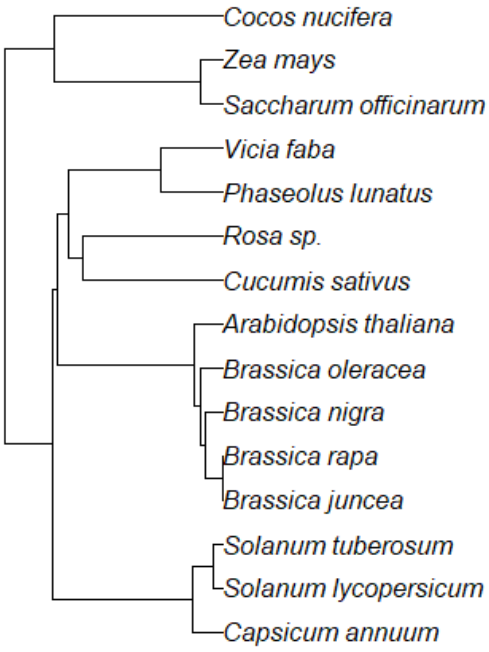
33930,Diaeretiella_rapae:284.08633930)Node13:202.61931980)Node10:250.66051460)Node9:041.127701660)Node7:002.76103250)Node6:123.49274190,((Cycloneda_sanguinea:874.23143660,Chrysoperla_externa:874.23143660)Node15:018.031414170)Node14:012.484798920)Node5:009.4460345750,(Orius_insidiosus:593.46908030,(Nesidiocoris_tenuis:375.69936530,(Campyloneuropsis_infumatus:235.26919660,(Engytatus_varians:141.2769380,(Macrolophus_basicornis:082.21682780,Macrolophus_caliginosus:082.1682780,Macrolophus_pygmaeus:082.1682780)Node20:059.108659980)Node19:093.99225864)Node18:140.43016870) Node17:217.7697150) Node16:320.72460390) Node4:085.806315750) Node1;



Phylogeny tree of plant species

The phylogenetic matrix of plants (including divergence times between species) was obtained from the TimeTree.org Database. The constructed phylogenies contain all the species included in our research project.

(((Capsicum_annuum:21.33925000,(Solanum_lycopersicum:7.08005000,Solanum_tuberosum:7.08005000)'14':14.25920000)'13':103.78035000,(((((Brassica_juncea:0.09165000,Brassica_rapa:0.09165000)'25':12.48925000,Brassica_nigra:12.58090000)'37':3.56623000,Brassica_oleracea:16.14713000)'36':4.44006000,Arabidopsis_thaliana:20.58719000)'35':100.77645000,((Cucumis_sativus:102.61000000,Rosa_sp.:102.61000000)'34':10.50942000,((Phaseolus_lunatus:7.71295000)'43':37.30881000,Vicia_faba:45.02176000)'33':68.09766000)'51':8.244220000)'50':3.75596000)'49':34.49316000,((Saccharum_officinarum:16.31763000,Zea_mays:16.31763000)'57':107.14192000,Cocos_nucifera:123.45955000)'60':36.15321000);



Tables

Table S3. Equations used to calculate the Odds Ratio (OR) [31] and its respective sampling variance and confidence intervals (LL - lower limit, UL - upper limit) [32]. A: single-infested plant. B: multiple-infested plant. C and D: 50% chance of random choice.

Odds Ratio			
$OR = \frac{AD}{BC}$	*eqn 1*		
Sampling variance		**Sampling error**	
$V_{\ln(OR)} = \frac{1}{A} + \frac{1}{B} + \frac{1}{C} + \frac{1}{D}$	*eqn 2*	$SE_{\ln(OR)} = \sqrt{V_{\ln(OR)}}$	*eqn 3*
Confidence Intervals			
$LL = \ln(OR) - 1.96 \times SE_{\ln(OR)}$	*eqn 4*	$UL = \ln(OR) + 1.96 \times SE_{\ln(OR)}$	*eqn 5*

Table S4. Results from all multi-level meta-analytic models run. Categories represent estimates from each meta-analytic model run for each subgroup. Specialist NE's (parasitoid or predator) response to plants infested with a host and a nonhost of distinct feeding guilds vs. plants infested with host. Generalist NE's (parasitoid or predator) response to A- plants infested with two host of distinct feeding guilds vs. plants infested with a host, B- plants infested with two host of the same feeding guild vs. plants infested with a host. Specialist NE's response to plants infested with host and nonhost vs. plants infested with nonhost. Effect sizes in bold are considered statistically significantly different from 0, as the 95% credible interval did not overlap 0. k -number of effect sizes, n -number of NEs species, I^2 -heterogeneity. Results that showed a positive effect sizes (log OR) indicated NEs preference for single-infested plants, while negative effect sizes indicate NEs preference for multiple-infested plants. All models were fitted using the rma.mv function from the metafor package.

Categories	Analysis log (OR)						Moderator	Egger's Regression				Heterogeneity (%)	
	k	n	Estimate	Lower 95% CI	Upper 95%CI	*P*	*P*	Estimate	± SE	t value	*P*		
												I^2 total	58.72
Specialist NE	37	9	0.07	-0.38	0.52	0.77						**Plant phylogeny**	33.38
								-0.01	0.16	-0.09	0.93	**NE phylogeny**	12.25
Specialist Parasitoid	26	7	0.17	-0.39	0.72	0.55	0.51					**Herbivore species**	< 0.01
Specialist Predator	11	2	-0.05	-0.66	0.55	0.86						**Study identity**	13.09
Generalist NE	33	14	-0.22	-0.57	0.13	0.22						**I^2 total**	64.45
Generalist Parasitoid	8	3	-0.02	-0.58	0.54	0.95	0.39					**Plant phylogeny**	< 0.01
Generalist Predator	25	11	-0.32	-0.73	0.09	0.13		-0.28	0.16	-1.78	0.08	**NE phylogeny**	9.80
Different guild	15	8	-0.44	-0.88	<0.01	0.05	0.13					**Herbivore species**	54.65
Same guild	18	6	-0.10	-0.46	0.27	0.60						**Study identity**	< 0.01
Specialist NE	24	2	-0.81	-1.08	-0.53	**<0.01**		-0.68	0.33	-2.02	0.06	**I^2 total**	30.99

	Plant phylogeny	< 0.01
	NE phylogeny	< 0.01
	Herbivore species	< 0.01
	Study identity	30.99

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ARTIGO 2

Normas do periódico Ecological Frontiers (versão preliminar)

Defense responses in coffee plants: species-specific effects of single and multiple phloem-feeding infestation on tritrophic interactions

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Abstract

Plant responses to herbivory are influenced by both the number and identity of herbivores involved. Herbivory by multiple phloem-feeding insects can trigger distinct plant defense responses compared to single infestation. This study examines how colonization by *Coccus viridis* (green scales) and *Planococcus citri* (citrus mealybugs) affects host selection, herbivore performance, and induced plant defenses in *Coffea arabica*, as well as their attractiveness to the ladybug predator *Cryptolaemus montrouzieri*. While initial infestation by either herbivore did not influence subsequent host selection, co-infestation significantly reduced mealybug performance, likely due to enhanced salicylic acid (SA) signaling. Mealybug infestation induced higher levels of phytohormones, including jasmonic acid (JA) and its derivatives (JA-Ile, OH-JA, COOH-JA-Ile), as well as increased concentrations of non-volatile metabolites such as caffeine, catechin, and neochlorogenic acid, compared to green scale infestation. Multiple infestation altered volatile organic compound (VOC) profiles, reducing the attraction of ladybugs to plants infested by both herbivores. Green scales suppressed JA derivatives through SA expression, while mealybugs uniquely activated both SA and JA pathways, disrupting the typical JA-SA antagonism observed in phloem-feeding herbivores. This dual activation shaped ecological interactions, with mealybugs playing a dominant role in plant defense induction despite experiencing reduced performance in multiple-infested plants. These findings highlight the complexity of plant defense mechanisms in multiple infestation systems and underscore species-specific interactions that shape plant-herbivore-natural enemy dynamics. By improving our understanding of plant resistance strategies, this study provides valuable insights for ecological and agricultural applications.

Keywords: natural enemy, plant volatiles**,** cross-talk, herbivory competition, secondary metabolites, predator, coccoidae.

Introduction

Plants are commonly attacked by multiple herbivore arthropods simultaneously and must defend themselves against these attacks to survive. Plants have evolved constitutive and inducible defenses mechanisms that provide resistance against herbivores. Constitutive and induced defense defenses rely on the synthesis of secondary metabolites, which are present in plants as regulators of growth and development or are inducible in response to herbivore attacks [1]. Plants attacked by herbivores can produce toxic, deterrent or anti-nutritive compounds that directly affect the physiology and/or behavior of insect herbivores [2,3]. Additionally, they can emit a distinct blend of volatile organic compounds (VOCs), known as herbivore-induced plant volatiles (HIPVs), that attract natural enemies of the herbivores [4,5].

Plants recognize the herbivore attack or the imminence of herbivory by herbivore-associated cues, such as enzymes in herbivore saliva, frass, honeydew, oviposition secretions and volatiles emitted by neighboring plants [6,7]. Herbivory activates specific metabolic pathways responsible for synthesizing defense compounds [8,9]. The synthesis of herbivore-induced plant defenses is mainly regulated by the orchestration of phytohormones, such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and ethylene. In particular, the activation of the JA signaling pathway plays a crucial role in augmenting herbivore-induced defenses against herbivores in general. These hormonals signaling pathways appear to be modulated according to the herbivore's feeding modes, ensuring a tailored response to the attacking herbivore [6,10,11]. For example, plant damage inflicted by chewing insects typically activates the jasmonate signaling pathway [6,12], whereas phloem-feeding insects trigger the salicylate pathway [6,13]. These two pathways can act antagonistically; the accumulation of SA may inhibit the JA signaling pathway, rendering plants more susceptible to insect attack [14,15]. This negative cross-talk between SA and JA has been identified as a strategy employed by phloem-feeding insects, such as aphids and mealybugs, to suppress JA-dependent plant defenses [16,17], facilitating their feeding and establishment. Consequently, this suppression can favor the colonization by herbivores from different guild (e.g., chewers), in a density-dependent manner [18–21].

In natural settings, plants often experience co-infestation by different herbivores, leading to complex ecological interactions. Co-occurring herbivores may avoid competition by temporal displacement or altering spatial distribution on the plant [22–25]. The outcomes of such interactions seem to depend on herbivore feeding guilds, order of arrival, and herbivore density [18,26–28]. However, while the dominant herbivores may improve their own

performance by suppressing plant defenses, this suppression can limit the performance of subsequent herbivores [28–31]. Asymmetrical facilitation can be observed among phloem-feeding insects that share the same plant, with only one species negatively affecting the performance of a later-arriving species [17,28–30,32]. Co-infestation by multiple phloem-feeding insects can induce distinct chemical defense profiles in plants, including alterations in production of phytohormones, secondary metabolites, and volatile organic compounds (VOCs) [29,30,33]. These herbivore dynamics influence plant cascade responses, including HIPVs and their attractiveness to natural enemies, potentially affecting their foraging efficiency in the presence of herbivores from different guilds or a non-host species [34–37].

Natural enemies associate specific HIPVs with the presence of their hosts, enhancing their foraging efficiency for reliable cues [38,39]. The profile of HIPVs varies depending on the identity of the herbivores, as well as the species, age, and physiological state of the plant [5,40–42]. This variation leads to the emission of qualitatively distinct volatile blends and phytohormone activation level [41,43]. The presence of multiple herbivores can modify the composition of HIPV blends [22,44–46], and the presence of non-host may negatively affect the foraging of some natural enemies to locate their host [34,36,37]. However, natural enemies do not necessarily lose their ability to locate host in multiple-infested plants, they remain attracted to the HIPVs produced by their specific host or prey [47], suggesting stable chemical communication through search template composition despite the complexity of herbivore communities [39,47,48]. Conversely, the presence of multiple hosts herbivores does not necessarily result in improved attraction of natural enemies [45,47,49,50].

Coffee (*Coffea arabica* L.) (Rubiaceae), a crop of significant economic importance, is chemically rich and contains a diverse array of secondary metabolites, including alkaloids, flavonoids, terpenoids, xanthone compounds and phenolic compounds [51–53]. Coffee plants are susceptible to the infestation of various herbivores, which are considered pests in the agronomic context. Among phloem-feeding insects, members of the superfamily Coccoidea, such as the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) and the green scale *Coccus viridis* (Green) (Hemiptera: Coccidae), are notable pests [54–56]. These insects cause both direct and indirect damage to coffee plants, including stunting, virus transmission, defoliation, and reduced photosynthetic efficiency [57,58]. Their dispersal to select feeding site on the plant occur along nymphal stages (N1 and N2) with greater mobility, once settled they exhibit a sedentary lifestyle during their adult female stage [57,59,60]. In addition, *P. citri* produces a protective wax layer that covers its body and ovisacs [61]. Similar

to other hemipterans, the mealybug employs detoxifying enzymes in its saliva, and harbors symbiotic microorganisms that facilitate feeding [62–64]. Previous studies have demonstrated that infestation by this mealybug induces accumulation of both JA and SA in coffee plants, rendering plants more resistant to subsequent attack by the red spider mite *Oligonychus ilicis* [34]. Additionally, the sedentary behavior of these phloem-feeding insects, combined with continuous feeding, leads to honeydew accumulation on leaves, promoting sooty mold development [65]. The honeydew deposit by itself induces plant defense responses, act as a chemical signal from the host, and exhibit chemical properties that serve as host cues for natural enemies [35,66,67].

In the case of *Co. viridis*, honeydew accumulation promotes the growth of sooty mold, forming black superficial colonies that significantly reduce photosynthetic activity [68]. Green scales have been reported to inject toxins into vascular tissues, leading to phloem cell rupture and significant alterations in coffee plant metabolism [54,57,69]. Interestingly, certain coffee plant compounds, such as chlorogenic acid and caffeine, stimulate the locomotion of green scales without significantly increasing mortality, potentially enhancing their dispersal [70,71].

Cryptolaemus montrouzieri Mulsant (Coleoptera: Coccinellidae) is a coccidophagous predator widely introduced for the biological control of mealybugs, with a broad prey range [72–74]. *Cryptolaemus montrouzieri* has demonstrated the ability to detect plant volatiles associated with prey infestation [75] and to distinguish between plants infested by its prey and those infested by non-prey species [34]. The presence of non-prey herbivores, such as mites, on plants co-infested with mealybugs resulted in a distinct repellent effect on *Cr. montrouzieri*. The ladybug exhibited a clear aversion to the volatile blend emitted by presence of non-prey arthropods [34]. This suggests that the herbivory of multiple species can disrupt the attraction of *Cr. montrouzieri*, hindering its ability to locate mealybugs effectively, which ultimately reduces the predator's efficiency in controlling mealybug populations.

This study investigated the performance of two phloem-feeding sharing coffee plants, focusing on the cascade effect on the induction of chemical defense (phytohormones metabolites, volatiles and non-volatiles) and the tritrophic interaction with the mealybug’s predator, *Cr. montrouzieri*. The study explores four key hypotheses related to these interactions: (1) herbivory by phloem-feeding insects facilitates the colonization and establishment of a subsequent non-conspecific phloem-feeding insect by activating the salicylate signaling pathway and suppressing the jasmonate signaling pathway due to antagonistic interactions, which results in unaltering levels of herbivore-induced plant defenses; (2) multiple infestation

by the two phloem-feeding insect species in coffee plants has synergistic effects on herbivore-induced defenses compared to single infestation; (3) the ladybug prefers volatile blends emitted by plants infested with its preferred prey; and (4) the predatory ladybug is equally attracted to volatiles from plants infested by two phloem-feeding herbivores and plants infested by a single prey.

Materials and Methods

Plants and insects

Seeds of *C. arabica* cv. Mundo Novo were planted in polyethylene bags (20 cm x 10 cm) filled with a mixture of soil, commercial substrate (Tropstrato HA - Vida Verde), and sand in a 2:1:1 ratio, respectively. The plants were kept in a greenhouse (Lavras, MG, Brazil), under natural fluctuations of temperature and light (July 2021 through January 2022 and June 2022 through December 2023). All bioassays were conducted using coffee plants aged between 7 and 9 months. Founding colonies of the citrus mealybug *P. citri* were collected from cocoa plants (*Theobroma cacao* L.) in Lavras, MG, Brazil. The green scales *Co. viridis* were collected from coffee plants in Lavras, MG, Brazil. These colonies were maintained in the laboratory on sanitized pumpkin fruits (*Cucurbita maxima* L. cv. Cabotchá), which were clean with neutral soap and sodium hypochlorite and kept in cages. The *Cr. montrouzieri* stock colony started with ~500 individuals obtained from rearing facility at Embrapa Mandioca e Fruticultura (Cruz das Almas, BA, Brazil). The ladybugs were kept in 20 L transparent plastic containers with side openings covered with voile to allow air circulation. Their diet consisted of *P. citri*, provided on pumpkins that had been infested for one month in the laboratory rearing. All insects' colonies were kept at controlled temperature and light (25 ± 2°C, 70 ± 10% RH, 12 h light: 12 h dark).

Plant treatments

Mealybug-infested plants were obtained by infesting the mid-third leaf pair of coffee plants with 50 mealybugs (nymphs and adults) collected from the pumpkins in the rearing [76]. Therefore, to obtain green scale-infested plants, a leaf from plants in the rearing containing about 50 green scales (nymphs and adults) was clipped to the mid-third leaf pair of the coffee plant. Unlike mealybugs, green scales are much less mobile and typically keeps its stylets inserted into the leaf unless the leaf dries out [70]. After approximately 20 days, the green scales colonized the coffee plants. Since neither green scales nor mealybugs exhibited a preference for infesting plants already colonized by the others (see results for dual choice assays), multiple-

infested coffee plants received both mealybugs and green scales simultaneously using the same methods as for single infestation (Figure 1A). To prevent insects from escaping, each infested plant was individually covered with fine-mesh fabric bags and the plants were used approximately 30 days after infestation. Uninfested plants were also bagged but kept insect-free in the same room as the infested treatments for the same duration.

Host preference by phloem-feeding insects

To evaluate whether herbivory by non-conspecific phloem-feeders hinders or facilitates colonization by the other, a two-choice assay was conducted to assess the preference of mealybugs and green scales for uninfested and infested coffee plants (Figure 1B). Without excising it from the plant, a single leaf from the mid-third leaf pair on each plant was isolated from the petiole using a 1:1 mixture of entomological glue and lanolin, preventing tested insects from spreading across the plant. This leaf served as the connection point for the insect species to choose between the two highlighted plants. To evaluate mealybug host preference, a detached coffee leaf infested with approximately 50 mealybugs (nymphs and adults) was clipped to the isolated leaf of both an uninfested coffee plant and a scale-infested plant, positioned on opposite sides. Twenty replicates were performed in the greenhouse (under natural conditions of temperature, light and humidity). The scale host preference for uninfested and mealybug-infested coffee plants was assessed using a similar experimental setup, but the detached coffee leaf clipped to the leaf of each plant carrying approximately 50 green scales (nymphs and adults). Twenty-five replicates were performed in the greenhouse (under natural conditions of temperature, light and humidity). The number of insects moving to each plant was recorded by counting the individuals present on each plant after 3, 6, and 9 days.

Phloem-feeding insects' performance

The performance of phloem-feeding insects, both in co-infestation and alone, was evaluated by counting the number of individuals on the plants after 30 days from infestation. The mid-third leaf pair of coffee plants were initially infested with 50 mealybugs (nymphs and adults) or using leaves with about 50 green scales (nymphs and adults), following the infestation method previously described. For assessing the performance of the two phloem-feeding insects in co-infestation, coffee plants were infested with the same numbers as in the single-infestation treatments (Figure 1C). Twelve replicates were performed in the greenhouse (under natural

conditions of temperature, light and humidity). The plants were then enclosed with fine-mesh fabric bags to prevent the insects from escaping, and after 30 days of infestation, the numbers of nymphs and adults of each species present on plants were counted.

Phytohormones and Target analysis of secondary metabolites

The fourth leaf pair from uninfested, mealybug-infested, scale-infested and multiple-infested coffee plants (mealybug and scale infestation) were collected 30 days after insect infestation. The leaves were thoroughly cleaned using cotton moistened with distilled water to remove any adhering insects and immediately froze in liquid nitrogen. They were lyophilized for 27 hours at -45 ºC in a freeze dryer (L101, LIOTOP, LIOBRAS, São Carlos, Brazil). The dried leaves were ground into a fine powder and stored in microtubes. For analysis of putative defensive secondary metabolites, an aliquot of 50 mg of freeze-dried leaf tissue was diluted in 500 µL of 99.8% methanol (Fisher Scientific, UK). The mixture was subjected to a sonication bath for 25 minutes to facilitate the extraction of phytochemicals. After sonication, the sample was centrifuged at 30,000 rpm for 10 minutes to separate the solid residues from the liquid extract. The supernatant was filtered through glass fiber to remove any remaining particulate matter. To standardize different filtered volumes, the sample was concentrated using a nitrogen concentrator (Blowdown Evaporator). Following concentration, 500 µl of methanol was added, and the mixture was sonicated for an additional 25 minutes. A 3 µL aliquot of the final solution was injected into the LC-MS system for analysis. A caffeine standard solution at 100 ng. μL^{-1} was used as an external standard. The mass spectra of coffee plant metabolites were compared with the authentic standard available (caffeine), those reported by [51] and in the PubChem library [77]. Individual compounds were quantified by the peak area relative to the external standard and corrected by the sample weight. LCMS analysis was carried out using an Acquity ultra-high-pressure liquid chromatography (UPLC) system coupled to a Synapt G2Si Q-Tof mass spectrometer with an electrospray ionization source (Waters, UK). The system was controlled through Masslynx 4.1 software (Waters). The mobile phase consisted of solvent A (0.01% formic acid v/v, water) and solvent B (0.01% formic acid v/v in methanol 99,8% (Fisher Scientific, UK) (Cambon et al 2023).

For the phytohormonal analyses, a 30-mg aliquot of freeze-dried leaf tissue was used. A total of 1.5 ml of methanol and 4 µl of internal standard (D6JA, D4SA, D6ABA: 10 ng. μl^{-1}, and D6-JA-Ile: 2 ng. μl^{-1} in MeOH) were added to each sample, which was shaken for 30

minutes and centrifuged at 13,000 rpm at 4°C for 20 minutes. The supernatant was transferred to new 2 ml Eppendorf tubes on ice. The pellet from the first extraction was resuspended in 500 µl of methanol, shaken, and centrifuged again. The resulting supernatant was combined with the first extract. The supernatant was concentrated by vacuum centrifugation. After concentration, 500 µl of methanol was added, and the mixture was vortexed and centrifuged at 16,000 rpm at 4°C for 7 minutes. A 400 µl aliquot of the supernatant was collected for analysis. Phytohormone analysis was performed by LC-MS/MS as described in Heyer et al. (2018), using an Agilent 1260 series HPLC system (Agilent Technologies) coupled to a QTRAP 6500 tandem mass spectrometer (SCIEX, Darmstadt, Germany). Chromatographic separation was carried out on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies), with water containing 0.05% formic acid (mobile phase A) and acetonitrile (mobile phase B). The elution profile was: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min, 90–100% B; 4.02–4.5 min, 100% B; 4.51–7.0 min, 10% B. The flow rate was 1.1 ml/min, and the column temperature was maintained at 25°C. The mass spectrometer was operated in negative ionization mode with a Turbo Spray ion source. The ion spray voltage was -4,500 eV, and the turbo gas temperature was set to 650°C. Nebulizing gas was set at 60 psi, curtain gas at 40 psi, heating gas at 60 psi, and collision gas at "medium". Multiple reaction monitoring (MRM) mode was used. Since both D6-labeled JA and D6-labeled JA-Ile standards contained 40% D5-labeled compounds, the peak areas of both D5- and D6-compounds were summed for quantification.

Prey preference of *Cr. montrouzieri*

The predator preference for consuming mealybugs and green scales was assessed using both non-choice and dual-choice assays (Figure 1D). In the non-choice assay, a single adult female ladybug, previously isolated from the laboratory colony and starved for 48 hours, was introduced into the center of a rectangular plastic arena (24 × 15 cm) containing an excised coffee leaf carrying 50 individuals (nymphs and adults) of either mealybugs (17 replicates) or green scales (23 replicates), placed at the center of the arena. In the dual-choice assays, the setup was similar except that two coffee leaves were positioned on the opposite sides of the arena – one bearing mealybugs and the other green scales individuals – allowing *Cr. montrouzieri* female to choose between them when introduced at the arena center. Twenty replicates were performed in the laboratory at controlled temperature and light (25 ± 2°C, 70 ± 10% RH, 12 h light: 12 h dark). The ladybugs were removed after 24 hours, and the number of insects consumed was estimated by subtracting the remaining mealybugs and green scales from

the initial numbers at the start of the assay. In the dual-choice assay, the proportion of each phloem-feeding insect consumed was estimated relative to the total of insects provided.

Olfactory preference of *Cr. montrouzieri* to plant volatiles

The olfactory preference of *Cr. montrouzieri* females for volatiles emitted from uninfested and infested coffee plants was evaluated in Y-tube olfactometry bioassays. A glass Y-tube olfactometer with equal side arms (15 cm long and 4 cm in external diameter) was vertically positioned in a cardboard box (Figure 1E). The vertical approach was applied due to the negative geotropism [34] whereas the box aimed at minimizing visual cues. Coffee plants were individually enclosed in polyester bags (41 cm x 33 cm) with openings at the top to connect the airflow tubes. To eliminate any potential visual stimuli for the ladybugs, the plant bags containing them were covered with white paper. Charcoal-filtered and humidified air was pulled from the room into the olfactometer system by a pump at a flow rate of 1.5 $L.min^{-1}$.

Prior to assays, 10-days-old adult female ladybugs were individualized from the laboratory colony and starved for 24 hours. Each female was then released into the central arm of the Y-tube, and its choice was recorded when it crossed a line located in the distal third of one of the lateral arms of the olfactometer (10 cm from the center) within a 5-min period. Each insect was tested only once. Insects that did not make a choice within the 5 min, was considered as “non-responsive" and excluded from the analysis. To avoid directional bias (left/right), the placement of the treatments was switched after each trial. Additionally, to reduce bias by low- and/or non-volatile chemical cues left by the ladybugs on the surface of the glass (insect footprints), Y-tube olfactometer was replaced with a clean unit after every five insects tested. The bioassays were performed in the laboratory at controlled temperature and light (25 ± 2°C, 70 ± 10% RH). The olfactory preference of ladybugs was assessed for the following types of infestation (=treatment combinations): (i) uninfested plant *vs.* mealybug-infested plant; (ii) uninfested plant *vs.* scale-infested plant; (iii) scale-infested plant *vs.* mealybug-infested plant; (iv) multiple-infested plant *vs.* mealybug-infested plant; (v) multiple-infested plant *vs.* scale-infested plant; and (vi) multiple-infested plant *vs.* uninfested plant. For each assay, six plants pairs were used, and ten adult female ladybugs were tested per pair.

Plant volatiles

Volatile emissions from mealybug-infested, scale-infested, multiple-infested, as well as uninfested plants, were collected using a push-pull aeration system for eight hours during the photophase (from 8:00 a.m. to 4:00 p.m.) in the laboratory. Plants were maintained under artificial light (UV LED Full Spectrum Grow Light, 2,000 lux). Each plant was enclosed in polystyrene bags (41 cm x 33 cm) and connected to the aeration system with an incoming airflow of 0.5 L/min, filtered through activated charcoal and a triple filter (TRIO-02, ARFUSION, Americana, São Paulo, Brazil). Volatiles were captured using thin glass tubes filled with 30 mg of HayeSep-Q® adsorbent polymer (Hayes Separation Inc., Bandera, TX, USA), connected to the bags with the plants. Air was drawn through the filters at 0.25 L/min using a vacuum pump (SKC Pocket Pump TOUCH, SKC Ltd., Blandford Forum, UK). The adsorbent filters were eluted with 100 µL of hexane (Sigma Aldrich, St. Louis, USA). A 2 µL volume of a 10 ng/µL solution of nonyl acetate was added to each sample as an internal standard. The extracts were stored in sealed glass vials at -20°C freezer.

A 2-µL aliquot of each extract was injected at 250 °C into a gas chromatograph with a flame ionization detector (GC-FID; Shimadzu GC-2010, Shimadzu Corp., Kyoto, Japan) equipped with an Rtx-1 column (30 m × 0.25 µm × 0.25 mm; RESTEK, Bellefonte, PA, USA) using helium as the carrier gas at a linear velocity of 30.3 cm/sec. The column temperature was held at 40 °C for 5 minutes, then increased to 150 °C at 5 °C/min, and finally raised to 250 °C at 20 °C/min and held for 30 minutes.

A 2-µL aliquot of each representative sample was also run in a gas chromatograph coupled to a mass spectrometer (GC-MS; GCQP-2010 Ultra, Shimadzu Corp., Kyoto, Japan) equipped with an Rtx-1MS column (30 m × 0.25 µm × 0.25 mm; Restek, Bellefonte, PA, USA), using a similar method to the GC-FID. The quadrupole ion source and transfer line were maintained at 250 °C for electron impact analysis at 70 eV (35–300 m/z). Total volatile emission was estimated by summing all peak areas in the GC-FID chromatogram, and individual compounds were quantified as relative percentage areas. The mass spectra and Kovats indices (using alkanes C7-C30) of the compounds were compared with those in the NIST11 library and available synthetic standards for compound identification.

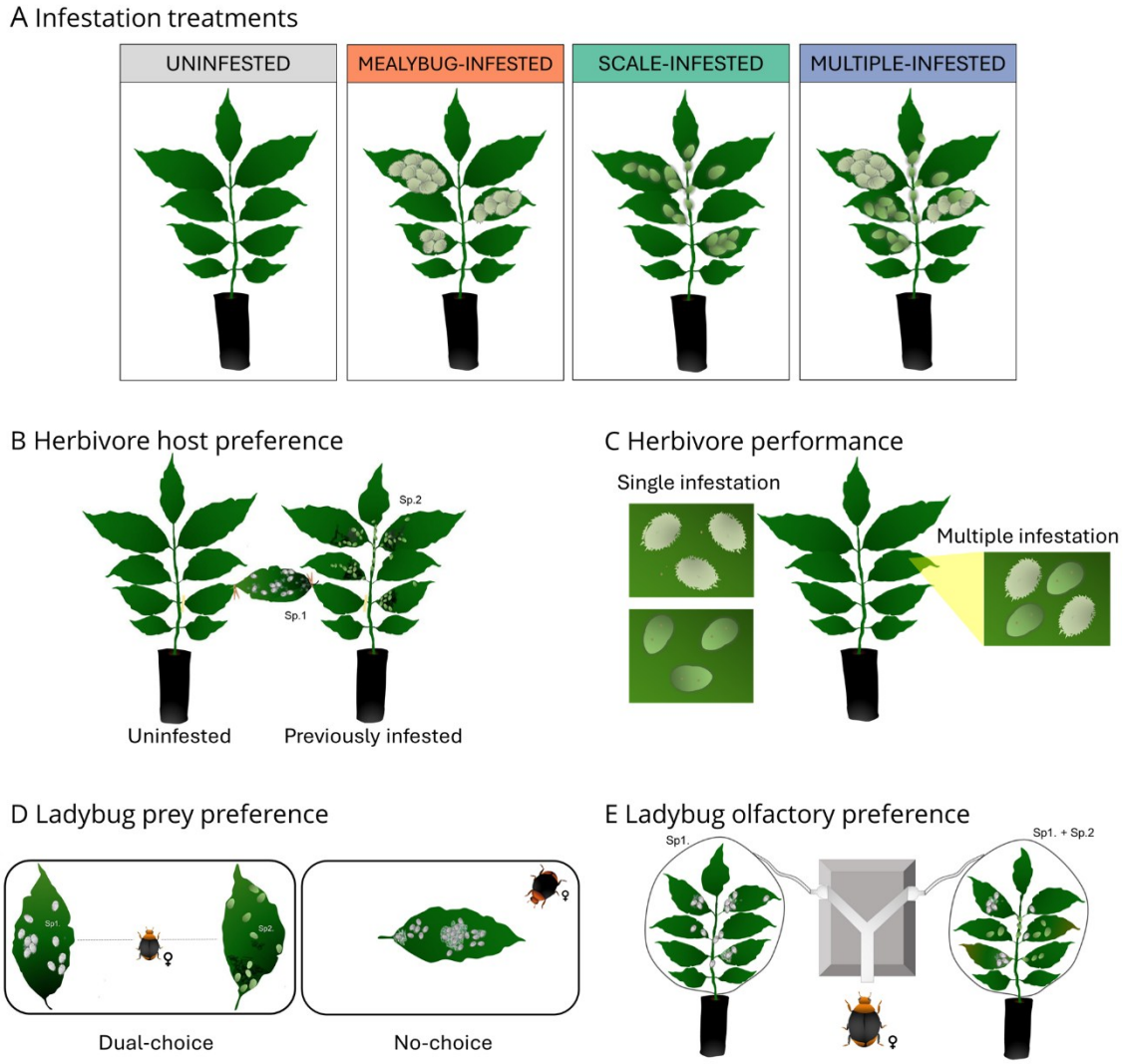

Figure 1. Schematic overview of the experimental bioassay. (A) Illustrative summary of coffee plant infestation treatments: uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (scale+mealybug). (B) Phloem-feeding insect preference between uninfested and interspecifically infested *Coffea arabica* plants. (C) Phloem-feeding insect colonization performance on uninfested plant (single infested) and multiple-infested plants (multiple infested). (D) Female ladybug (*Cryptolaemus montrouzieri*) prey preference in a dual-choice assay and a non-choice assay arena. (E) Ladybug HIPVs olfactory preference in a Y-tube olfactometer, between different levels of phloem-feeding insect coffee plant infested. Sp.1 *Planococcus citri*, Sp.2 *Coccus viridis*.

Data analyses

Host preferences were analyzed using generalized linear mixed models (GLMM) with a *Poisson* distribution, employing the *glmer* function from the *lme4* package. Overdispersion was assessed using *check_overdispersion* function from the *performance* package [79]. When overdispersion was detected, models were refitted using GLMM *negative binomial* distribution. Model significance was evaluated using AIC comparison. Phloem-feeding insect performance was analyzed using generalized linear models (GLM) with a *Poisson* distribution, with model significance assessed by comparison to a null model. Overdispersion was checked using Half-Normal Plot (HNP) envelope plots for deviance residuals, using the *hnp* function from the *hnp* package [78] and *check_overdispersion* function from the *performance* package [79]. If detected, data were analyzed using *quasipoisson* or *negative binomial* distribution, using the *glm.nb* function in the *MASS* package. For ladybug prey preference assays, non-choice test data were modeled using GLM with a *Poisson* distribution, while dual-choice test data were analyzed using GLM with a *binomial distribution*. Choice data on the olfactometer assays were analyzed using GLMM with a *binomial* distribution, employing the *glmer* function from the *lme4* package. Plant pairs were included as a random factor to account for pseudoreplication. Volatile composition and the leaf secondary metabolites profiles were compared across treatments using multivariate approaches. Data were standardized using log-transformation and pareto scaling [80]. Differences in volatile composition between groups were analyzed using permutational multivariate analysis of variance (PERMANOVA) based on a Euclidean distance matrix (999 permutations). Permutational multivariate within-group dispersion analysis (PERMDISP) was performed using the *adonis* and *betadisper* functions from the *Vegan* package. Post-hoc pairwise comparisons were conducted using the *pairwise.adonis2* function from the *pairwiseAdonis* package. Principal component analysis (PCA) was performed using the *prcomp* function from the *stats* package. The phytohormone levels, individual volatile components, and leaf secondary metabolites were compared using ANOVA with log-transformed data. Post-hoc multiple comparisons were conducted using Tukey's Honest Significant Differences (HSD) test, implemented via the *TukeyHSD* function from the *stats* package. All analyses were conducted using *R* software version 4.4.1 [81].

Results

Host preference by phloem-feeding insects

Prior colonization by one phloem-feeding insect did not affect plant's attractiveness to the other species. The scale did not show preference between uninfested plants and those infested by mealybugs (χ^2 = 0.65, df = 1; p = 0.42, Figure 2A). Similarly, mealybugs exhibited no preference between uninfested plants and those infested by green scales (χ^2 = 0.58, df = 1; p = 0.44, Figure 2B).

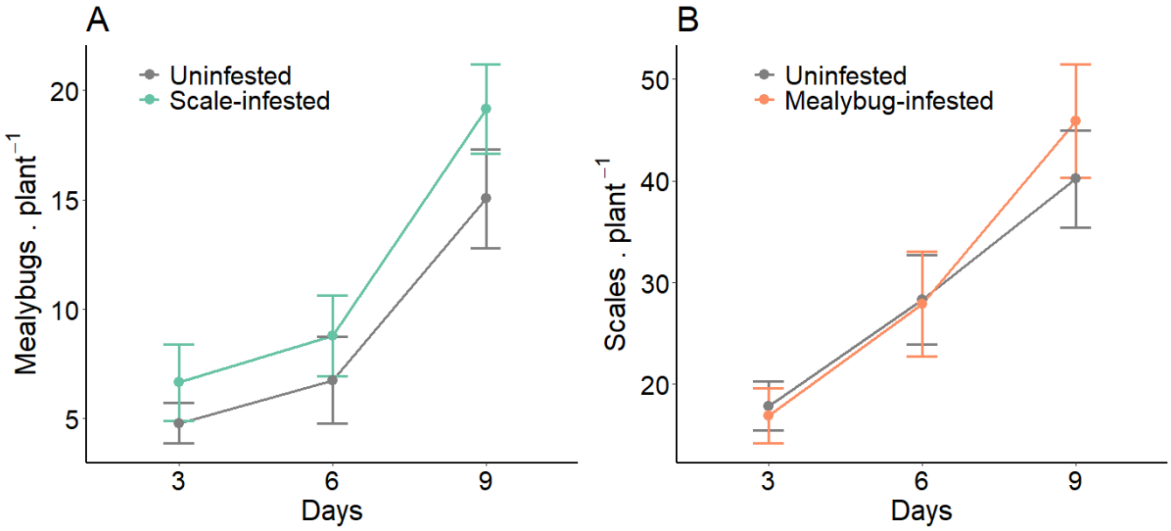

Figure 2. Host preference of phloem-feeding insects in dual-choice assays for uninfested *versus* heterospecific-infested *Coffea arabica* plants over time. A) mealybug (*Planococcus citri*); B) Scales (*Coccus viridis*). Dots represent the mean numbers ± SE of green scales or mealybugs on the plant treatment at a time point.

Phloem-feeding insects' performance

The performance of mealybugs was poorer on coffee plants co-infested by green scales compared to uninfested plants. There were approximately 22% more adults and 24% more nymphs of mealybugs on uninfested plants than on plants co-infested with green scales (adults: χ^2 = 6.33, df = 1,12; p = 0.01, nymphs: χ^2 = 3.99, df = 1,22; p = 0.04, Figure 3A).

On the other hand, the presence of mealybugs did not affect the performance of green scales on coffee plants. The number of adult green scales showed no significant variation, with a slight reduction of approximately 6% on plants co-infested with mealybugs compared to uninfested plants (χ^2 = 0.40, df = 1,22; p = 0.84, Figure 3B). Similarly, the number of nymph

green scales was statistically similar between uninfested plants and those multiple-infested with mealybugs ($\chi^2 = 3.33$, df = 1,22; $p = 0.07$, Figure 3B).

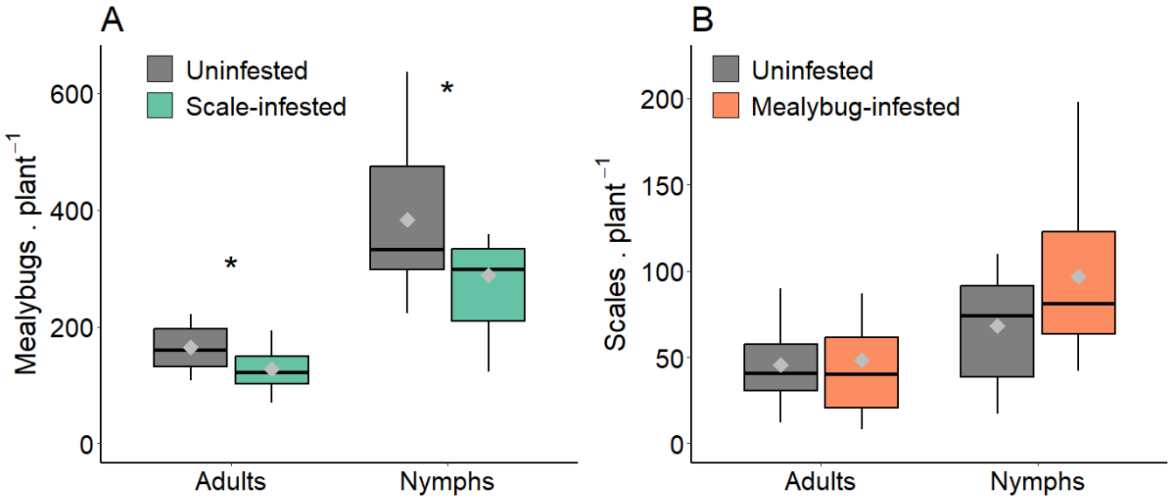

Figure 3. Performance of phloem-feeding insects in *Coffea arabica* plants multiple-infested with heteroespecifics or uninfested. A) mealybug (*Planococcus citri*); B) Scales (*Coccus viridis*) performances based on numbers of adults and nymphs found on coffee plants after 30 days of infestation. * Indicates significant differences according to Tukey's Test ($p < 0.05$).

Phytohormones

Even though the amounts of cis-(+)-12-oxophytodienoic acid (cis-OPDA), the precursor of jasmonates, were similar across all treatments ($F_{3,39} = 1.33$, $p = 0.28$, Figure 4A), the levels of JA and its derivatives JA-Ile and COOH-JA-Ile were significantly greater in coffee plants infested with mealybugs or multiple-infested with green scales compared to uninfested and scale-infested plants (JA: $F_{3,39} = 111.30$, $p < 0.001$; JA-Ile: $F_{3,39} = 37.47$, $p < 0.001$; COOH-JA-Ile: $F_{3,39} = 15.75$, $p < 0.001$) (Figure 4, Table S1). Levels of OH-JA in coffee plants, in turn, were only affected by green scale infestation, showing a downregulation compared to the other treatments ($F_{3,39} = 4.62$, $p < 0.01$, Figure 4D). SA levels were upregulated in plants infested either with green scales or mealybugs compared to uninfested plants ($F_{3,39} = 4.14$, $p < 0.001$, Figure 4F), with no significant differences relative to multiple-infested plants. ABA levels were significantly reduced only by scale infestation compared to uninfested and the others infestation treatments ($F_{3,39} = 18.14$, $p < 0.001$, Figure 4G).

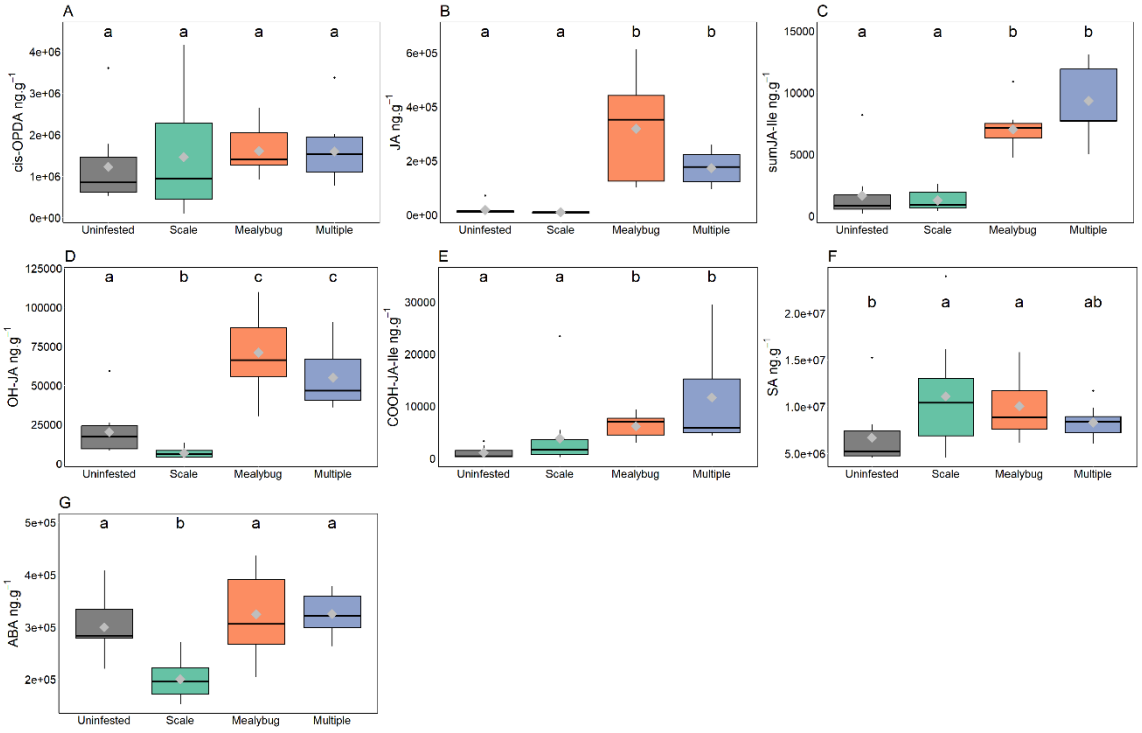

Figure 4. Levels (ng. g^{-1} dry weight) of phytohormones in leaves of uninfested, scale (*Coccus viridis*), mealybug (*Planococcus citri*), and multiple-infested (scale+mealybug) *Coffea arabica* plants. A) cis-12-oxo-phytodienoic acid (OPDA); B) jasmonic acid (JA); C) sum of jasmonic acid isoleucine (sumJA-Ile); D) hydroxylated jasmonic acid (OH-JA); E) jasmonoyl-isoleucine (COOH-JA-Ile); F) salicylic acid (SA); G) Abscisic-acid (ABA). Different letters above bars indicate significant differences according to Tukey's Test ($p < 0.05$).

Targeted analysis of secondary metabolites

Higher concentrations of caffeine ($F_{3,43} = 53.23$, $p < 0.01$, Figure 5A), catechin ($F_{3,43} = 16.34$, $p < 0.01$, Figure 5B), epicatechin ($F_{3,43} = 14.27$, $p < 0.01$, Figure 5C) and neochlorogenic acid ($F_{3,43} = 19.02$, $p < 0.01$, Figure 5E) were observed in plants infested with mealybugs and co-infested with mealybugs and green scales, compared to uninfested plants and those infested with green scales alone (Figure 5, Table S2). The presence of theophylline was exclusively detected in green scale-infested plants ($F_{3,43} = 15.55$, $p < 0.01$ Figure 5D). Additionally, higher levels of tiliroside were detected on multiple-infested plants ($F_{3,43} = 4.95$, $p < 0.01$ Figure 5F) compared to uninfested and scale-infested plants but were similar to levels in mealybug-infested plants. Significant compositional differences were observed across all treatments ($F_{3,43} = 17.01$, $R^2 = 0.54$, $p < 0.01$), with no significant variance within groups ($F_{3,43} = 1.27$, $p = 0.29$). However,

metabolite composition of mealybug-infested and multiple-infested plants were similar ($F_{3,43}$ = 2.55, R^2 = 0.11, p = 0.12, Table S3).

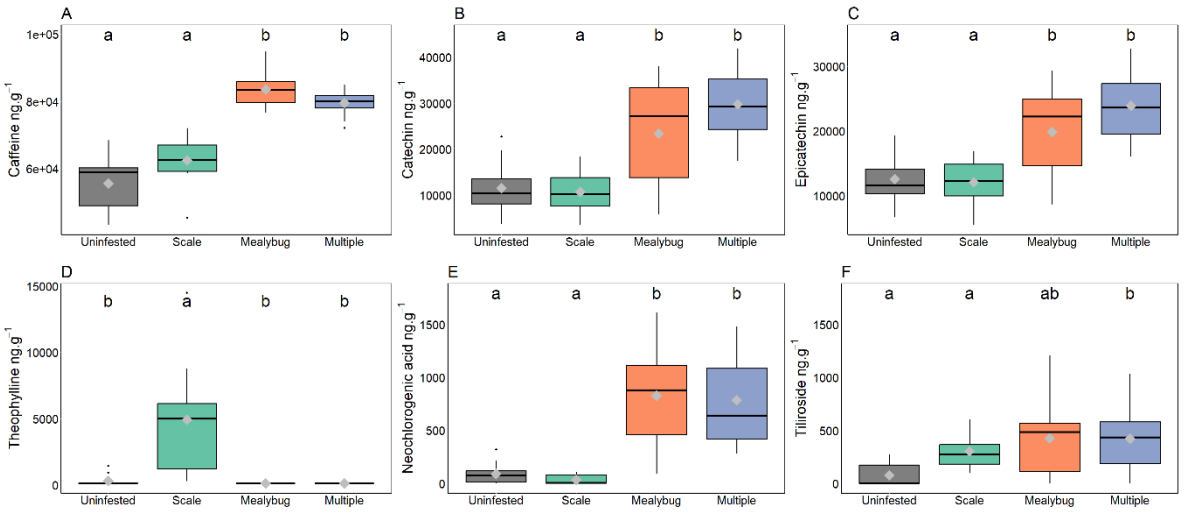

Figure 5. Amounts (ng. g^{-1} dry tissue) of secondary plant metabolites in leaves of: uninfested, scale (*Coccus viridis*), mealybug (*Planococcus citri*), and multiple-infested (mealybug + scale) infested *Coffea arabica* plants. A) Caffeine; B) Catechin; C) Epicatechin; D) Theophylline; E) Neochlorogenic acid; F) Tiliroside. Different letters above bars indicate significant differences according to Tukey's Test (p <0.05).

Prey preference of *Cr. montrouzieri*

The ladybug *Cr. montrouzieri* consumed similar numbers of mealybugs and green scales adults when provided separately (non-choice assay) (χ^2 = 1.18, df = 1,38; p = 0.28, Figure 6A). However, in the dual-choice assay, where ladybugs could choose between the two prey species, they showed a preference for consuming mealybugs (χ^2 = 4.00, df = 1,38; p = 0.04, Figure 6B). The mean consumption was 23.8 ± 1.98 SE mealybugs compared to 9.6 ± 1.7 SE green scales, with ladybugs preying 2.4 times more mealybugs than green scales.

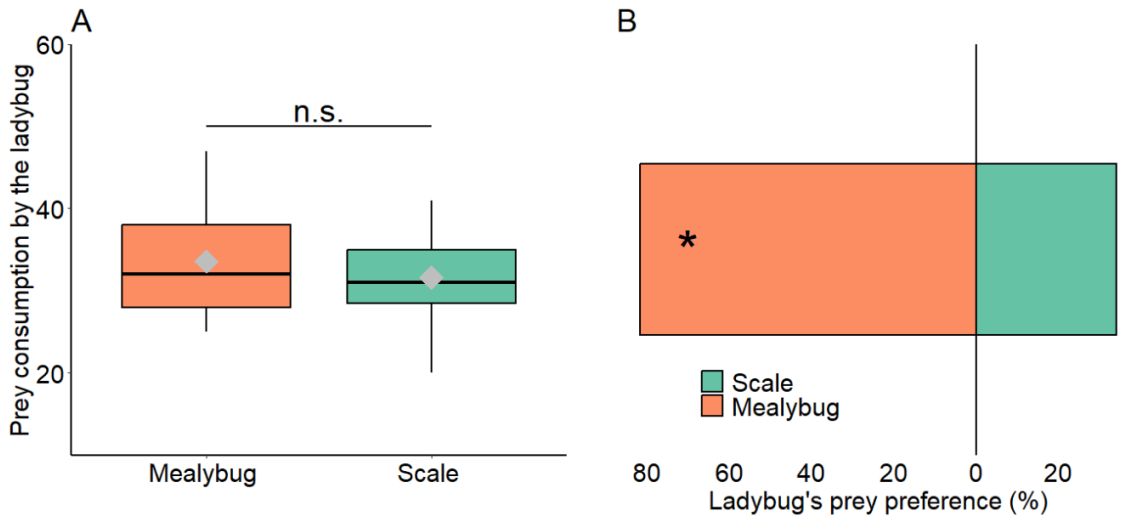

Figure 6 Prey preference of *Cryptolaemus montrouzieri* females for *Planococcus citri* (mealybug) and *Coccus viridis* (scale) in (A) a non-choice assay; (B) a dual-choice assay. 'n.s.' indicates no statistically significant difference between treatments, while * indicates significant differences according to Tukey's Test ($p < 0.05$).

Olfactory preference of *Cr. montrouzieri* to plant volatiles

In olfactometer assays, the ladybugs *Cr. montrouzieri* were more attracted to volatiles from plants infested with either *P. citri* (mealybug) or *Co. viridis* (scale) compared to those from uninfested plants (mealybug: $\chi^2 = 8.88$, df = 1,95; $p < 0.01$; green scales: $\chi^2 = 6.62$, df = 1,113; $p = 0.01$, Figure 7). However, when exposed to volatiles from mealybug-infested and green scale-infested plants, the ladybugs did not differentiate between the two treatments ($\chi^2 = 0.15$, df = 1,101; $p = 0.70$, Figure 7c). Volatiles from multiple-infested plants were less attractive to *Cr. montrouzieri* than those from mealybug-infested plants ($\chi^2 = 3.89$, df = 1,79; $p = 0.04$, Figure 7d). However, the ladybugs did not differentiate between volatile emissions from scale-infested and multiple-infested plants ($\chi^2 = 1.84$, df = 1,103; $p = 0.18$, Figure 7e). Similarly, *Cr. montrouzieri* showed no preference between volatiles from uninfested and multiple-infested plants ($\chi^2 = 0.08$, df = 1,107; $p = 0.85$, Figure 7f).

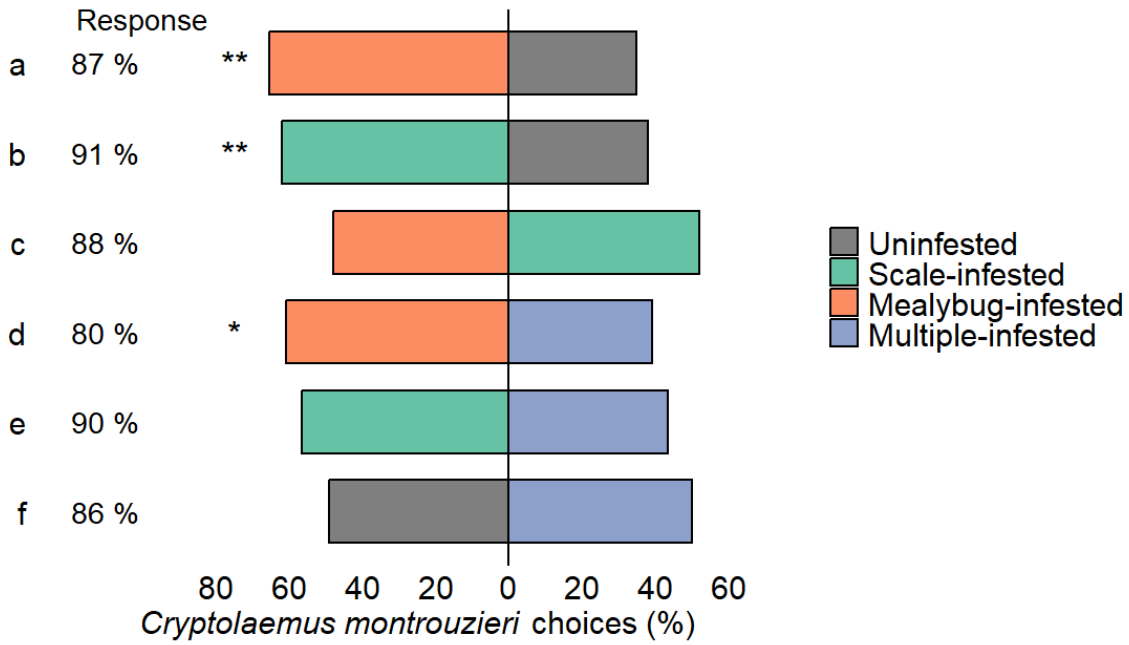

Figure 7. Olfactory preference of the predatory ladybug *Cryptolaemus montrouzieri* in two-choice olfactometer assays for volatiles emitted from uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) *Coffea arabica* plants. n = 6 plant pairs for each combination. * $p < 0.05$ ** $p < 0.01$ according to Tukey's test.

Plant volatiles

A total of nine VOCs were identified in the headspace of coffee plants under different treatments, comprising two terpenes, three benzenoids, two alkanes, and two unidentified compounds (Table 1). Significant differences in volatile compositions were observed among treatments ($F_{3,18} = 8.10$, $R^2 = 0.57$, $p < 0.01$), with no significant variance within groups ($F_{3,18} = 0.65$, $p = 0.61$). The PCA clearly separated the volatile blend of uninfested plants from those of the infestation treatments along PC1 and PC2 similar results, but the volatile blend of multiple-infested plant partially separated from those of mealybug-infested or scale-infested plants (Figure 7, Table S4).

The univariate analysis revealed that scale insect infestation, either alone or in combination with mealybugs, resulted in higher total amounts of volatiles compared to uninfested plants. In contrast, mealybug infestation alone induced an intermediate total amount of volatiles, which did not significantly differ from either uninfested plants or the other infestation treatments (Table 1). In terms of individual compounds, scale infestation elicited

higher emissions of the terpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and supraene compared to uninfested and mealybug-infested plants. When green scales co-infested plants with mealybugs, the volatile blend contained DMNT in similar quantities to those emitted from scale-infested plants. Additionally, ethyl benzoate and the unknown compound 2 were exclusively detected in the volatile emissions of scale-infested and multiple-infested plants. Mealybug infestation alone resulted in larger emissions of 3,7-dimethyldecane compared to uninfested plants. Furthermore, the volatile blend emitted from mealybug-infested plants contained the fungal-associated compound 1-octen-3-ol at higher levels than uninfested and scale-infested plants, but at levels similar to those emitted from multiple-infested plants. When comparing the total amounts of chemical groups, scale infestation, either alone or in combination with mealybugs, induced larger emissions of terpenes and benzenoids in the volatile blend compared to uninfested plants. Conversely, mealybug infestation alone resulted in a blend with terpenes and benzenoids similar to those of uninfested plants and intermediate between the levels in the blends emitted from scale-infested or multiple-infested plants. In contrast, the total amounts of alkanes did not differ significantly across treatments.

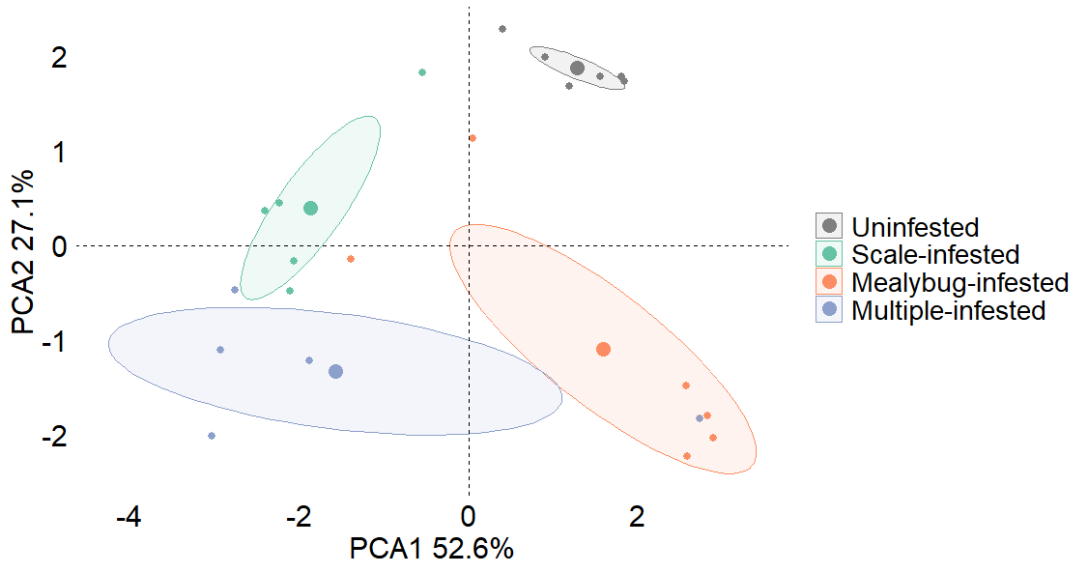

Figure 8. Principal components analysis (PCA) of the relative amounts of chemical compounds found in coffee plants, *Coffea arabica* under different treatments: uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) plants. Bigger dots represent the treatments centroids and smaller dots represent the samples: n = 5–6 plants. See Table 1 for the full list of compounds.

Table 1. Volatile organic compounds detected in the headspace of differently infested coffee plants, *Coffea arabica*: Uninfested, scale infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) plants. Amounts are reported in mean relative amount ± SE.ng^{-1}.

Compounds	KI	Uninfested	Scale-infested	Mealybug-infested	Multiple-infested	F	*p*
Terpenes		0.31±0.05 c	7.47±2.70a	0.50±0.28bc	6.07±2.68ab	7.21	**<0.01**
DMNT	1106	0.27±0.04b	3.94±1.38a	0.24±0.06b	4.80±2.12a	3.50	**0.04**
Supraene	1572	0.04±0.02b	3.52±1.36b	0.26±0.23bc	1.48±0.50ac	8.75	**<0.01**
Benzenoids		1.26±0.16c	4.29±0.64ab	1.93±0.25bc	5.59±1.87a	11.05	**<0.01**
Methyl salicylate	1170	1.26±0.16	4.07±0.62	1.22±0.26	4.95±1.69	3.47	0.07
Ethyl benzoate	1147	0.00±0.00b	0.13±0.02a	0.01±0.00b	0.23±0.09a	11.44	**<0.01**
1-octen-3-ol	967	0.00±0.00a	0.10±0.05ab	0.70±0.22c	0.41±0.17bc	11.15	**<0.01**
Alkanes		1.08±0.14	1.05±0.09	0.76±0.07	0.85±0.17	1.62	0.22
Isobutyl nonane	1097	0.08±0.01	0.07±0.00	0.05±0.00	0.06±0.01	1.91	0.16
3,7-dimethyldecane	1067	1.01±0.13	0.97±0.09	0.71±0.07	0.80±0.16	1.27	0.31
Unknowns							
Unknown 1	1117	0.10±0.02	0.06±0.01	0.05±0.01	0.05±0.00	3.09	0.06
Unknown 2	1265	0.00±0.00b	0.00±0.00b	0.29±0.13a	0.13±0.04a	11.79	**<0.01**
Total production		2.76±0.37b	12.86±3.30a	3.52±0.34ab	12.7±4.69a	7.08	**<0.01**

DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatrien

Discussion

Interactions between the green scale and citrus mealybug in coffee plants

Plants must deal with attacks of multiple herbivores while investing in growth and reproduction. Once under attack, they can exhibit either a specific or generalized induced response to the herbivore's identity or feeding guild, influencing plant resistance or susceptibility to subsequent herbivores via induced defenses [18,26–28]. In general, attack by phloem-feeding insects induces responses mediated by the activation of the SA signaling pathway, which can antagonistically interact with the jasmonate signaling pathway [43,82]. While this negative cross-talk between SA and JA signaling pathways is expected to facilitate infestation by subsequent herbivores, it may, in some cases, enhance resistance, suggesting that these interactions are highly specific to the herbivore species [26,27,31,83,84].

Here we investigated how the infestation by two taxonomically related phloem-feeding insects (Superfamily Coccoidea), *Co. viridis* (green scales) and *P. citri* (mealybugs), induced plant responses and influenced interactions between these two herbivores. First-instar nymphs of both herbivores actively locate and select feeding sites before establishing colonies [57,59,60,69], whereas adult females are much less mobile or even stationary [57,59]. Thus, the ability of first-instar nymphs to select a suitable host or of elevated quality is important for colony establishment. Our results revealed that the arrival of either green scales or mealybugs on coffee plants did not influence the likelihood of subsequent arrival by the other herbivore. However, when both phloem-feeding insects shared the same plant, mealybug performance was negatively impacted.

Despite both insects belonging to the phloem feeding guild, their infestation triggered distinct plant hormonal responses. While scale herbivory led to the accumulation of SA and suppression of ABA, mealybug feeding activated both SA and JA signaling pathways, as previously reported for mealybug infestation in coffee plants [34]. The suppression of ABA accumulation observed in scale-infested plants may have been caused by the activation of SA signaling pathways, which are pathways that can interact antagonistically [85,86]. Moreover, the activation of SA signaling by scale infestation likely impeded the accumulation of jasmonates (both active and inactive forms) through the regulation of transcription factors that mediate SA-JA crosstalk [16], illustrating the antagonist interaction between these pathways. While this hormonal regulation often enhances herbivore performance among herbivores within the same feeding guild [17,30,87], we observed that scale infestation did not facilitate mealybug colonization and

establishment. This aligns with evidence that increased salicylate signaling induced by a phloem-feeding herbivore can negatively impact the performance of another herbivore within the same guild [17,29,30].

The distinct phytohormonal regulation caused by green scale and mealybug infestation resulted in a different profile of putative defensive secondary metabolites. Notably, mealybug infestation triggered a more pronounced change, increasing the levels of most of the assessed secondary metabolites compared to that induced by scale infestation, likely due to the elevated levels of both SA and JA, including its derivatives such as JA-Ile, OH-JA, and COOH-JA-Ile. These included the alkaloid caffeine, the flavonoids catechin and epicatechin, and the phenolic compound neochlorogenic acid. Among them, caffeine and chlorogenic acid have been suggested as deterrents to the scale *Co. viridis*, inducing locomotor activity and reducing feeding [57,71]. Similarly, catechin and epicatechin have shown significant deterrent effects against herbivores [88–91]. Tiliroside, which was discretely induced by mealybug infestation, acts as an oviposition deterrent for the coffee leaf miner (*Leucoptera coffeella*) [92]. The upregulation of these putative defensive compounds in mealybug-infested plants may enhance their resistance. However, our study showed that green scales remained unaffected by these chemical changes, in co-infestation with mealybug-infested plants, indicating that green scales can cope with these defensive compounds in coffee plants.

In contrast, scale infestation induced an increase only in the alkaloid theophylline in coffee plants, likely as a result of the activation of the salicylate pathway alone. Like caffeine, theophylline is a methylxanthine, but it serves as a precursor in caffeine biosynthesis and is a less stable form [93–95]. Although studies on the role of theophylline as an antiherbivore defense compound are scarce, alkaloids are broadly recognized for their defensive properties, exhibiting species-specific effects on herbivores [96,97]. The accumulation of this single compound, among the assessed secondary metabolites, coincides with reduced mealybug performance in scale-infested plants, and thereby deserves future studies on its potential activity against the mealybug.

Mealybugs were numerically more abundant on plants when infested alone (single-infested) or in co-infestation (multiple-infested) with green scales. This numerical difference can be attributed to their shorter life cycle (about 40 to 52 days) compared to the green scales (about 50 to 70 days) [57,58,69]. Multiple-infested plants exhibited higher mealybug infestation (85% adults and 78% nymphs' mealybugs on multiple-infested plants), highlighting the species'

dominance in shaping both the phytohormonal and metabolite profile as they resembled those induced by mealybug infestation rather than scale infestation. This likely reflects an adaptive plant resistance mechanism to the infestation of the most prevalent and damaging herbivore [31,98].

Interactions among green scales, mealybugs and the predator

The predatory ladybug *Cr. montrouzieri* has been widely introduced as a biocontrol agent against Pseudococcidae, primarily targeting the mealybug *P. citri* [74,99]. However, it is also effective in controlling other hemipteran species, including *Co. viridis* [73,100,101]. Our study showed that *Cr. montrouzieri* consumed both mealybugs and green scales at similar rates when offered separately but preferred mealybugs when given a choice. This preference may be linked to the wax production of mealybugs, which is absent in green scales. It has been suggested that *Cr. montrouzieri* shows a preference for wax-producing prey or specific wax-producing developmental stages, as the chemical cues and structure of wax filament stimulates ladybug oviposition [102,103]. Interestingly, this preference for mealybugs did not translate into an olfactory preference of *Cr. montrouzieri* for volatile emissions from plants infested with either green scales or mealybugs, being equally attracted to HIPVs from plants carrying mealybugs or green scales. Thus, HIPV emissions from singly infested plants effectively signal prey presence to *Cr. montrouzieri*, although they do not provide specific information about prey quality, which is likely greater or easier to consume in mealybugs.

Even though the ladybug did not differentiate between the volatile blends emitted from mealybug-infested and scale-infested plants, the composition of these blends was significantly distinct. This difference was primarily quantitative, as only one compound (unknown 2) was exclusive to plants carrying mealybugs, while scale-infested plants emitted higher amounts of several compounds, including terpenes and benzenoids. The overlap of a single or of a subset of compounds in the blends, regardless of concentration, may have served as a reliable signal of prey presence [48,104], leading to the equal attractiveness of *Cr. montrouzieri* to both treatments. Although no study has specifically tested the activity of isolated plant volatile compounds in *Cr. montrouzieri* behavior or antennal physiology, some volatiles detected in the headspace of coffee plants are known as attractants for other ladybugs, such as DMNT [105,106] and methyl salicylate (MeSA) [107,108]. Additionally, other compounds, such as squalene, may also play a role, as it has been shown to attract other natural enemies [109,110], although its effects remain unproven for ladybugs. Further research is needed to investigate the biological activity of these compounds to *Cr. montrouzieri*.

The recognition and attractiveness of volatile emissions from plants infested by a single prey species to natural enemies have been well-documented [38,40,111,112]. However, in natural settings, plants are attacked by a community of herbivores, which can interfere with the chemical communication among plants, prey herbivores, and natural enemies [22]. We hypothesize that plants infested by multiple herbivores from the same feeding guild inducing similar plant defense pathway [11] would amplify the expression of induced plant defenses, enhancing the attractiveness of HIPVs to natural enemies.

While we observed that *Cr. montrouzieri* was attracted to volatile emissions from plants carrying a single prey species compared to uninfested plants, it was not attracted to volatile emissions from plants carrying both prey species, suggesting a disruption in chemically mediated tritrophic interaction. When given a choice between odors from mealybug-infested and multiple-infested plants, *Cr. montrouzieri* was attracted to mealybug-infested plants, despite the lower number of prey individuals in these plants compared to multiple-infested plants, which contained both mealybugs and green scales. Since scale-infested plants emitted volatiles that attracted the ladybug, we expected a similar outcome as mealybug-infested plants when compared to multiple-infested plants. However, the ladybug did not differentiate between scale-infested plants and multiple-infested plants. In this specific olfactory context, the ladybug was either equally attracted to both treatments or was unable to perceive the volatile blend emitted by scale-infested plants as attractive. Indeed, the volatile analysis revealed that the blend emitted by multiple-infested plants resembles that by scale-infested plants rather than mealybug-infested plants. For example, amounts of DMNT, supraene and ethyl benzoate were present in the blend from both scale-infested and multiple-infested plants at higher amounts than the other treatments. Although not significant, the total amount of MeSA, a volatile benzenoid induced by herbivory, including mealybug infestation [34,106], was about three times more abundant in the blend of scale- and multiple-infested plants compared to mealybug-infested plants. MeSA is known as an attractant to natural enemies [113,114], including ladybugs [107,108] although its effectiveness can be concentration-dependent and inconsistent in some cases [108,115,116].

Notably, the emission of multiple-infested plants also contained some compounds at similar amounts as the blend emitted by mealybug-infested plant, like the alcohol 1-octen-3-ol. This compound is derived from fungal activity and likely results from the fermentation of hemipteran honeydew [117,118], which can signal a carbohydrate source for natural enemies, occasionally being used by *Cr. montrouzieri* [66,119]. It has been shown that feeding on the honeydew

enhances predator mobility and prey consumption [120]. Despite the blend from multiple-infested plants containing several potential attractive compounds, as mentioned above, overlapping with the composition of both scale-infested and mealybug-infested plants, the ladybug did not discriminate between it and the uninfested plant. It is likely that the lack of attractiveness might be due to a unique combination of compounds at a specific ratio, which is not recognized by the ladybug as a signal of prey presence. From the compounds detected in coffee plant headspace, only ethyl benzoate, present in scale- and multiple-infested plants, has been reported for repellency and insecticidal properties against insects [34,121,122]. We encourage future studies to elucidate the reason underlying the lack of attractiveness of the volatile blend emitted by plants carrying both prey species to *Cr. montrouzieri* through olfactory and electroantennography tests with the isolate compounds as well as in mixtures.

Conclusion

Our results emphasize the specificity of plant defenses in multitrophic systems, particularly among phloem-feeding herbivores, which are expected to share similar hormonal manipulation mechanisms. Despite belonging to the same feeding guild and being closely related, our findings demonstrate that infestation by *Co. viridis* and *P. citri* trigger distinct profiles of phytohormones, volatile and non-volatile secondary metabolites, which modulate complex ecological interactions, including herbivore performance and the attraction of *Cr. montrouzieri*.

The dominance of mealybugs in shaping plant hormonal and metabolic profiles reflects their adaptive capacity and the interplay between SA and jasmonic acid JA pathways, though this did not lead an increase in the performance of a subsequent herbivore [30,76]. In contrast, green scales demonstrated a greater ability to alter plant volatile emissions, potentially masking the volatile foraging cues used by natural enemies—an advantage for herbivores.

Although multiple infestation amplified certain plant physiological responses, such as increased levels of MeSA and DMNT, the plant attractiveness to the predator was equal or lower compared to single infestation. Further research is required to identify whether specific VOCs or their mixtures influence the behavior of *Cr. montrouzieri* and how these blends could be optimized to improve the attraction of natural enemies.

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Data Availability

All data are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Enggel B. S. Carmo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Illustration, Project administration, Writing - Original Draft, Writing - Review & Editing. **Renato C. Macedo-Rego:** Writing - Original Draft, Writing - Review & Editing, Supervision, Funding Acquisition. **Arodí P. Favaris** and **José Mauricio Simões Bento:** Gas chromatographic analyses, Data curation, Funding Acquisition. **Alexander N. Borg** and **John C. Caulfield:** Liquid chromatographic analyses of target metabolites, Data curation, Funding Acquisition. **Axel Mithöfer** and **Bruna Correa da Silva:** Liquid chromatographic analyses of phytohormones, Data curation, Funding Acquisition. **Maria Fernanda G. V. Peñaflor:** Conceptualization, Project administration, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding Acquisition.

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Supplementary material

Table S1. Levels (mean ±SE ng. g^{-1} dry tissue) of phytohormones in leaves of uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) infested *Coffea arabica* plants.

Treatments	Uninfested	Scale-infested	Mealybug-infested	Multiple-infested	F	*P*
SA	6674.00±946.00b	11110.00±1571.00a	10053.00±1060.00ab	8293.00±484.00ab	4.62	**<0.01**
JA	18.20±5.52a	9.98±1.02a	319.00±59.80b	173.00±18.00b	26.96	**<0.01**
ABA	300.00±16.20a	200.00±11.40a	324.00±25.00b	325.00±12.00b	28.84	**<0.01**
sum JA-Ile	1.64±0.69	1.27±0.22	7.02±0.61	9.33±0.81	1.93	0.14
cis-OPDA	1228.00±271.00	1466.00±380.00	1613.00±208.00	1598.00±219.00	0.64	0.59
OH-JA	20.10±4.40a	6.71±0.86b	71.00±8.60ac	55.00±5.88ac	8.74	**<0.01**
COOH-JA-Ile	1.08±0.32a	3.82±1.86b	6.16±0.73c	11.70±2.94c	6.24	**<0.01**

SA- Salicilic acid, JA- Jasmonic acid, ABA – Abscisic acid, JA-Ile – Jasmonic acid isoleucine, cis-OPDA - cis-12-oxo-phytodienoic acid (OPDA); OH-JA hydroxylated jasmonic acid, COOH-JA-Ile jasmonoyl-isoleucine.

Table S2. Amounts (mean ±SE ng. g^{-1} dry tissue) of secondary plant metabolites in leaves of: uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) infested *Coffea arabica* plants.

Treatments	Uninfested	Scale-infested	Mealybug-infested	Multiple-infested	F	*P*
Theophylline	222.00±138.00b	4939.00±1178.00a	00.00±00.00b	00.00±00.00b	15.55	**<0.01**
Caffeine	55582.00±2299a	62456±2019a	83543±1583b	79539±1171b	53.23	**<0.01**
Catechin	11530±183a	10725±1266a	23421±3269b	28789±2482b	16.34	**<0.01**
Epicatechin	12566±1100a	12083±989a	19880±2059b	23944±1687b	14.27	**<0.01**
Tiliroside	78.40±30.60b	306.00±45.40ab	428.00±98.50a	422.00±98.8a	4.95	**<0.01**
Neochlorogenic acid	90.7±28.3b	33.3±13.00b	829.00±148.00a	786.00±133.00a	19.02	**<0.01**

Table S3. PERMANOVA, PERMUTEST, and pairwise tests of the relative amounts of secondary plant metabolites found in coffee plants, *Coffea arabica*, across different treatments: uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) plants.

Pairwise test	**F**	**R²**	***P***
Mealybugs vs. Uninfested	39.9	0.63	**<0.01**
Scales vs. Uninfested	4.12	0.16	**0.02**
Scales vs. Mealybugs	27.11	0.55	**<0.01**
Mealybugs vs. Multiple infested	2.55	0.11	0.12
Scales vs. Multiple infested	44.82	0.69	**<0.01**
Uninfested vs. Multiple infested	53.53	0.72	**<0.01**
PERMANOVA	17.01	0.54	**<0.01**
PERMUTEST	1.27		0.29

Table S4. PERMANOVA, PERMUTEST, and pairwise tests of the relative amounts of chemical compounds found in coffee plants, *Coffea arabica*, across different treatments: uninfested, scale-infested (*Coccus viridis*), mealybug-infested (*Planococcus citri*), and multiple-infested (mealybug + scale) plants.

Pairwise test	**F**	**R²**	***P***
Mealybugs vs. Uninfested	5.92	0.37	**0.01**
Scales vs. Uninfested	24.52	24.52	**<0.01**
Scales vs. Mealybugs	13.39	13.39	**0.01**
Mealybugs vs. Multiple infested	3.68	0.29	0.06
Scales vs. Multiple infested	2.78	2.79	**0.04**
Uninfested vs. Multiple infested	10.21	10.21	**<0.01**
PERMANOVA	8.09	0.57	**<0.01**
PERMUTEST	0.65		0.61

TERCEIRA PARTE

CONSIDERAÇÕES FINAIS

Os resultados apresentados nesta tese demonstram a complexidade das interações quando a planta é atacada por mais de uma espécie. Nossa meta-análise revelou que a herbivoria múltipla por insetos hospedeiros/presas e não-hospedeiros/presas não impacta a atratividade dos voláteis induzidos por herbivoria (HIPVs) para inimigos naturais (INs). Embora estudos anteriores tenham relatado efeitos variados da infestação múltipla sobre a eficiência dos INs, nossos resultados sugerem que esses impactos são específicos para determinados sistemas ecológicos. A estabilidade da comunicação química nos sistemas tritróficos permite que os INs localizem seus hospedeiros, mesmo na presença de herbívoros não-hospedeiros. A análise também revelou lacunas no conhecimento, incluindo a influência da experiência prévia dos INs na sua resposta a voláteis de plantas infestadas por múltiplos herbívoros e diferenças entre especialistas e generalistas. Além disso, os sistemas de estudo utilizados foram limitados a modelos clássicos, destacando a necessidade de diversificação para se compreender melhor como a herbivoria múltipla afeta as interações tritróficas. Estudos futuros devem considerar a ordem de chegada dos herbívoros na planta, bem como a densidade e diversidade de herbívoros não-hospedeiros que podem modificar a atratividade dos HIPVs para os NEs.

No caso do sistema estudado, envolvendo cochonilhas-verdes (*Coccus viridis*) e cochonilhas-farinhentas (*Planococcus citri*) em plantas de café, observou-se que a infestação simples por uma das espécies resultou em padrões distintos nos perfis hormonais e metabólicos da planta, com implicações diretas para o desempenho dos herbívoros e a atração de inimigos naturais. Apesar dessas respostas químicas complexas, a infestação múltipla não influenciou a seleção de hospedeiros pelos herbívoros, mas afetou negativamente o desempenho das cochonilhas-farinhentas quando em co-infestação com cochonilhas-verdes. Esse resultado sugere que a competição intra-guilda mediada por alterações nas defesas da planta pode ser um fator crucial na dinâmica populacional desses herbívoros.

No que diz respeito à atração de inimigos naturais, os resultados revelaram que a joaninha predadora *Cryptolaemus montrouzieri* foi capaz de reconhecer plantas infestadas por *Co. viridis* ou *P. citri*, mas não apresentou preferência por plantas co-infestadas por ambas as espécies.

Esses resultados destacam a complexidade das interações tritróficas em sistemas agrícolas e a importância de se considerar a identidade e a combinação de herbívoros ao estudar as respostas de defesa das plantas. A ativação simultânea de vias de defesa mediadas por ácido salicílico (SA) e ácido jasmônico (JA), bem como a indução de metabólitos secundários específicos, sugere que as plantas de café possuem mecanismos sofisticados para lidar com a herbivoria múltipla. No entanto, a eficácia dessas respostas na redução do desempenho dos herbívoros e na atração de inimigos naturais pode variar conforme o contexto ecológico.

Em síntese, este estudo contribui para a compreensão dos mecanismos de defesa das plantas em resposta à herbivoria múltipla e destaca a importância de abordagens integradas que considerem tanto as interações planta-herbívoro quanto às interações herbívoros-inimigos naturais. Futuras pesquisas devem explorar como essas respostas variam em diferentes condições ambientais e como podem ser manipuladas para melhorar o controle biológico de pragas em sistemas agrícolas. Além disso, a investigação dos papéis específicos de metabólitos secundários, como cafeína e catequina, na defesa das plantas e na dinâmica tritrófica pode abrir novas perspectivas para o manejo sustentável de pragas.

APÊNDICE A – Imagens dos bioensaios

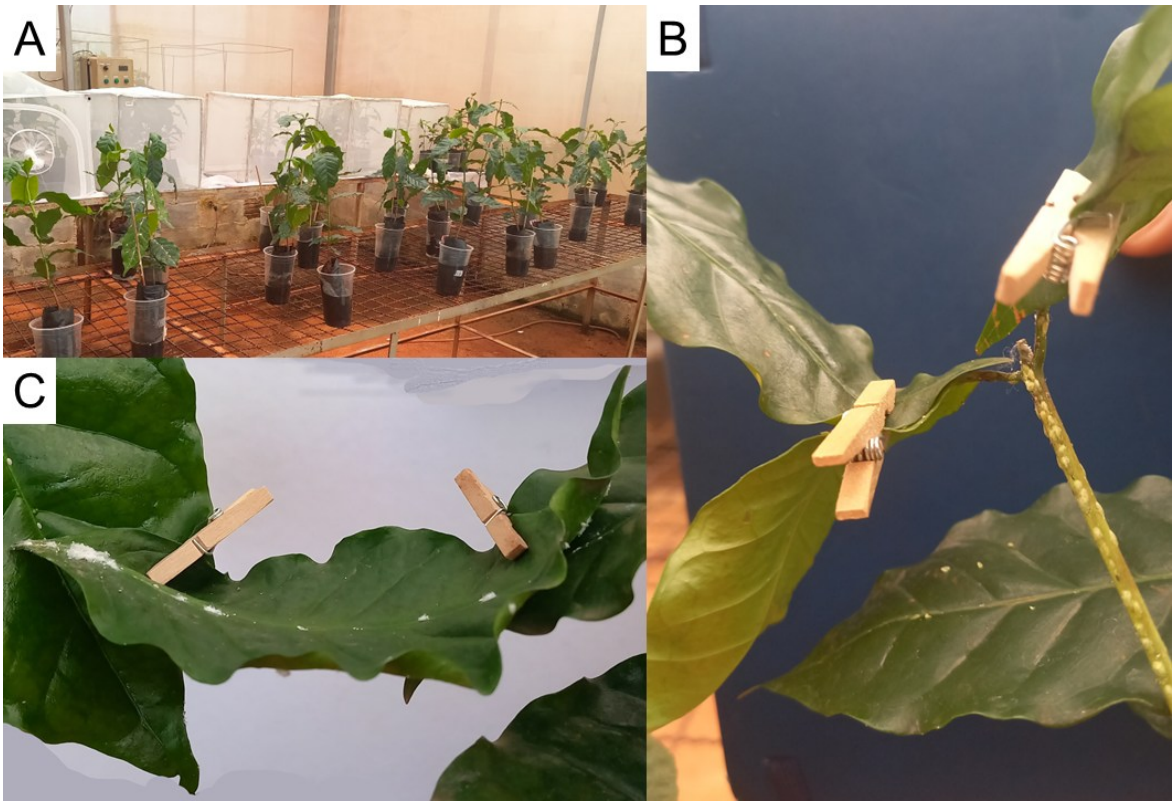

Figura 1. Bioensaio de preferência de planta hospedeira por parte das cochonilhas *Planococcus citri* e *Coccus viridis*, realizado entre plantas de café não infestadas e plantas infestadas por cochonilhas heteroespecíficas. (A) Estrutura das repetições do bioensaio em casa de vegetação; (B) Conexão do galho previamente infestado com *Co. viridis* entre plantas não infestadas e plantas infestadas com *P. citri*; e (C) Conexão da folha previamente infestado com *P. citri* entre plantas não infestadas e plantas infestadas com *Co. viridis*.

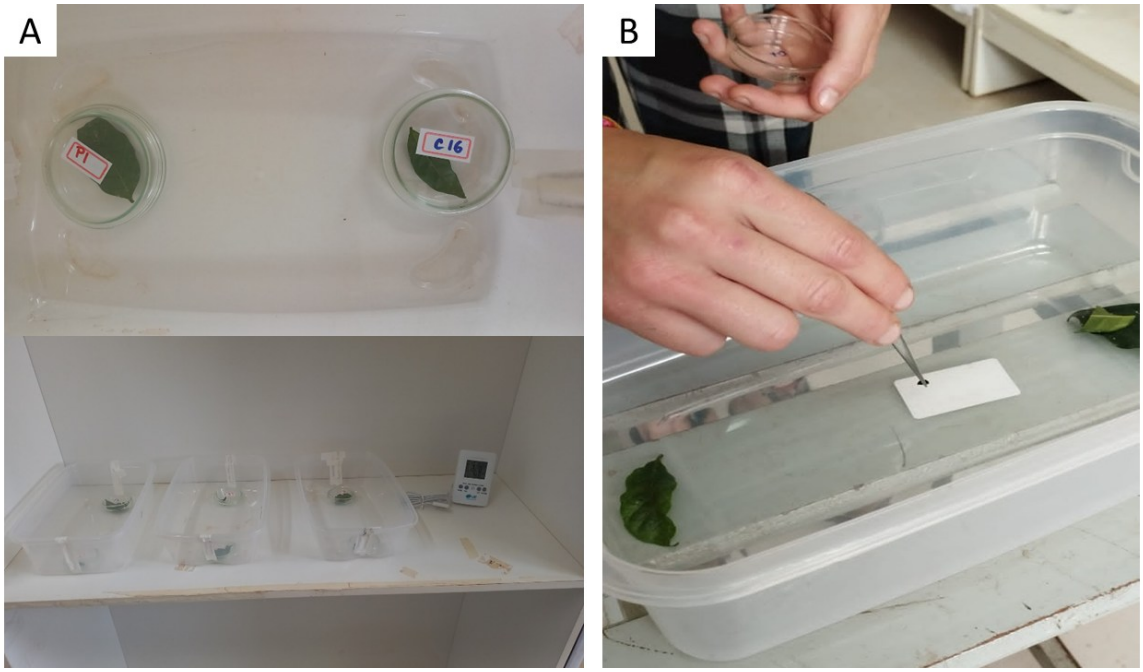

Figura 2. Bioensaio de preferência e consumo de presas pela joaninha *Cryptolaemus montrouzieri*, entre as cochonilhas *Planococcus citri* e *Coccus viridis*. (A) Estrutura das repetições do bioensaio em laboratório, em caixas de plástico (24 × 15 cm); e (B) Liberação da joaninha *Cr. montrouzieri* na arena de bioensaio para o teste com chance de escolha.

Figura 3. Bioensaio de preferência olfativa da joaninha *Cryptolaemus montrouzieri* entre plantas de café com diferentes níveis de infestação (simples e múltipla) por cochonilhas *Planococcus citri* e *Coccus viridis*. A imagem mostra a liberação da joaninha no sistema com olfatômetro em tubo em 'Y' coberto com caixa para ocultar pistas visuais e luminosa.

APÊNDICE B – Material de divulgação científica

Meio digital: Instagram

Título: Cryptolaemus montrouzieri ou @cryptolaemus_montrouzieri

Autora: Enggel Carmo

Link: https://www.instagram.com/cryptolaemus_montrouzieri/

Desde 2016, quando ainda era bolsista de iniciação científica (PIBIC) no Programa de Pós-Graduação em Entomologia Universidade Federal Rural de Pernambuco (PPGE/UFRPE), venho participando de pesquisas sobre joaninhas predadoras de cochonilhas. No início, meu foco foi estudar a biologia das espécies *Tenuivalvae notata* (Mulsant) e *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), e, posteriormente, passei a avaliar as interações interespecíficas mediadas por pistas químicas liberadas por essas espécies.

Por exigência da matriz curricular do Programa de Pós-Graduação em Entomologia da Universidade Federal de Lavras (PPGEN/UFLA), e pela facilidade de exposição do conteúdo e alcance a diferentes públicos, decidi criar em 2023 uma página no *Instagram* para compartilhar meus conhecimentos e os resultados das pesquisas relacionadas a utilização de joaninhas no controle biológico.

Nessa página, por meio de textos e publicações redigidos em português, compartilho vídeos mostrando as metodologias de criação dos organismos, dicas sobre técnicas de pesquisa e divulgo estudos sobre a biologia, ecologia e evolução de espécies de insetos, especialmente sobre a joaninha *Cryptolaemus montrouzieri*. Ademais, compartilho dicas sobre técnicas de pesquisa; e exponho minhas habilidades em ilustração científica, coleta e análise de dados, além dos meus próprios trabalhos acadêmicos. Por fim, trata-se de uma excelente maneira de manter contato com a comunidade científica por meio do engajamento das postagens publicadas.

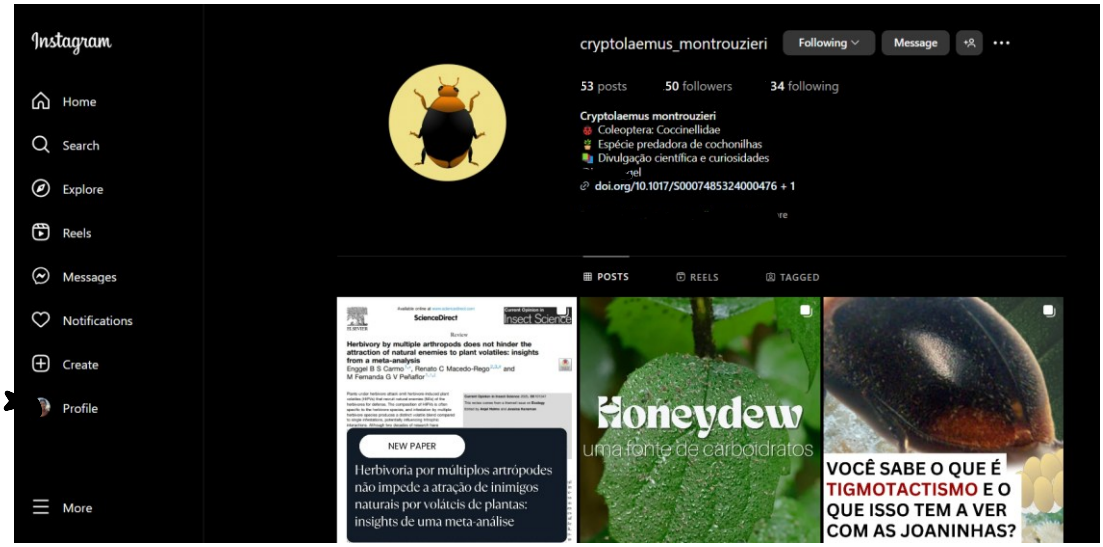

Figura 1. Captura de tela (“print screen”) da página de divulgação científica “@cryptolaemus_montrouzieri” no Instagram. A página foca em conhecimentos sobre joaninhas e suas presas, com foco especial na joaninha predadora de cochonilhas, *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae). Captura de tela realizada em 10 mar. 2025.

APÊNDICE C – Sistema de coleta de voláteis “push-pull” LEQIIP

Em 2023, meses antes do meu estágio doutoral na Inglaterra (Doutorado Sanduíche), precisei realizar coleta de voláteis das plantas de café sob diferentes tratamentos de infestação: não-infestadas, infestação simples por *Co. viridis* ou *P. citri*, e infestação múltipla por *Co. viridis* + *P. citri*. Para isso, desenvolvemos um sistema de coleta de voláteis no Laboratório de Ecologia Química da Interações Inseto-Planta (LEQIIP), utilizando um sistema de aeração "push-pull" (ARS, Gainesville, FL, EUA), de acordo com a metodologia descrita por Peñaflor et al. (2016). As plantas foram acondicionadas individualmente em sacos de poliestireno (41 cm x 33 cm) e esses sacos foram conectados ao sistema de aeração com um fluxo de entrada de ar de 0, 5L.min^{-1}, precedido por um sistema de filtragem de ar com carvão ativado e um filtro de ar triplo odontológico - ARFUSION (Figura 1). Os filtros para a coleta dos voláteis consistiam em um tubo de vidro fino contendo 30 mg de polímero adsorvente HayeSep-Q® (Hayes Separation Inc., Bandera, TX, EUA), que foram inseridos nos sacos. O ar foi aspirado a 0,25 L.min^{-1} por meio de uma bomba de vácuo (SKC Pocket Pump TOUCH) conectada aos filtros. Vapores de sacos vazios foram coletados em paralelo para monitorar contaminantes do sistema (Figura 1).

Após 8 horas de aeração (das 8h às 16h), os filtros de polímero foram eluídos com 100 µL de hexano, e os extratos foram acondicionados em frascos de vidro vedados e armazenados em freezer.

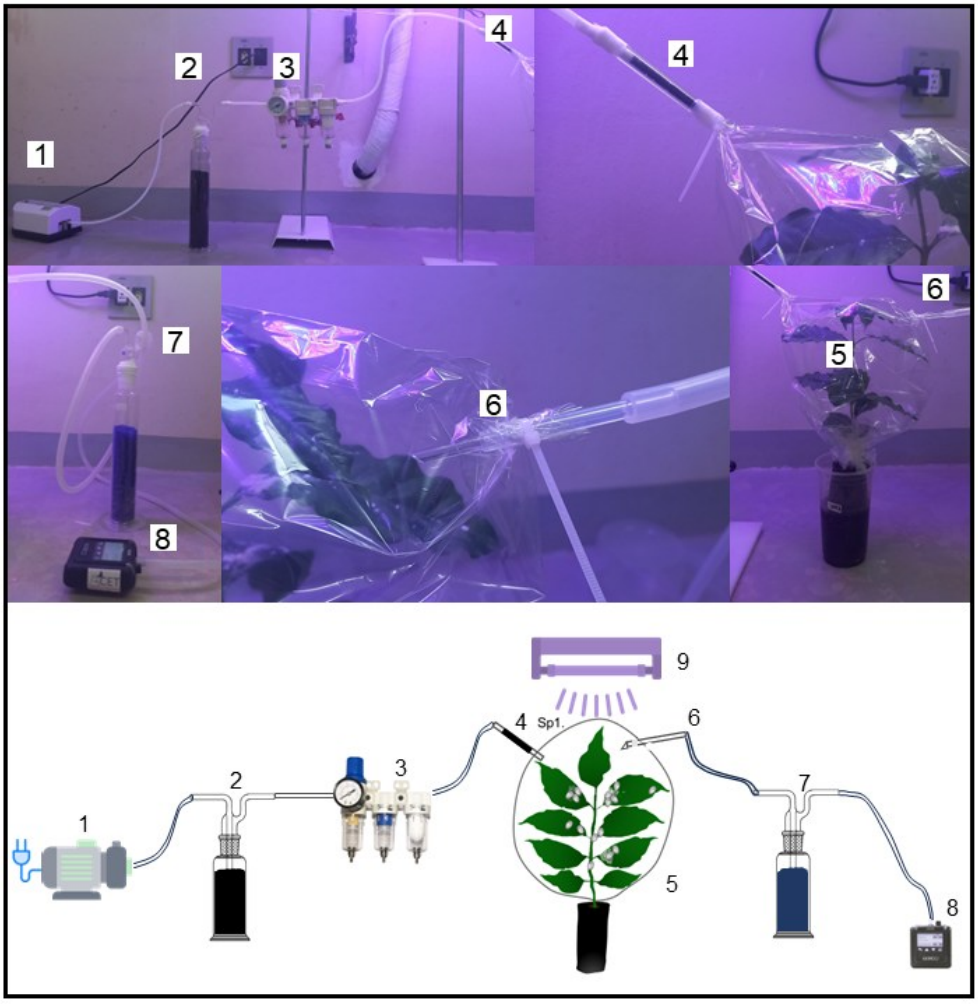

Figura 1. Sistema de aeração “push-pull” para coleta voláteis: 1- Bomba de aquário (Fluxo de ar 0.5 $L.min^{-1}$), 2- Filtro de carvão ativado, 3- Filtro De Ar Triplo (Odontológico - ARFUSION); 4- Mini-filtro (12 cm) (lã de vidro + carvão ativado), 5 – Planta de café em sacola de poliéster para aeração, 6- Filtro de vidro com 30g de polímero adsorvente HayeSep-Q® (Hayes Separation Inc., Bandera, TX, USA) para coleta de voláteis, 7- Desumidificador do sistema – Sílica azul, 8 – Bomba à vácuo 0.25 $L.min^{-1}$ (SKC Pocket Pump TOUCH), 9- Painel de luz UV – Função VEG.