FERNANDA MOREIRA ANDRADE

MODULATION OF CHEMICAL PLANT DEFENSES AGAINST TWO ARTHROPOD HERBIVORES SHAPES SUBSEQUENT ECOLOGICAL INTERACTIONS

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ecologia Aplicada, área de concentração Ecologia e Conservação de Recursos em Paisagens Fragmentadas e Agrossistemas, para obtenção do título de Doutor.

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APROVADA em 17 de março de 2023
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RESUMO

As plantas sob herbivoria têm suas defesas induzidas ativadas, que atuam diretamente sobre o desenvolvimento e sobrevivência do herbívoro através da produção de compostos tóxicos ou antinutritivos. As plantas realocam energia para a síntese dessas defesas, o que pode acarretar mudanças no seu metabolismo primário. No entanto, herbívoros de diferentes guildas alimentares desenvolveram estratégias a fim de superar essas defesas dos hospedeiros, que podem ou não facilitar ataques de herbívoros subsequentes. Nesse contexto, a presente tese buscou investigar o efeito da herbivoria do ácaro-vermelho-do-cafeeiro (*Oligonychus ilicis*) e do bicho-mineiro-do-cafeeiro (*Leucoptera coffeella*) nas defesas induzidas do caffeeiro (*Coffea arabica*) e suas consequências ecológicas para coespecíficos e heteroespecíficos. Foram realizados ensaios comportamentais com os herbívoros em laboratório, análises *targeted* e *untargerted* do metabolismo primário e secundário das plantas de café, bem como análises do seu perfil fitohormonal. No primeiro capítulo, observou-se que plantas infestadas pelo ácaro foram mais atrativas e promoveram uma maior taxa de oviposição de coespecíficos que plantas não infestadas. Além disso, os alcaloides cafeína, teofilina e trigonelina pareceram não exercer função de defesa das plantas de *C. arabica* contra o ácaro. Porém, o composto fenólico ácido clorogênico foi um forte candidato a cumprir esse papel. Embora não seja possível afirmar que o ácaro suprimiu as defesas das plantas de café, sua alimentação acarretou um aumento nas concentrações de ácido salicílico (AS) e de ácido 12-oxo-fitodienóico (OPDA), mas não do ácido jasmônico (AJ) e ácido jasmônico-isoleucina (AJ-Ile). Sugerimos que a conversão de OPDA em AJ e AJ-Ile não foi bem-sucedida nas plantas infestadas pelo ácaro, o que pode estar relacionado à maior suscetibilidade dessas plantas. No segundo capítulo, mostramos que a infestação pela larva minadora de *L. coffeella* tornou a planta de café melhor hospedeira para a infestação subsequente de coespecíficos, mas não de heteroespecíficos. Em contrapartida, *L. coffeella* não discriminou entre plantas infestadas pelo ácaro e não infestadas, porém as plantas infestadas pelo heteroespecífico afetaram negativamente o desempenho da larva minadora que consumiu menos tecido foliar, em relação às plantas não infestadas. Os resultados das análises químicas mostraram que os herbívoros induziram respostas distintas no perfil metabólico das plantas quando se alimentaram separadamente (infestações simples) e simultaneamente (infestação múltipla). Ademais, enquanto a infestação simples promoveu um aumento nas concentrações de *features* (compostos não identificados) significativos, a infestação múltipla as suprimiu. Esses resultados parecem refletir a diferente regulação fitohormonal das defesas das plantas pelos herbívoros. As plantas infestadas pela larva minadora apresentaram acúmulo de AS e ácido abscísico (ABA), mas não AJ. E, as plantas infestadas pelo ácaro, apresentaram acúmulo de AS e de OPDA, mas não de AJ e AJ-Ile. Nossos resultados sugerem que a larva minadora de *L. coffeella* e o ácaro-vermelho-do-cafeeiro, *O. ilicis*, suprimem defesas da planta de café que favorecem o estabelecimento de coespecíficos, mas não de heteroespecíficos. Apesar de ambos os herbívoros reduzirem as defesas moduladas pelo AJ, o mecanismo pelo qual essa supressão ocorre parece ser distinto entre os dois e ainda não foi reportado na literatura.

Palavras-chave: *Coffea arabica***.** Defesas induzidas pela herbivoria. Interação planta-herbívoro. *Leucoptera coffeella*. Metabolômica. *Oligonychus ilicis*. Supressão de defesas por herbívoros.

ABSTRACT

Plants under herbivory have their induced defenses activated, which act directly on the development and survival of the herbivore by the production of toxic or antinutritive compounds. Plants reallocate energy to synthesize these defenses, which can lead to changes in their primary metabolism. However, herbivores from different feeding guilds have developed strategies to overcome these host defenses, which may or may not facilitate attacks by subsequent herbivores. Hence, this thesis aims to investigate the effect of herbivory of the southern red mite (*Oligonychus ilicis*) and the coffee leaf miner (*Leucoptera coffeella*) on the induced defenses of coffee plants (*Coffea arabica*) and its ecological consequences for conspecifics and heterospecifics. Behavioral tests with herbivores were carried out in the laboratory, and targeted and untargeted analysis of the primary and secondary metabolism of coffee plants, as well as analysis of the phytohormonal profile. In the first chapter, it was shown that plants infested by the mite were more attractive and increased oviposition of conspecifics than non-infested plants. Apparently, the alkaloids caffeine, theophylline, and trigonelline did not appear to exert a defense function in *C. arabica* plants against the mite. However the phenolic compound, chlorogenic acid was a strong candidate for that role. Although it was not possible to affirm that the mite suppresses coffee plant defenses, its feeding increased the concentrations of salicylic acid (SA) and 12-oxophytodienoic acid (OPDA), but not jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile). We suggest that the conversion of the precursor, OPDA, into jasmonates, JA, and JA-Ile, is not successful in plants infested with the mite, which could be related to the greater susceptibility of these plants. In the second chapter, the coffee leaf miner infestation made the coffee plant a better host for the subsequent infestation of conspecifics, but not heterospecifics. On the other hand, *L. coffeella* did not discriminated between mite-infested and non-infested, however the leaf miner was negatively affected and consumed less leaf tissue, compared to non-infested ones. The results of the chemical analysis showed that herbivores induced distinct responses in the metabolic profile (primary and secondary) of plants under single infestations and simultaneously (multiple infestation). Furthermore, while the single infestation upregulated the concentrations of greater number of features (unidentified compounds), the multiple infestation triggered suppressed them. These results seem to reflect the different phytohormonal regulation of plant defenses by herbivores. Leaf miner-infested plants showed accumulation of SA and abscisic acid (ABA), but not JA. Mite-infested plants showed accumulation of SA and OPDA, but not JA and JA-Ile. Our results suggest that the southern red mite and the coffee leaf miner suppress coffee plant defenses that favor the establishment and development of conspecifics, but not heterospecifics. Although both herbivores reduce the defenses modulated by JA, the mechanism by which this suppression occurs seems to be different between the two and has not yet been reported in the literature.

Key words: *Coffea arabica.* Defenses suppression by herbivores. Herbivory induced defenses. *Leucoptera coffeella*. Plant-herbivore interactions. Metabolomics. *Oligonychus ilicis*.

SUMÁRIO

PRIMEIRA PARTE

1. INTRODUÇÃO GERAL

Em um cenário coevolutivo, as plantas desenvolvem sofisticados mecanismos de defesas que as tornam resistentes a ataques de herbívoros especialistas que, em contrapartida, desenvolvem estratégias para superá-las. Assim, é estabelecida uma relação íntima entre plantas e artrópodes herbívoros, cujas respostas do hospedeiro são espécie-específicas com consequências ecológicas para coespecíficos do herbívoro (indivíduos da mesma espécie) e heteroespecíficos (herbívoros de outra espécie), bem como níveis tróficos superiores.

Para lidar com a constante ameaça de herbivoria, as plantas contam com mecanismos de defesa constitutivos, que independem da ação dos herbívoros para sua expressão, e induzidos, que são desencadeados apenas sob herbivoria ou outros estímulos associados à herbivoria (WALLING, 2000). As defesas induzidas podem afetar negativamente os herbívoros de maneira direta, como por meio de compostos tóxicos ou antinutritivos que prejudicam o seu desenvolvimento e sobrevivência (KESSLER; BALDWIN, 2002), e de maneira indireta, através da liberação de compostos orgânicos voláteis atrativos para os inimigos naturais dos herbívoros (predadores e parasitoides) (DICKE, 2009).

A ativação das defesas induzidas pela herbivoria desencadeia a síntese de novos metabólitos secundários, além do aumento daqueles já existentes, como terpenos, alcaloides, fenóis, glucosinolatos, dentre outros (MITHÖFER; BOLAND, 2012; WAR et a., 2018). Esses compostos não atuam no crescimento e desenvolvimento das plantas, mas as protegem atuando negativamente no desenvolvimento e sobrevivência dos herbívoros e outras ameaças, como patógenos, diante suas características tóxicas, deterrentes ou antinutritivas (BENNETT; WALLSGROVE, 1994; CROTEAU et al., 2000). Porém, o metabolismo das plantas é mantido de maneira homeostática (em equilíbrio) e qualquer modificação na cadeia metabólica resultante da herbivoria pode gerar mudanças no seu fenótipo químico. Nesse contexto, o perfil de metabólitos primários, que engloba os compostos responsáveis pela produção e síntese de compostos essenciais para seu crescimento e desenvolvimento (FERNIE; PICHERSKY, 2015), também é modificado (ZHOU et al., 2015). Assim, açúcares e aminoácidos, além de necessários para a planta, também o são para a sobrevivência dos consumidores, tendo também um papel fundamental nas interações planta-herbívoro (ZHOU et al., 2015). Diante disso, os metabólitos primários e secundários são importantes fontes de informação que indicam a qualidade do hospedeiro para benefício do próprio herbívoro e de sua prole (AWMACK; LEATHER, 2002; MITHÖFER; BOLAND, 2012; ZHOU et al., 2015).

Essas mudanças no perfil químico induzidas pela herbivoria são espécie-específicas, o que significa que são dependentes da espécie de planta e identidade do herbívoro (HOWE; JANDER, 2008). De modo geral, o reconhecimento da herbivoria ocorre pelo padrão de dano físico e a interação com substâncias específicas da secreção oral do herbívoro com receptores celulares (HOWE; JANDER, 2008). Após esse reconhecimento, é desencadeada a ativação de cascatas (ou rotas) de sinalização dos fitohormônios, que são metabólitos envolvidos em processos de regulação das plantas (DAVIES, 2004). Essas cascatas de sinalização, que compreendem uma série de oxidações e reações enzimáticas, vão gerar moléculas ativas dos fitohormônios que, ao se ligarem em complexos repressores no núcleo celular, desencadearão a transcrição e posterior ativação de genes de defesa, resultando na produção dos metabólitos secundários (HARTMANN, 2007; HOWE; JANDER, 2008; WU; BALDWIN, 2010).

As defesas induzidas são moduladas pelos fitohormônios e sua ativação, em geral, está relacionada à guilda alimentar à qual o herbívoro pertence. Assim, herbívoros mastigadores ativam a via do ácido jasmônico (AJ) (HOWE; JANDER, 2008), enquanto sugadores de floema ativam a via do ácido salicílico (AS) (KALOSHIAN; WALLING, 2005). Sugadores de conteúdo celular extravasado (e.g., ácaros) e insetos consumidores de parênquima foliar (e.g., minadores de folha) ativam ambas essas vias, a do AJ e AS (SCHIMMEL; ATAIDE; KANT, 2017; YANG et al., 2021). Além dos fitohormônios citados, outros moduladores também podem atuar nas defesas das plantas contra herbivoria, como o etileno (ET), o ácido abscísico (ABA), citocinina e giberelina (GA) (KAHL et al., 2000; PIETERSE et al., 2009). Apesar da alta especificidade das respostas das plantas contra a herbivoria, é importante destacar que o metabolismo das plantas é complexo e as rotas de sinalização, principalmente do AJ e AS, podem ser ativadas simultaneamente ou suprimir a ação umas das outras (*crosstalk* negativo), o que pode influenciar interações subsequentes (PIETERSE et al., 2009).

Para driblar as defesas induzidas e permanecer na planta, alguns artrópodes herbívoros desenvolveram estratégias como a supressão das defesas induzidas. Essas estratégias têm sido reportadas em diversos sistemas envolvendo herbívoros de variadas guildas alimentares. Por exemplo, a lagarta *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) suprime a produção de nicotina em *Nicotiana tabacum* L. (Solanaceae) a partir de enzimas salivares (MUSSER et al., 2002). Já no caso do inseto sugador de floema *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), sua alimentação desencadeia a ativação da via do AS, o que leva à supressão das defesas relacionadas ao AJ de *Phaseolus lunatus* L. (Fabaceae) através do antagonismo das rotas do AS-AJ (*crosstalk* negativo) (ZHANG et al., 2009). Por outro lado, a alimentação da

larva minadora do microlepidóptero *Phyllonorycter blancardella* (Fabricius) (Lepidoptera: Gracillariidae) modifica o perfil fitohormonal de *Malus domestica* (Suckow) (Rosaceae) ao elevar os níveis de citoquininas e causar efeito antagônico entre AJ-AS (ZHANG et al., 2016). Diferentemente de insetos, algumas espécies de ácaros fitófagos (aqueles que se alimentam de plantas) suprimem a expressão de genes responsivos aos moduladores de defesa AJ e AS (ALBA et al., 2015). Portanto, é notável que existem diversas maneiras pelas quais os herbívoros suprimem as defesas de seus hospedeiros e isso pode estar relacionado às particularidades de cada sistema oriundos do histórico evolutivo que compartilharam.

Herbívoros minadores têm uma longa história coevolutiva com seus hospedeiros. Após sua eclosão, as larvas entram no mesofilo e se alimentam do tecido interno da folha formando minas de diversas formas que podem facilmente ser observadas na face externa (SINCLAIR; HUGHES, 2010). Diante desse hábito tão característico, esses herbívoros adaptaram estratégias a fim de lidar com as defesas das plantas e melhorar as condições do seu micro-habitat. Essas estratégias envolvem a reconfiguração do hospedeiro nos diversos níveis, seja no metabolismo primário, secundário e fitohormonal, ou no transcricional (expressão e supressão de genes) (GIRON et al., 2007; SINCLAIR; HUGHES, 2008, KAISER et al., 2010; KAWAZU et al., 2012), que podem refletir em alterações locais (na mina ou folha minada) ou sistêmicas (na planta como um todo) (ROUMANI et al., 2022; ZHANG et al., 2016). No entanto, quando comparado aos herbívoros que se alimentam na parte externa das folhas, pouco se sabe sobre as interações entre herbívoros minadores e seus hospedeiros mediadas por compostos químicos, principalmente em ambientes tropicais. Além disso, pouco é explorado sobre os efeitos da herbivoria de insetos dessa guilda alimentar nas interações subsequentes com coespecíficos e heteroespecíficos.

Por outro lado, o número de estudos sobre as interações entre ácaros fitófagos e seus hospedeiros nas últimas décadas é crescente. Esses pequenos artrópodes perfuram as células das folhas de seus hospedeiros e sugam o líquido que é extravasado causando a morte celular (HELLE; SABELIS, 1985). Esse dano, embora pequeno, pode tanto acarretar a indução de defesas em seus hospedeiros moduladas pelo AJ e AS (WALLING, 2000; LEITNER et al., 2005; GRINBERG et al., 2005; ARENA et al., 2018), quanto suprimir as defesas do hospedeiro (KANT et al., 2008; SARMENTO et al., 2011; GLAS et al., 2014; ALBA et al., 2015). Essa supressão foi primeiramente descrita por Sarmento et al. (2011) que observaram um aumento significativo da taxa de reprodução de coespecíficos e um maior desenvolvimento da sua prole em plantas previamente infestadas. Posteriormente, foi mostrado que algumas espécies e

populações são capazes de silenciar as defesas dos hospedeiros através da supressão de genes marcadores de defesas, independente do acúmulo de fitohormônios, o que influencia nas interações subsequentes com coespecíficos e heteroespecíficos (GLAS et al., 2014; ALBA et al., 2015; GODINHO et al., 2016, BLAAZER et al., 2018). Assim, tem sido observado que os ácaros utilizam de diversos meios para driblar as defesas de seus hospedeiros, que podem variar até mesmo entre populações da mesma espécie (KANT et al., 2008, GLAS et al., 2014; ALBA et al., 2015; ARENA et al., 2018; SCHIMMEL et al., 2018; ZHANG et al., 2020), o que torna difícil o estabelecimento de padrões.

Essas reconfigurações que as plantas passam após a infestação de herbívoros têm efeitos nas interações com herbívoros subsequentes, sejam eles coespecíficos ou heteroespecíficos. No geral, a herbivoria induz a síntese de defesas químicas, tornando o hospedeiro mais resistente a futuros ataques (KARBAN, 2011). Em contrapartida, a herbivoria por supressores de defesas pode gerar uma cascata de consequências ecológicas por tornarem seus hospedeiros mais suscetíveis, o que varia desde a facilitação para uma segunda espécie (o que favorece a herbivoria múltipla), gerando muitas vezes a competição por recursos, até a alteração da atratividade dos voláteis aos inimigos naturais dos herbívoros (STAM et al., 2014; BLAAZER et al., 2018). No entanto, herbívoros podem responder de maneiras distintas a uma dada situação, o que faz do seu modo de alimentação e seu grau de especialização no hospedeiro importantes fatores para compreensão das interações observadas no ambiente.

No ambiente agrícola, observamos com frequência o estabelecimento de dois ou mais herbívoros se beneficiando da mesma planta. Por exemplo, em plantios de café (*Coffea arabica* L.) (Rubiaceae), o ácaro-vermelho-do-cafeeiro *Oligonychus ilicis* (McGregor) (Acari: Tetranychidae) e o bicho-mineiro *Leucoptera coffeella* (Guérin-Méneville) (Lepidoptera: Lyonetiidae) são herbívoros frequentemente encontrados em altas densidades populacionais, causando sérios danos às plantas (SOUZA; REIS; RIGITANO, 1998; FRANCO et al., 2008). O ácaro-vermelho-do-cafeeiro perfura as células da epiderme e mesófilo foliar e suga o conteúdo celular extravasado, dando as folhas da planta de café o aspecto bronzeado, o que consequentemente diminui a área disponível para fotossíntese (REIS, 1997). Mesmo sendo nativo dos EUA, essa espécie é encontrada principalmente em plantios de café no Brasil (FRANCO et al., 2008). Já a larva do bicho-mineiro se alimenta das células do parênquima paliçádico do tecido foliar, formando galerias que levam à redução da área disponível para fotossíntese e à queda prematura das folhas (SOUZA; REIS; RIGITANO, 1998). Nativo da África, o centro de origem do café, esse herbívoro se alimenta exclusivamente desta planta

(DANTAS et al., 2021). Apesar de não compartilharem um longo histórico coevolutivo, esses herbívoros interagem nos plantios de café, sendo encontrados em uma mesma área, bem como na mesma planta ou folha (TEODORO; TSCHARNTKE; KLEIN, 2009).

As respostas da planta de café frente ao ataque dos herbívoros e os efeitos sobre as suas populações são pouco exploradas, mesmo com a grande importância dessa *commodity* no cenário nacional e internacional. Recentemente, um estudo mostrou que plantas infestadas por *O. ilicis* são mais atrativas e suscetíveis à infestação pela cochonilha-branca, *Planococcus minor*, propiciando a ocorrência de herbivoria múltipla (PEÑAFLOR et al., 2019). Esses resultados sugerem que o ácaro pode suprimir as defesas da planta de café, tornando-a mais suscetível a outros herbívoros. Em relação ao bicho-mineiro, sabe-se que a concentração de cafeína na planta de café (metabólito secundário com propriedade tóxica para herbívoros) está relacionada positivamente com a sua infestação, indicando que é um composto estimulante e não tóxico para a espécie (MAGALHÃES et al., 2008). No entanto, não se sabe as alterações metabólicas que a larva minadora de *L. coffeella* desencadeia nas plantas de café que podem influenciar as interações com seus coespecíficos e heteroespecíficos, observadas tanto em condições de campo quanto em laboratório.

Nos últimos anos, estudos multidisciplinares têm apresentado importantes resultados nas diversas áreas do conhecimento, mostrando a importância dessa complementariedade de abordagens na solução de questionamentos e problemas. Nesse sentido, os trabalhos envolvendo a ecologia e a metabolômica (*ecometabolomics*) têm ganhado bastante visibilidade ao buscar as principais alterações no metabolismo primário, secundário e fitohormonal de hospedeiros frente ao dano de herbívoros que possam explicar as interações observadas (PETERS et al., 2018, VAN; VAN DER, 2018). Além disso, os resultados obtidos podem ser utilizados em programas futuros de estratégias de controle de herbívoros-praga no campo, visando a diminuição do uso de produtos químicos através do desenvolvimento de cultivares mais resistentes ou tolerantes.

Diante o exposto, a presente tese buscou investigar o efeito da herbivoria de *O. ilicis* e *L. coffeella* nas defesas induzidas de *C. arabica* e suas consequências ecológicas para coespecíficos e heteroespecíficos. Foram realizados ensaios comportamentais e da biologia de ambos os herbívoros em condições de laboratório e análises químicas de componentes do perfil metabólico das plantas de café. Esperamos que os resultados aqui apresentados contribuam para um melhor entendimento sobre as interações ecológicas mediadas por compostos químicos e

que possam ser utilizados no futuro para o desenvolvimento de uma agricultura mais sustentável.

2. CONCLUSÃO GERAL

O primeiro artigo mostra que as plantas infestadas por *O. ilicis* foram mais atrativas e de melhor qualidade que plantas não infestadas, o que propiciou uma maior taxa de oviposição aos coespecíficos. As análises químicas sugerem que os alcaloides não exercem função de defesa nas plantas de *C. arabica* contra *O. ilicis*, no entanto, o composto fenólico ácido clorogênico é um forte candidato a exercer essa função. Essas diferenças observadas entre plantas infestadas por *O. ilicis* e plantas não infestadas possivelmente estão relacionadas ao acúmulo do fitohormônio AS e do precursor OPDA, mas não dos jasmonatos, AJ e AJ-Ile. Embora não seja possível afirmar que *O. ilicis* suprime as defesas das plantas de café, sugerimos que a conversão do OPDA em AJ e AJ-Ile não é bem sucedida nas plantas sob sua infestação, o que ainda não foi reportado na literatura envolvendo ácaros fitófagos e supressão de defesas.

No segundo artigo, mostramos que o bicho-mineiro (*L. coffeella*) desencadeou alterações nas plantas de café que beneficiaram o desenvolvimento de coespecíficos, mas não de heteroespecíficos (*O. ilicis*). Similarmente, a infestação pelo ácaro também não favoreceu o heteroespecífico, *L. coffeella*. As análises químicas mostraram que os herbívoros acarretaram respostas distintas no perfil metabólico (primário e secundário) das plantas sob infestações simples (uma espécie de herbívoro) e dupla (duas espécies de herbívoros). No geral, as plantas sob infestação simultânea da larva minadora e do ácaro não acarretaram um efeito aditivo nas concentrações de metabólitos secundários (análise *targeted*) e fitohormônios, mas uma supressão nas concentrações de diversos *features* (compostos não identificados pela análise *untargeted*). Esses resultados parecem refletir as diferentes ativações de defesas das plantas pelos herbívoros mediadas pelos fitohormônios. Enquanto as plantas infestadas pelo bicho-mineiro acumularam AS e ABA, as plantas infestadas pelo ácaro apresentaram acúmulo de AS e OPDA. No entanto, em ambos os casos não houve acúmulo dos jasmonatos AJ e AJ-Ile. Os mecanismos que desencadeiam essa possível supressão da via do AJ por ambos os herbívoros favorecem apenas os coespecíficos e não os heteroespecíficos. Além disso, esses mecanismos parecem ser distintos, já que as plantas infestadas pelo bicho-mineiro não apresentaram acúmulo de OPDA como as plantas infestadas pelo ácaro.

Os resultados aqui apresentados são relevantes no aspecto científico ao reportar possíveis supressões de defesa do hospedeiro desencadeadas de maneiras distintas por herbívoros de diferentes guildas alimentares que influenciam nas interações subsequentes; no aspecto tecnológico, ao integrar os estudos ecológicos e metabolômicos para compreender as

interações em um sistema de alta relevância para a região e para o país; e no aspecto econômico, ao gerar informações que possam ser utilizadas em futuros programas de controle de pragas mais sustentáveis e economicamente vantajosos na cultura cafeeira.

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SEGUNDA PARTE

ARTIGOS DE ACORDO COM IDENTIFICAÇÃO DO PERIÓDICO

ARTIGO 1

Mite herbivory disrupts conversion of 12-Oxophytodienoic acid (OPDA) into jasmonic acid and increases plant suitability for conspecifics

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Main Conclusion

Feeding by *Oligonychus ilicis* induced alterations on the coffee plant's phytohormone profile and secondary metabolites that turned the plants more suitable to conspecifics promoting an increase in plant attractiveness and higher oviposition rate of conspecifics.

Abstract

Some phytophagous mites can suppress plant defenses promoting greater susceptibility to new individuals not by phytohormonal levels as phloem-feeding insects, but at gene level. We have previously shown that arrival of *Oligonychus ilicis* in coffee facilitates the subsequent colonization by white mealybugs, but not the reverse direction. In this context, our study aimed at investigating the consequences of the herbivory by *O. ilicis* in the regulation of plant defenses and colonization by conspecifics. In the dual-choice tests, we found mite-infested plants were preferred by conspecifics over uninfested plants. The mite-infested plants were better hosts to mites as females deposited greater number of eggs on them than on uninfested plants. Metabolomic analyses revealed that mite feeding increased the concentration of caffeine and theophylline but suppressed the theobromine level, and reduced the chlorogenic acid level, when compared to uninfested plants. Furthermore, mite-infested plants have higher accumulation of SA (Salicylic acid) and OPDA (12-Oxophytodienoic acid), but not JA (Jasmonic acid) and JA-Ile (Jasmonic acid-isoleucine), suggesting that this mite does not induce JA defenses in coffee plants by disrupting the conversion of OPDA into JA forms. In summary, the results of this study highlight that *O. ilicis* feeding on coffee plants increases the susceptibility of the host to conspecifics that is likely related to the suppression of the JA related defenses. However, it remains to be investigated whether suppression of coffee defenses by *O. Ilicis* does occur downstream of JA and JA-Ile accumulation, in the transcript levels of JA-related defensive genes.

Keywords

Coffee plant; phytohormones; plant secondary metabolites; plant-arthropod interactions.

Introduction

The selection of suitable hosts for feeding and oviposition by arthropod herbivores is crucial for their fitness (Awmack and Leather 2002). Although host selection is a complex process that is influenced by plant cues of different nature, chemical cues play a fundamental role (Dicke 2000). The plant metabolic profile comprises diverse chemical groups that convey valuable information about host suitability for arthropod herbivores, such as plant nutritional and defense status, and presence of other herbivores (Stout et al. 2006; Mithöfer and Boland 2012).

Plants attacked by arthropod herbivores reconfigure their metabolism by reallocating energy to produce defenses and deal more intensely against the stressor. Herbivory induces plant defenses which comprise the upregulation of pre-existent secondary metabolites and the synthesis of novel anti-herbivore metabolites (Howe and Jander 2008; Mithöfer and Boland 2012). Herbivore-induced plant defenses are elicited by the perception of herbivore cues, such as those derived from oral secretions and damaged tissue (herbivore associated molecular patterns or HAMPs) (Boller and Felix 2009; Acevedo et al. 2015). After this initial recognition, a series of oxidations and enzymatic reactions are triggered that will generate active molecules of phytohormones (Wu and Baldwin 2010; Couto and Zipfel 2016). When these molecules bound in repressor complexes in the cell nucleus, they trigger the activation and subsequent transcription of defense genes, resulting in the expression of enzymes and consequently in the production of secondary metabolites and volatile compounds (Hartmann 2007; Howe and Jander 2008; Wu and Baldwin 2010). Phytohormones are metabolites involved in plant regulation processes, and the jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are the main modulators of herbivore-induced plant defenses (Pieterse et al. 2009; Erb et al. 2012). In general, herbivory by chewing insects activates the JA pathway, while phloem-feeders elicit the SA pathway, and cell-content feeders, such as mites, trigger both JA and SA pathways (Howe and Jander 2008; Kaloshian and Walling 2005; Kant et al. 2008; Schimmel et al. 2017). Despite that general pattern, chemical changes in the metabolic profile of plants elicited by herbivory are specific according to the identity of the herbivore since there are other signaling molecules and interaction among them that regulate the expression of induced defenses (Kessler and Halitschke 2007; McCormick et al. 2012).

In this context, herbivore-damaged plants are expected to be more resistant to subsequent infestations by herbivores, reducing their fitness due to higher concentrations of

toxic compounds and defense proteins (Walling 2000; Karban 2011; Mithöfer and Boland 2012; Kant et al. 2015). However, herbivores may prefer host plants previously colonized with herbivores because of ecological factors and changes in plant chemical profile (Horiuchi et al. 2003; Zhang et al. 2011; Martini et al. 2014; Sun et al. 2014; Su et al. 2018; Xu and Turlings 2018; Silva et al. 2021). Anti-herbivore secondary plant metabolites, such as alkaloids, terpenes and phenols, can be toxic, anti-nutritive, deterrent or repellent to herbivores (Karban 2011; Mithöfer and Boland 2012). However, in a coevolutionary context, herbivores that feed on a restricted range of hosts have developed adaptations to deal with these compounds. Thus, compounds that are toxic to a given herbivore can be attractive or neutral to others (War et al. 2018). In addition, herbivores have also developed ways to circumvent this arsenal of plant defenses, such as suppressing the expression of induced plant defenses (Ali and Agrawal 2012; Kant et al. 2015).

This suppression can occur at phytohormonal and/or gene levels, making plants more susceptible for new attackers. Some phloem-feeders can suppress JA-modulated defenses by activating the SA pathway, this occurs due to the negative interaction between both signaling routes (Zhang et al. 2009; Zhang et al. 2011). However, phytophagous mites (cell-content feeders) have effectors in their saliva that suppress induced plant defenses by disrupting the expression of SA or JA-sensitive genes independently of hormonal crosstalk (Kant et al. 2008; Sarmento et al. 2011; Glas et al. 2014; Alba et al. 2015; Godinho et al. 2016; Villarroel et al., 2016; Schimmel et al. 2017). Consequently, mite herbivory can benefit conspecifics or heterospecifics feeding on the same plant (Sarmento et al. 2011; Alba et al. 2015; Blaazer et al. 2018).

Recently, Peñaflor et al. (2019) found that coffee plants infested by the southern red mite *Oligonychus ilicis* (McGregor) (Acari: Tetranychidae) became more susceptible to subsequent herbivore infestation and suggested that herbivory by this tetranychid mite may suppress plant defenses in coffee plants, similar to what has been shown in other systems (Kant et al. 2008; Sarmento et al. 2011; Glas et al. 2014; Alba et al. 2015; et al. 2016). *Oligonychus ilicis* is not considered a specialist phytophagous mite, but in South America this species is mostly associated with coffee crops (*Coffea arabica* and *Coffea canephora*) (Reis et al.1997). It usually feeds on the adaxial surface of coffee leaves by puncturing the cells with its stylet causing damage to the cell wall and membrane, thus reducing photosynthetic area (Reis et al. 1997). Therefore, when the population reaches higher levels, *O. ilicis* can decrease crop

productivity, and its presence in the field is a reason to alert growers, who still use chemical methods to control this species.

Coffee is a commodity of great importance, but chemical ecology studies of interactions involving the coffee plant and its associated herbivores are still little explored. Considering the ability of some phytophagous mites in making plants more susceptible to herbivory and the results recently reported by Peñaflor et al. (2019), the aim of this study was to investigate the consequences of the herbivory by *O. ilicis* in *C. arabica* plants to its conspecifics. We hypothesized that herbivory by *O. ilicis* suppresses induced coffee plant defense, consequently facilitating subsequent infestation by conspecifics. We conducted behavioral assays to investigate the host preference of *O. ilicis* between uninfested and conspecific-infested plants, and its performance in terms of egg production. In line with this goal, we also quantified the level of the most important phytohormones, and secondary metabolites involved in plant defense against spider mites.

Materials and methods

Plants and insects.

Coffee seeds (*C. arabica* L. cultivar Mundo Novo) were sown in plastic seedling bags (10 x 20 cm) containing soil, substrate, and sand (2:1:1 ratio) in an insect-free greenhouse under natural oscillations of temperature and light (Lavras, MG, Brazil). Plants were watered every day and at two months old they were fertilized with macronutrients (nitrogen, phosphorus, and potassium) and micronutrients (iron, manganese, copper, and magnesium). Micronutrients were diluted at 2.5 mL. L^{-1} and macronutrients at 1 mL.L^{-1}. The solutions were sprayed at twilight to prevent leaf burn every fifteen days. Coffee plants were 7- or 8-months-old (seven or eight leaf pairs) when used in assays.

The southern red mite (*O. ilicis*) was reared in coffee leaves collected from coffee plantations in the field. In the laboratory, leaves were washed, placed in Petri dishes above water-soaked foam, and surrounded with moistened cotton to prevent mites from escaping (Reis et al., 1997). The leaves were renewed every seven to nine days. The mite colonies were maintained at 25 ± 2 ºC and 12h light.

Treatments.

Three leaves from different pairs located in the medium third of a mite-infested plant were infested with 25 *O. ilicis* females each, totalizing 75 mites per plant, which were kept for seven days in the greenhouse. The petiole of each leaf pair received a mixture of insect glue (Biocontrole, Indaiatuba, São Paulo SP) and lanolin (1:1 ratio) to prevent mites from escaping. The petiole of three leaf pairs of uninfested plants also received the glue-lanolin mixture and were kept under the same conditions but did not have mites. Mite-infested and uninfested plants were caged (50cm x 50cm) to avoid mite infestation on uninfested plants.

Host preference.

The preference of *O. ilicis* females to mite-infested and uninfested plants was assessed in dual choice tests performed in the laboratory, in dark conditions, to avoid directional bias from light incidence, and temperature of 23 ± 2 ºC. The arena used in the tests was built with a Petri dish bottom (5 cm diameter), in which a wooden T-shaped stick (18 cm width, 9 cm height) was attached to the center. A pair of uninfested and mite-infested plants were positioned in the opposite sides of the T-shaped stick. An uninfested leaf of mite-infested plant and a corresponding leaf of an uninfested plant touched the side arms of the T-shaped stick, allowing the mites released in the Petri dish bottom to reach the plants by climbing on the stick (Figure S1). Each replicate consisted in the choice of twenty *O. ilicis* females released in the Petri dish after 1h, 2h, 4h, 6h, 8h, and 24h.

To verify wether *O. ilicis* females were guided by cues from the mite-infested plant or from conspecifics, we conduct an additional host selection assay using the same methodology but testing mite-infested plant *vs.* uninfested plant + mites. The latter treatment was obtained by putting three fake leaves (green leaf-shaped paper card with a glue-lanolin mixture on the border to prevent mites from scaping) carrying 25 *O. ilicis* females on it (i.e., total of 75 mites per plant), simulating the same number of individuals and distribution of the mite-infested plant treatment (Figure S2). As mites were less mobile and died without food after 7h, we established that this experiment would be evaluated at 1h, 2h, 4h and 6h after release of 20 mites in the Petri dish.

Performance.

The performance of *O. ilicis* was measured in terms of the oviposition in non-choice tests with mite-infested plants or uninfested plants. This assay was performed in the laboratory

at 21 ± 2 ºC and 12h light. A circular area (2-cm diameter) in the uninfested leaves of the three leaf pairs of mite-infested plants and the correspondent leaves of uninfested plants were delimited by the mixture of insect glue and lanolin (Figure S3). Each area received six 3-day-old *O. ilicis* females. The number of eggs and alive females was evaluated daily for four days using a pocket magnifier (10x magnification).

Metabolomics.

Two pairs of leaves of the middle third of 12-mite-infested and uninfested plants were detached and immediately frozen in liquid nitrogen. Each leaf was individually loaded into identified pots which were taken to the lyophilizer. The leaves were lyophilized for 27h and then individually ground to a fine powder.

We analyzed secondary metabolites of mite-infested and uninfested plants using an adapted methodology from Lisec et al. (2006). Briefly, an aliquot of 10 mg of lyophilized leaf tissue was transferred to Eppendorf® tubes (2 ml capacity), where it was added 1400 µl of 80% Methanol (containing 12 µg/ml of umbelliferone, 8.46µg/ml Ribitol, and 8.46 µg/ml L-Norvaline). The Eppendorf® tubes were vortexed for 10s and shacked for 10min at 70ºC in a thermomixer at 950 r.p.m. After these processes, the microtubes were centrifuged for 10min at 11.000g and 200 µl of the supernatant were transferred to a glass vial (2 mL) with insert for analysis. The secondary metabolites were separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH C18 column (100 mm x 2.1 mm particle size of 1 µm). 7 µm) maintained at 55°C and a 20min acetonitrile aqueous gradient at a flow rate of 250 µl/min. Were used a solvent gradient HPLC grade water with 0.1% formic acid (A) and HPLC grade acetonitrile with 0.1% formic acid (B). The initial condition was 97% A and 3% B, increasing to 45% B at 10min, 75% B at 12min where it was maintained at 75% B until 17.5min before returning to the initial conditions. The eluate was transferred to a 5600 (QTOF) TripleTOF using a font where it was ionized by electrospray ionization, Duospray™ ions (all AB Sciex, Framingham, MA), in positive and negative modes. We identified and quantified five secondary metabolites from leaf tissue of mite-infested and uninfested coffee plants: caffeine (1,3,7-Trimethylxanthine), theobromine (3,7-Dimethylxanthine), theophylline (1,3-Dimethylxanthine), trigonelline (N-methylnicotinic acid), and chlorogenic acid (3-Caffeoylquinic Acid). The levels of caffeic acid were not enough for detection and quantification. Final concentration of alkaloids was quantified based on

known concentrations of the standards in MassHunter software. The amounts of the alkaloids were also normalized by the dried weight of the plant sample.

We analyzed phytohormone levels of mite-infested and uninfested plants using an adapted methodology from Christensen et al. (2014). A 10 mg aliquot of lyophilized tissue was placed in Eppendorf® tubes and added 50 µl of the internal standard solution, 750 µl of ammonium Acetate solution, and 560 µl of MeOH. Each Eppendorf® tubes was vortexed for 15-20s to fully suspend the ground tissue and placed in the sonicator for 15min. The Eppendorf® tubes were centrifuged for 10min at 20.000 RCF at room temperature and 1 mL of the supernatant was transferred to a new Eppendorf® tubes and speed-vacuum for 6-8h. The resulting material was resuspended in 100 µl of 0.01% formic acid in water, vortexed for at least 30s and placed on ice for 10min. The Eppendorf® tubes were then centrifuged ate 20.000 RCF for 10min at 4ºC and 90 µl of the supernatant was transferred to a glass vial (2 mL) with insert for analysis in a liquid chromatographer-mass spectrometer (Agilent Technologies 6550 iFunnel Q-TOF LC/MS) with a RRHD Zorbax Eclipse Plus-C18 column (100 mm length, 2.1 mm diameter, 1.8 µm particle size). The solvent gradient used was 99% A (milli-Q water + 0.1% formic acid) to 99.5% B (acetonitrile + 0.1% formic acid) over 8min with a flow rate of 0.6 ml/min. We identified and quantified the following phytohormones from leaf tissue of mite-infested and uninfested coffee plants: salicylic acid (SA - 2-hydroxybenzoic acid), OPDA (12-Oxophytodienoic Acid), jasmonic acid (JA - 3-oxo-2-2′-cis-pentenyl-cyclopentane-1-acetic acid), jasmonic acid-isoleucine (JA-Ile - (±)-Jasmonic Acid-Isoleucine) and abscisic acid (ABA - (2Z,4E)-5-[(1R)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl]-3-methylpenta-2,4-dienoic acid). Final concentrations of the detected phytohormones were quantified in the MassHunter software relative to the recovery of their internal standard (SA, ABA, JA, and JA-Ile – the former quantified based on d5-JA) or relative to the calibration curve of unlabeled standards (OPDA). Amounts of phytohormones were also normalized by the dried weight of the plant sample.

Statistical analyses.

Prior to analysis, data were checked for normality and homogeneity of variances trough Shapiro-Wilk and Barlett's tests, respectively. Mite choices in the host preference assays were analyzed by general linear mixed effect model (GLMM) with Negative Binomial distribution, including time and block as random factors and the interaction treatment*time as a fixed effect.

Mite oviposition in the performance assay were analyzed by a linear mixed effect model (LME). The data of the mean number of eggs per female per day was transformed (log1p) and included in the model as a response variable and the interaction treatment*day as the fixed effect. The variables day, plant and arena were included as random factors. We used the same analysis (LME) to analyze data of each day of experiment with treatment as the fixed effect. The amounts of secondary metabolites and phytohormones were transformed (log) when necessary and were analyzed by LME. When the data did not meet the assumptions for parametric analyses even after transformation, we used general linear mixed effect model (GLMM) with Gamma distribution. The amounts of each compound were response variables, and treatment was included as fixed effect in the model. The variables position, repetition/leaf, batch, and extraction batch were included as random effects. The analyzes were performed in software R Studio (R Core Team, 2022).

Results

Host preference

In dual-choice tests, *O. ilicis* females preferred mite-infested plants than uninfested plants as a host and this result was consistent over the time evaluated (Fig. 1, GLMM, treatment: $P < 0.001$, chisq = 22.42; time: $P < 0.001$, chisq = 144.66; treatment*time: $P = 0.99$, chisq = 0.36). When time points were analyzed individually, *O. ilicis* preferred mite-infested plants at 2h, 8h, and 24h (Table S1).

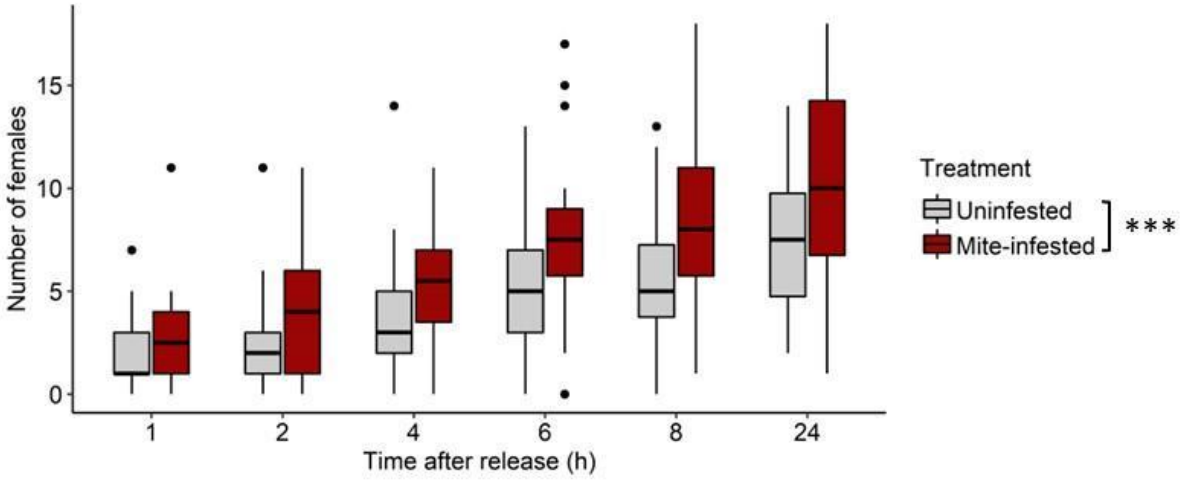

Figure 1 – Host preference of the southern red mite (*Oligonychus ilicis*) females to uninfested and mite-infested coffee plants along the time course in dual choice tests (number of females) (n = 24).

To evaluate wether *O. ilicis* preference to mite-infested plants was guided by chemical cues derived from plants or by the mites themselves, we conducted an additional host preference assay. Females did not show preference between the treatments uninfested + mite and uninfested coffee plants along the time course or at any time point (Fig. 2 and Table S2, GLMM, treatment: $P = 0.427$, chisq = 0.63; time: $P < 0.001$, chisq = 57.44; treatment*time: $P = 0.990$, chisq = 0.11). This result confirmed that the preference of *O. ilicis* females to mite-infested plants were not influenced by odors emitted from conspecifics.

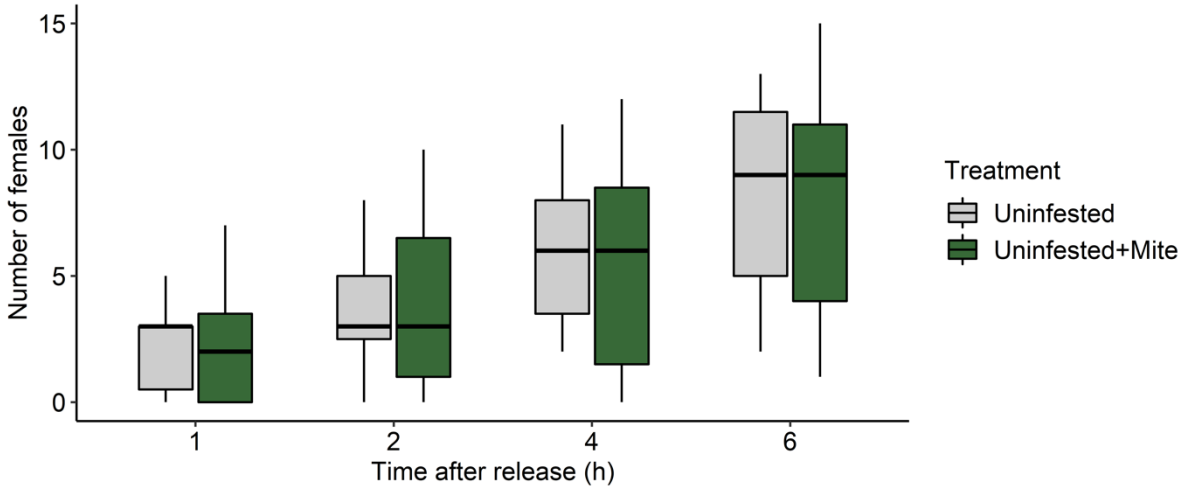

Figure 2 – Host preference of the southern red mite (*Olygonichus ilicis*) females between uninfested and uninfested plants + mite during the time in dual choice tests (n = 15).

Performance

Females of *O. ilicis* deposited more eggs per day on mite-infested plants than uninfested plants along the time course (Fig. 3, LME, treatment: $P < 0.01$, chisq = 8.82; time: $P < 0.05$, Chisq = 8.00; treatment*time: $P = 0.36$, chisq = 3.20). When days were analyzed individually, the oviposition was greater on mite-infested plants at day 2 (LME; day 1: $P = 0.88$; day 2: $P < 0.001$; day 3: $P = 0.080$; day 4: $P = 0.134$).

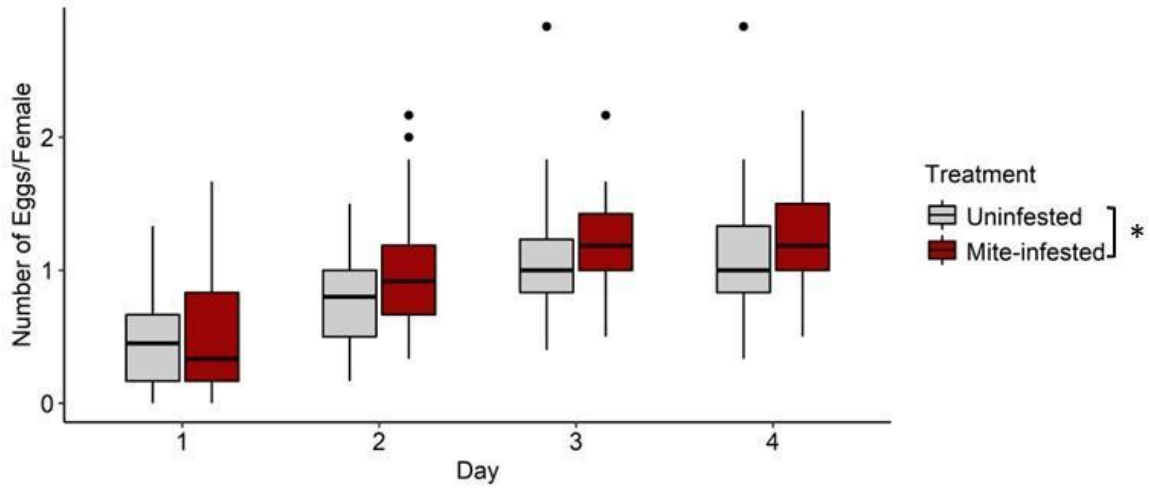

Figure 3 – Performance of the southern red mite (*Olygonichus ilicis*) females assessed in terms of number of eggs per female along four days in mite-infested and uninfested coffee plants in no-choice assays (n = 12).

Secondary metabolites

Mite-infested plants produced higher levels of caffeine and theophylline than uninfested plants (Fig. 4A, LME, caffeine: $P < 0.001$; Fig. 4C, theophylline: $P < 0.001$). However, mite-infested plants had lower amounts of theobromine (0.01673 ± 0.001) compared to uninfested (0.0196 ± 0.0009) plants (Fig. 4B, LME, theobromine: $P < 0.05$). A similar trend was observed for chlorogenic acid, which was down-regulated by 22% in mite-infested plants, even though the difference was not significant (Fig. 4E, GLMM, P = 0.059). Mite-infested and uninfested contained similar amounts of trigonelline (Fig. 4D, LME; $P = 0.63$).

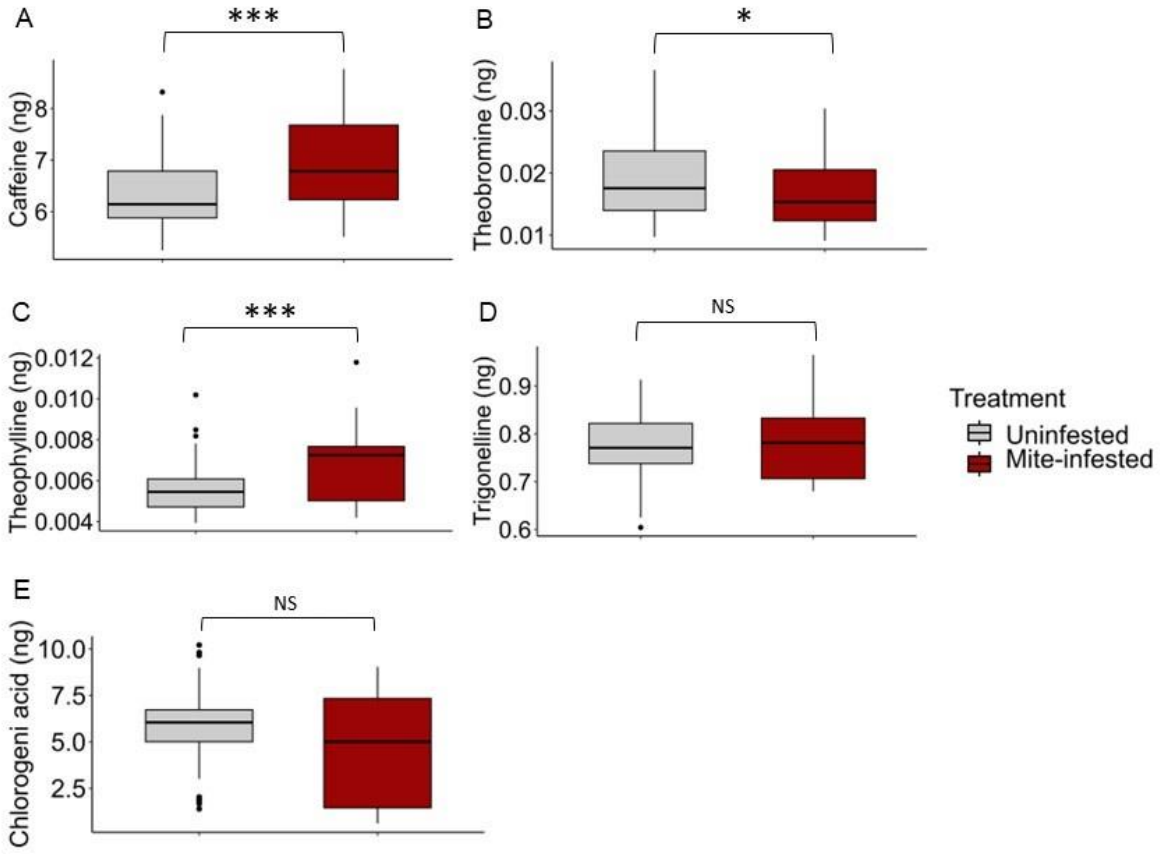

Figure 4 – Secondary metabolites content (ng. g^{-1}) of uninfested and mite-infested coffee plants (n = 12). A) Caffeine; B) Theobromine; C) Theophylline; D) Trigonelline; E) Chlorogenic acid.
NS = non significant, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, according to linear mixed effect model (LME).

Phytohormones

Mite-infested plants had increased levels of SA relative to uninfested plants (Fig. 5A, LME, $P < 0.001$), but the amount of abscisic acid was similar between treatments (Fig. 5B, LME, $P = 0.16$). Feeding by *O. ilicis* also caused accumulation of the JA precursor OPDA (Fig. 5C, LME, $P < 0.05$) but did not alter levels of JA and JA-Ile (Fig 5D, LME, JA: $P = 0.88$; Fig 5E, LME, JA-Ile: $P = 0.52$).

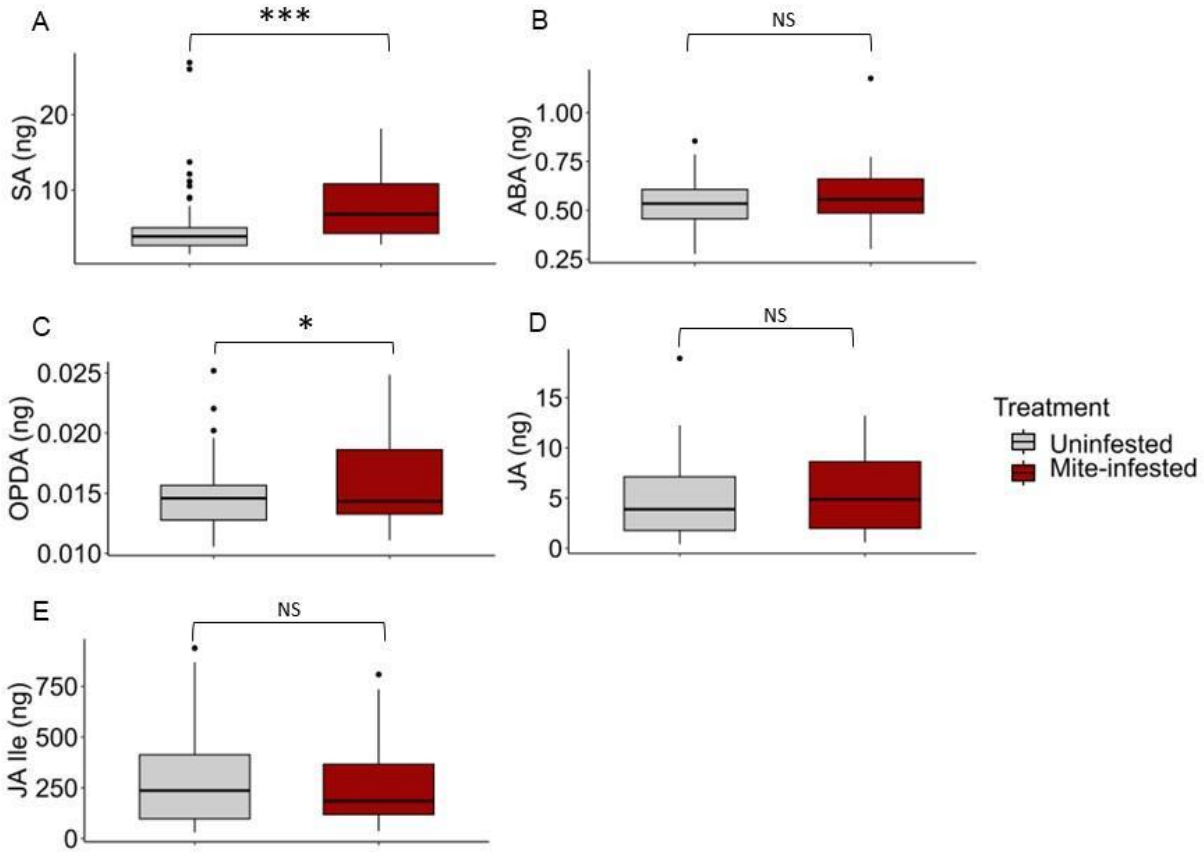

Figure 5 –Levels (ng.g^{-1}) of phytohormones and precursors in uninfested and mite-infested coffee plants (n = 12). A) Salicylic acid (SA); B) Abscisic acid (ABA); C) 12-oxophytodienoic acid (OPDA); D) Jasmonic acid (JA); E) Jasmonic acid isoleucine (JA-Ile). NS = non significant, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, according to linear mixed effect model (LME).

Discussion

The literature has shown that herbivory by phytophagous mites facilitates infestation by conspecific and heterospecific individuals (Sarmento et al. 2011; Glas et al. 2014; Alba et al. 2015; Godinho et al. 2016). However, several mechanisms may be involved in that susceptibility, such as the specificity of the plant's response to the herbivore, the communication between signaling pathways mediated by phytohormones, and the suppression of defenses (Musser et al. 2002; Zarate et al. 2007; Kant et al. 2015). We previously found that *O. ilicis*-infested coffee plant benefited the white mealybug *Planococcus minor* (Hemiptera: Pseudococcidae) (Peñaflor et al., 2019), but neither changes in plant chemistry nor the effects for the conspecifics themselves have been addressed. Here, we found that feeding by *O. ilicis* increased the susceptibility of coffee plants to conspecifics and altered the concentration of

some alkaloids and the chlorogenic acid, likely modulated by the accumulation of SA, but not of the active form of JA.

Because aggregations of spider mites feeding on the same leaf are commonly observed (Helle and Sabelis 1985; Yano 2012), we confirmed that the attraction of *O. ilicis* to mite-infested plants was not influenced by conspecific-derived odors in dual choice assays testing the attractiveness of uninfested coffee plants added by southern red mites. Herbivory by *O. ilicis* in coffee plants elicits a distinct composition of volatile emission that is highly attractive to a predatory mite (Andrade FM, manuscript in preparation). Although we did not test olfactory response of *O. ilicis* to coffee plant volatile emissions separately from gustatory and tactile cues, our results indicate that volatile emission of mite-infested plants is likely attractive to *O. ilicis* females, since they preferentially selected them at early time points in the assay, a preference that was maintained along the time course.

As a result of the activation of defenses against herbivory, there is also the production of non-volatile secondary metabolic compounds that can impair herbivore feeding, reproduction, and survival (Walling 2000; Howe and Jander 2008). We found that the herbivory of *O. ilicis* elicited the elevation of two of the four alkaloids evaluated, caffeine and theophylline, but suppressed the level of theobromine compared to uninfested plants. The concentration of the alkaloid trigonelline, which is a product of nicotinic acid biosynthesis (Ashihara et al. 2015), was not altered by the mite feeding. The alkaloids caffeine, theobromine, and theophylline are compounds involved in the biosynthesis of purine alkaloids (Ashihara and Crozier 1999). Theobromine is the substrate for the synthesis of caffeine (Ashihara and Crozier 1999), and its lower level in mite-infested plants is likely due to the higher level of caffeine. Theophylline is a product derived from caffeine catabolism, but it can be used in *de novo* synthesis of caffeine (Ashihara and Crozier 1999; Ashihara et al. 2008). Thus, an increase in caffeine levels is expected to result in higher levels of theophylline, as we observed in mite-infested plants. Although it is known for its repellent and toxic effect on several arthropod herbivores (Kim et al. 2006; Ashihara et al. 2008), caffeine is not considered a defensive compound of coffee plants against specialist herbivores, and it can even be an oviposition-stimulating plant chemical (Guerreiro Filho and Mazzafera 2003; Magalhães et al. 2008). As specialist herbivores of coffee plants, strategies to deal with caffeine have evolved as adaptations to it (Ceja-Navarro et al. 2015). Although *O. ilicis* is not considered a specialist herbivore of coffee plants, in Brazil its occurrence as a pest is limited to coffee crops (Reis et

al. 1997; Moraes and Flechtmann 2008). This intrinsic interaction between *O. ilicis* and coffee plants may have selected physiological adaptations or associations with symbiotic microorganisms that allow it to feed and survive even in host plants with higher caffeine concentrations. Therefore, our results suggest that this alkaloid does not seem to play a defensive role against *O. ilicis,* and future studies may investigate the mechanisms underlying this adaptation.

The suppression of defenses, such as the reduction of defensive compounds, can facilitate the establishment of subsequent herbivores in the plant (Kant et al. 2015). Besides alkaloids, phenols are important compounds that can act as defense against herbivores (War et al. 2018). The chlorogenic acid is a phenolic compound derived from the phenylpropanoid pathway (Niggeweg et al. 2004) and is associated with plant resistance against herbivores, including cell-content feeders, such as mites and thrips (Hoffland et al. 2000; Leiss et al. 2009; Hata et al. 2019). Interestingly, we observed a 22% reduction of the phenolic compound chlorogenic acid in mite-infested plants compared to uninfested plants. Although not significant, this reduction may reflect an improvement in the quality of *C. arabica* for *O. ilicis*, which led to a higher number of eggs in plants previously infested by their conspecifics.

The production of secondary compounds results from the activation of signaling pathways and their interactions. Evidence available in the literature shows that SA is related to higher levels of caffeine in cocoa leaves (Aneja and Gianfagna 2001), and in the transcription of genes involved in caffeine biosynthesis in *Coffea canephora* (Kumar et al. 2015). In contrast, activation of both SA and JA signaling pathways may be involved in the biosynthesis of phenolic compounds, although JA seems not to be involved in the regulation of chlorogenic acid synthesis (Nandi et al. 2003; Lee et al. 2017). As the herbivory by *O. ilicis* elicited high levels of SA, but not JA, it seems likely that the level of these phytohormones regulated the synthesis of caffeine and the reduction of amounts of acid chlorogenic acid.

In general, herbivory by cell-content feeders, such as mites, activates both the SA and JA signaling pathways in their hosts (Walling 2000; Leitner et al. 2005; Grinberg et al. 2005; Arena et al. 2018). In contrast, our results show that infestation by the red spider mite in coffee plants cause accumulation of SA, but not JA, suggesting a negative cross-talk between these two signaling pathways. Sap-sucking insects are well-known to induce the SA pathway leading to the suppression of the JA pathway as a strategy to reduce induced plant defenses (Zarate et al. 2007; Zhang et al. 2009; Zhang et al. 2015). Up to date, the known mechanism by which

herbivory by phytophagous mites suppress induced plant defenses is at the level of expression and transcription of JA-responsive genes, downstream of JA synthesis (Glas et al. 2014; Alba et al., 2015). Although we did not measure plant gene expression, our results suggest a novel mechanism underlying JA suppression by herbivory.

There are several different ways that the induction of salicylic acid can interfere with the JA signaling pathway (Pieterse et al. 2009; Leon-Reyes et al. 2010; Wei et al. 2014). Although the herbivory of *O. ilicis* did not elicit accumulation of JA and JA-Ile, it increased the level of the JA precursor OPDA compared to uninfested plants. OPDA, which is produced in the peroxisome, is reduced by the OPR3 (OPDA reductase3) enzyme, and subsequently occurs three beta oxidations (by the Carboxylic Acid Side Chain – ACX, MFP, KAT) until it is converted into JA (Wasternack and Strnad 2016). OPDA accumulation and the absence or low levels of JA are observed in mutant plants lacking the OPR3 enzyme, which are more susceptible to pathogens and herbivores (Bosch et al. 2014; Guo et al. 2014; Scalschi et al. 2015; Varsani et al. 2019). This similarity in JA and JA-Ile levels between uninfested and mite-infested coffee plants suggests that the conversion of OPDA to JA is not successful in the presence of *O. ilicis*. As JA is an important mediator of plant defenses against herbivores, including phytophagous mites (Ament et al. 2004; Luypaert et al. 2015), this condition of low level of jasmonates in the mite-infested plants is likely responsible for the susceptibility to conspecific mites as well as other herbivores (Peñaflor et al. 2019).

In summary, our study showed that *O. ilicis* feeding induces a greater susceptibility of coffee plants to conspecifics that coincides with a lack of accumulation of JA and a reduction of theobromine and chlorogenic acid. There are likely several other changes in the secondary metabolism of coffee plants that were not measured in our study. Here, we propose a novel mechanism of suppression of JA-regulated defenses by phytophagous mites, in which mite feeding down-regulates one or more enzymes involved in JA synthesis from OPDA. It is important that future work tests this hypothesis by quantifying gene expression involved in JA synthesis and those responsive to JA in *O. ilicis*-infested coffee plants.

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SUPPLEMENTARY INFORMATION

Figures

Figure S1- Arena used to test the preference of the southern red mite (*Oligonychus ilicis*) between conspecific-infested and uninfested coffee plants. A wooden T-shaped stick (18 cm width, 9 cm height) attached to the center of a Petri dish bottom (5 cm diameter).

Figure S2 - Arena used to test the preference of the southern red mite (*Oligonychus ilicis*) between uninfested and uninfested+mite plants. The treatment "uninfested + mite" consisted of three fake leaves (green leaf-shaped paper card with a glue-lanolin mixture on its border to prevent mites from escaping) each carrying 25 *O. ilicis* females on it. The uninfested plant received three fake leaves with no mites.

Figure S3 - A circular arena (2 cm diameter) delimited by a mixture of insect glue plus lanolin used in the performance assay of the southern red mite (*Oligonychus ilicis*). Each arena received six 3-day-old *O. ilicis* females and each plant received three arenas in three leaves.

Tables

Table S1. Mean number (±SE) of females in each treatment per time during host selection between uninfested and mite-infested plants.

Time (h)	Uninfested plant	Mite-infested	*P value*
1	1.833 ± 0.354	2.708 ± 0.505	0.089
2	**2.500 ± 0.481**	**3.916 ± 0.583**	**0.044 ***
4	3.750 ± 0.629	5.083 ± 0.696	0.160
6	5.041 ± 0.698	7.333 ± 0.865	0.052
8	**5.791 ± 0.653**	**8.541 ± 0.914**	**0.014 ***
24	**7.458 ± 0.787**	**10.416 ± 0.938**	**0.017 ***

Significant p-values are highlighted in bold according to GLMM with negative binomial distribution (* $p < 0.05$).

Table S2. Mean number (±SE) of females in each treatment per time during host selection between uninfested and uninfested plants + mite.

Time (h)	Uninfested	Uninfested + Mite	*P value*
1	2.200 ± 0.438	2.066 ± 0.556	0.858
2	3.666 ± 0.590	3.600 ± 0.838	0.930
4	5.866 ± 0.742	5.200 ± 1.065	0.633
6	8.333 ± 0.983	8.066 ± 1.177	0.868

ARTIGO 2

Simple and multiple infestations by a leaf miner and a mite induce distinct alterations in host plant metabolic profile affecting subsequent interactions

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(Versão preliminar do artigo redigido conforme norma do periódico científico *Functional Ecology*)

Abstract

1. Plants under herbivore infestation reconfigure their metabolism, which may or may not benefit conspecifics and heterospecifics. In general, leaf miner larvae are restricted to female host selection for oviposition, and therefore have adapted strategies to improve host conditions in their favor. However, little is known about plant-mediated effects caused by leaf miner herbivory on subsequent interactions with herbivores.
2. Our study investigated whether leaf miner *Leucoptera coffeella* herbivory favored colonization and establishment of conspecifics and heterospecifics (*Oligonychus ilicis*) in *Coffea arabica*, and possible changes in the metabolic and phytohormonal profile of the plant involved in the defense response to simple and multiple infestations.
3. Behavioral assays were performed with herbivores and targeted and untargeted analyzes of the metabolic profile, as well as the phytohormonal profile of coffee plants were conducted by liquid and gas chromatography-mass spectrometry.
4. Leaf miner infestation in coffee plants shortened the development period of conspecifics and negatively affected the mite oviposition. *L. coffeella* did not discriminated between plants infested with mites and non-infested, but mite-infested promoted lower food consumption by leaf miners. While leaf miner feeding induced the accumulation of SA and ABA, the mite induced the accumulation of SA and OPDA in the leaf tissue of coffee plants. Although the secondary metabolites investigated in the targeted analysis do not appear to exert defense functions for the two herbivores given their similar concentrations, it could be that the differential activation of phytohormones caused changes in the primary and secondary metabolic profiles observed in the untargeted analyzes. Furthermore, our results show that simultaneous infestation by leaf miner and mite promoted a greater number of features with lower concentrations than that observed for other treatments.
5. Our results showed that *L. coffeella* infestation in coffee plants favored their conspecifics, but not their heteroespecifics (*O. ilicis*). On the other hand, *L. coffeella* was also negatively affected by the heteroespecifics. We highlight the importance of metabolomics and ecology integration in plant-herbivore interactions studies and contributed to a better understanding of interactions between leaf miners and subsequent herbivores.

Key words: ecometabolomics, induced defenses, *Leucoptera coffeella*, primary and secondary metabolites, suppression of defenses, *Oligonychus ilicis*.

Introduction

Herbivores use chemical cues from plants as sources of information during host selection (Dicke, 2000; Anton & Cortesero, 2022). In general, after arrival, the herbivore evaluates the quality of the host in terms of nutritional suitability and levels of defense, to decide whether to stay or continue the search (Schoonhove et al., 2005). However, there are species that have different feeding habits during their development stages, such as some leaf miner insects, whose females do not feed on the host in which they lay their eggs. Therefore, larvae develop and feed inside the leaves of hosts chosen by the females during oviposition (Sinclair & Hughes, 2010). However, contrary to the preference-performance hypothesis that predicts that females choose hosts that maximize offspring success (Levins & MacArthur, 1969; Thompson, 1988), females do not always choose good quality hosts (Valladares & Lawton, 1991; Sotelo-Cardona et al., 2021).

Host plant quality involves not only the nutritional condition, but also its defense levels (Awmack & Leather, 2002; Mithöfer & Boland, 2012). Herbivory triggers the activation of induced defenses that act directly and indirectly on the herbivore by non-volatile and volatile chemical compounds (Chen, 2008; War et al., 2012). These defenses are modulated by phytohormone signaling pathways, with jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) being the most well-known. However, abscisic acid (ABA), cytokinins (CKs), gibberellins (GAs), among others, are also relevant in phytohormonal regulation during the activation of plant defenses against herbivory (Pieterse et al., 2009; Erb et al., 2012). Although plant defense responses are highly specific to the attacking herbivore, activation of signaling pathways is generally associated with the herbivore's feeding guild. Thus, JA predominantly acts in plant defenses against chewing herbivores, while SA predominantly acts in defenses against suckers (Zarate et al., 2007; Howe & Jander, 2008). Other herbivores, such as cell content feeders and leaf miners, seem to activate both pathways (Zhang et al., 2016; Schimmel et al., 2017; Zhang et al., 2020; Yang et al., 2021). Thus, plants previously colonized by herbivores have their metabolism reconfigured, which may or may not benefit subsequent attackers. Previous infestation by herbivores can induce the synthesis of compounds in their hosts that make them more resistant to new attacks (Poelman et al., 2008). In contrast, herbivory can suppress the synthesis of other secondary metabolites, leading to greater susceptibility to herbivores that are affected by such metabolites (Kant et al., 2015).

Because they have an intimate relationship with their host, leaf miner herbivores are expected to be able to reconfigure the metabolism of the host plant in their favor through phytohormonal regulation. These insects seem to have adapted strategies to make their microenvironment more favorable or to deal with potential challenging conditions. For example, leaf miners can cause reallocation of primary metabolites to the mined area, alterations of the phytohormonal profile, as well as the expression of responsive genes, and the symbiotic interactions with bacteria that help to reconfigure the metabolic profile of the hosts (Kawazu et al., 2012; Body et al., 2013; Zhang et al., 2012; Zhang et al., 2016). However, the effects of these strategies on subsequent herbivores, whether conspecific or heterospecific, have not yet been explored.

Larvae of *Leucoptera coffeella* Guérin-Méneville (Lepidoptera: Lyonetiidae) are leaf miners of plants of the genus *Coffea*. In addition to being a specialist herbivore, *L. coffeella* is also considered cosmopolitan, since its larvae cause serious damage to coffee production in *Coffea arabica* and *Coffea canephora* plantations, mainly in Neotropical producing regions (Souza et al., 1998; Pantoja-Gomez et al., 2019; Dantas et al., 2021). During its larval stage, *L. coffeella* feeds on the leaf parenchyma of coffee plants, forming mines and tunnels in the leaf mesophyll. Its feeding habits lead to a decrease in the available photosynthetic area of the plant, which can influence crop productivity (Souza et al., 1998; Guerreiro Filho, 2006). Although its control in the field is difficult, studies on chemical-mediated interactions between the coffee plant and *L. coffeella* are scarce, and more efforts can help in the development of new management tactics for this insect.

In addition to *L. coffeella*, the southern red mite, *Oligonychus ilicis* (McGregor) (Acari: Tetranychidae), is a spider mite commonly associated with coffee plants in Brazil (Reis et al., 1997; Franco et al., 2008). Although this phytophagous mite is not currently an important pest in coffee crops, regions with favorable climatic conditions for both species (dry and hot) promote their co-occurrence in the field (Pereira et al., 2007; Teodoro et al., 2009). Thus, they are herbivores that interact with each other in the field. Our work aimed to investigate whether herbivory by the leaf miner favors the colonization and establishment of conspecifics and heterospecifics (southern red-spider mite) in *C. arabica*, and what are the possible defense mechanisms and plant metabolites involved in the response of subsequent herbivores. As *O. ilicis* infested plants have already been reported in the literature to be more susceptible to another herbivore (Peñaflor et al., 2019), we also investigated the responses of *L. coffeella* by

C. arabica plants infested with the mite. In addition to behavioral assays with herbivores, we performed chemical analysis to identify metabolic changes in plants in simple and multiple infestations. The integration of ecology and metabolomics (ecometabolomics) has advanced in recent years and has shown that the global profile of plant metabolites is highly relevant to explain the interactions observed in the environment (Peters et al., 2018).

2. Materials and methods

2.1 Plants and herbivores

Coffea arabica cv Mundo Novo coffee plants with 7 to 8 months of age (7 to 8 pairs of fully expanded leaves) were used to perform the experiments. The planting and maintenance of the coffee seedlings were carried out in a greenhouse free from arthropods without light control (Lavras, MG, Brazil). Sowing was carried out in plastic bags (20 x 10 cm) containing a mixture of soil, sand, and substrate in a 2:1:1 proportion. The seedlings were irrigated daily and, from the first fully expanded pair of leaves, they were fertilized by foliar spraying with micro and macronutrients until the tests were performed.

The rearing of *L. coffeella* was initiated by adults that emerged from the mined leaves collected in the field. These adults were released into cages for oviposition on *C. arabica* (without any application of chemical products). Every day a sugar solution (honey + water) was provided to the adults. The rearing maintenance was carried out every seven days by adding new seedlings and cleaning the cages. Upon reaching the pupal stage, the insects were removed from the plants and placed in plastic pots with voile to the emergency. The new adults were released into cages for oviposition.

The rearing of *O. ilicis* was initiated by individuals from established rearing (EPAMIG). Mites were kept on coffee leaves collected in the field without the application of chemical products. After washing, the leaves were placed in foam soaked with water and surrounded with cotton to prevent the arthropods from escaping (Reis et al., 1997). After establishing the rearing, the adults were identified again by a specialist (Dr. Paulo Rebelles Reis). Every seven days, the leaves were replaced with new leaves. The rearing of *L. coffeella* and *O. ilicis* was kept in a room with controlled temperature (25 ± 3°C) with a photoperiod of 12 hours.

2.2 Treatments

To carry out the experiments, mite-infested plants, leaf miner-infested plants, multiple-infested plants (leaf miner + mite) and non-infested plants were used.

To obtain mite-infested plant treatment, three pairs of leaves from the middle third of the plants were isolated from the petiole with a mixture of entomological glue (Biocontrole, Indaiatuba, SP, Brazil) + lanolin in a 1:1 ratio, to restrict mites feeding on these leaves. One leaf of each pair was infested with 25 adult *O. ilicis* females, totaling 75 females per plant, which fed on the plants for seven days before the experiments. To obtain leaf miner-infested plants, three leaves of the three pairs of leaves in the middle third of the plants were placed in cages made of transparent plastic cups and voile (Figure S1). In each cage, 2 copulated females of *L. coffeella* were released for 48 hours, totaling 6 females per plant. After this period, the females were removed and the eggs were counted using a pocket magnifier (10x magnification). The number of 40 to 50 eggs per plant was standardized, with a maximum of 20 eggs on a single leaf. When necessary, excess eggs were removed using cotton moistened with water. The plants remained in a greenhouse and, after hatching, the larvae fed on the leaves for seven days prior to the experiments.

To obtain the multiple-infested treatment, plants were first exposed to *L. coffeella* oviposition, as explained in the previous treatment. From hatching, the other three leaves of the middle third were infested, each with 25 females of *O. ilicis*, as also described above. Both herbivores continued to feed simultaneously on the plants for seven days.

Non-infested plants did not receive any infestation (control). Compared to mite-infested plants and multiple-infested plants, the mixture of glue + lanolin was applied on the petiole of the leaves.

2.3 Experiments and statistical analysis

2.3.1 Host selection of *L. coffeella*

Host selection of *L. coffeella* was evaluated in dual-choice tests with the following combinations of treatments: (*i*) non-infested plant *vs.* leaf miner-infested plant and (*ii*) non-infested plant *vs.* mite-infested plant. To conduct the tests, couples of newly emerged adults (up to 48h) were separated for copulation for 24h. After this period, five couples were released into

each cage (70 cm high x 48 cm wide x 48 cm deep) containing a pair of plants (one of each treatment) for 48 hours. In the end, the plants were removed and all leaves were detached and taken under a stereoscopic microscope (40x) for egg counting. The data on the number of *L. coffeella* eggs did not meet the assumptions of normality and homoscedasticity and were, therefore, analyzed using generalized linear models (GLM) with Poisson family distribution with adjustment for overdispersion (quasipoisson). To verify the difference in the number of eggs between treatments, an analysis of variance was performed. The 'Car' package was used in the statistical software R (version 4.2.2).

2.3.2 Performance of *L. coffeella*

The performance of *L. coffeella* was evaluated through the development time from egg to adult and leaf area consumed by the larva in two tests with the following combinations of treatments: (*i*) non-infested plant *vs.* leaf miner-infested plant and (*ii*) non-infested plant *vs.* mite-infested plant. The three leaves without an infestation of pairs of leaves from leaf miner-infested plants and mite-infested plants and three leaves from the middle third of the non-infested plant were placed in cages made with transparent plastic cups (Fig. S1). In each cage, two previously copulated *L. coffeella* were released, totaling six females per plant. For this test, the time of 24 hours of oviposition was standardized to reduce the variation in the development time of each stage. After counting the eggs, a single egg was left on each leaf to avoid competition for resources among the larvae. Plants and insects were observed daily to assess the duration of each developmental stage of *L. coffeella* until adult emergence. After emergence, the leaves were detached and photographed to quantify the leaf mine size given by the area of necrotic tissue using ImageJ software. Data from egg count, days at each stage of development, and damaged leaf area by *L. coffeella* were tested for normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests, respectively. When the assumptions were met, mixed linear models (LME) were fitted. When not met, mixed generalized linear models (GLMM) with Poisson (link=log) and Gamma (link=log) distributions were adjusted. In all cases, the explanatory variable treatment was considered a fixed effect, and the block and leaf variables were considered random effects. To test the differences of the above-mentioned parameters between treatments, an analysis of variance was performed. The packages 'lme4', 'hnp', 'fitdistrplus', and 'Car' were used in the statistical software R (version 4.2.2).

2.3.3 Host selection of *O. ilicis*

Host selection of *O. ilicis* was evaluated over time in a dual choice test between non-infested plants *vs.* leaf miner-infested plants. A pair of leaves from each plant was connected by a T-shaped wooden structure (18 cm wide x 9 cm high) placed in a Petri dish (20 cm diameter) containing 20 mites (Fig. S2). The leaves of each plant connected by the T structure were isolated on the petiole with a mixture of glue+lanolin to avoid dispersion of the mites throughout the plant. The evaluation of the number of mites on each leaf was performed at 1h, 2h, 4h, 6h, 8h, and 24h after release. The test was carried out in a climate-controlled room at 24 ± 2ºC in the absence of light. The data obtained from the host selection of *O. ilicis* met the assumptions of normality and homoscedasticity and were analyzed by LME. The model that best fitted the data presented the explanatory variable treatment as a fixed effect and the variables time and block as random factors. To verify the difference in the number of mites in each treatment over time, an analysis of variance was performed. The packages 'lme4', 'hnp', and 'Car' were used in the statistical software R (version 4.2.2).

2.3.4 Performance of *O. ilicis*

The performance of *O. ilicis* was evaluated through the number of eggs per female over four days in non-infested plants *vs.* leaf miner-infested plants. Circular areas (2 cm diameter) were delimited with glue+lanolin on non-infested leaves (systemic effect) and on those with an area consumed by larvae of plants infested by *L. coffeella* (local effect), as well as on correspondent leaves of non-infested plants (Fig. S3). Each circular area received six adult females of *O. ilicis* at 2 to 4 days of age, and the number of eggs and females that survived was evaluated for four days. The data obtained did not meet the assumptions of normality and homoscedasticity. Thus, data transformation (x +1) and model adjustment (GLMM) were performed using the Gamma distribution. The model that best fitted these data presented the explanatory variable treatment as a fixed effect and the variables evaluation time, block, and sheet as random effects. To evaluate the difference in the number of eggs/female over the days between treatments, an analysis of variance was performed followed by the post-hoc Tukey test. The packages 'lme4', 'hnp', 'fitdistrplus', 'Car', and 'multcomp' were used in the statistical software R (version 4.2.2).

2.3.5 Metabolomics

A leaf from the middle third of the non-infested plants and an infested leaf in the corresponding position of mite-infested plants and leaf miner-infested plants were detached and immediately frozen in liquid nitrogen. For multiple-infested plant treatment, the corresponding pair of leaves was detached, including one leaf with leaf miner infestation and the other with mite infestation. After being frozen, the leaves were transferred to individual vials and placed in the lyophilizer. When completely dry (27 hours), the leaves were individually macerated to powder thickness and transferred to 2ml microtubes.

The amounts of the phytohormones salicylic acid (SA), jasmonic acid (JA) and its precursor, 12-oxophytodienoic acid (OPDA), and the derivative JA- Isoleucine (JA Ile) and abscisic acid (ABA) of the foliar samples of non-infested, leaf miner-infested, mite-infested, and multiple-infested plants were measured. Phytohormones were extracted by adding 650 ml of 100% methanol, 750 mL of 10 mM ammonium acetate, and 50 ml of 1ng/mL isotope-labeled standards (d6-ABA, d5-JA, d4-SA) to Eppendorf® tubes containing the lyophilized sample of coffee plants. The Eppendorf® tubes were vortexed for 20 sec, sonicated for 15 min, and centrifuged for 10 min at 20,000 g at room temperature. Subsequently, 1 mL of the supernatant was transferred to a new 2 mL Eppendorf® tube and dried under vacuum for 8 hours. After this period, the samples were resuspended in 100 mL of 0.1% formic acid and vortexed for 30 sec. Eppendorf® tubes were incubated on ice for 10 min and then centrifuged at 20,000 g for 10 min at room temperature. Finally, the samples were transferred to a 2 mL glass screw cap vial with insert for chemical analysis. After extraction, 4 µl of each sample was injected into the Q-TOF LC-MS (Agilent Technologies 6550 iFunnel) with a Zorbax Eclipse Plus-C18 RRHD column (100 mm length, 2.1 mm diameter, 1.8 µm of particle size). The solvent gradient used was from 99% A (milli-Q water + 0.1% formic acid) to 99.5% B (acetonitrile + 0.1% formic acid) over 8 minutes with a flow rate of 0.6 mL/min. Final concentrations of free phytohormones were quantified in the MassHunter software in relation to the recovery of its internal standard (SA, ABA, JA and JA-Ile) or in relation to the calibration curve of unlabeled standards (OPDA). The amounts of phytohormones were also corrected by the dry weight of the plant sample.

Metabolites from non-infested, leaf miner-infested, mite-infested and multiple-infested plants were extracted following a methodology adapted from Lisec et al. (2006). A 10 mg aliquot of lyophilized leaf tissue was transferred to Eppendorf® tubes (2 mL), where 1400 µl

of 80% Methanol (containing 12 µg/mL of umbelliferone, 8.46 µg/mL of Ribitol and 8.46 µg/mL L-Norvaline). The Eppendorf® tubes were vortexed for 10 sec and incubeted for 10 min at 70°C in a thermomixer at 950 r.p.m. After these processes, the Eppendorf® tubes were centrifuged for 10 min at 11,000g and 200 µl of the supernatant were transferred to a glass vial (2 mL) for analysis of secondary metabolites. For the analysis of primary metabolites, the remaining content in the Eppendorf® was resuspended and transferred to a glass vial (4 mL), where 1500 µl of chloroform (containing 10 µg/mL of docosanol) was added and vortexed for 20 sec. The vials were incubated at 50°C for 30 min and then vortexed for another 10 sec. The samples were again incubated at 50ºC for another 25 min and then 1000 µl of dH2O were added and vortexed for 10 sec. Another incubation process was performed at 50°C for 30 min. After this period, the vials were centrifuged for 15 min at 4000 rpm to separate the polar and non-polar phases. And then 1 mL of the polar phase was transferred to a 1.5 mL glass, which was taken to complete drying under vacuum for 3 hours, and 1 mL of the non-polar phase to another 1.5 mL glass, which was completely dried using nitrogen for 45 min. The apolar phase was resuspended in 70 µl of pyridine and derivatized with 30 µl of MSTFA+1%TMCS (Sigma-Aldrich), after incubation for 1 hour at 50°C, the sample was then transferred to a glass vial for analysis in the GC- MS. The polar phase was resuspended in pyridine containing 15 mg/mL methoxyamine-HCL, vortexed and sonicated between two incubation steps at 50 °C. The polar phase was derivatized with 50 µl of MSTFA+1%TMCS for 1 hour at 50 °C and then transferred to a new glass vial for GC-MS analysis. Polar and non-polar metabolites were analyzed using an Agilent 7890 GC attached to a 5975 MSD. Compounds were injected at 230 °C and separated on an HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness; Agilent) using the following temperature program: Starting at 70°C (for 5 min), the temperature was increased by 5 °C/min to 315 °C (for 12 min) final temperature. 1.0 mL of polar metabolites were injected using a split ratio of 2:1, while 0.3 µL of non-polar extraction phase was injected in splitless mode. Carrier gas helium was supplied at a constant flow rate of 1.0 mL/min. The mass spectrometer was operated in electron impact mode (70 eV: Transfer line: 250 °C (polar phase), 230 °C (non-polar phase): Source 230 °C: Quadropole 150 °C, scan range mass: 50–650). Deconvolution algorithms (extraction and correlation) were applied to the total ion chromatograms (TICs) of the samples (MassHunter Workstation, Qualitative and Quantitative Analysis software B.06.00; Agilent Technologies). Compounds were identified by comparing deconvoluted mass spectra with spectra in the NIST14 (National Institute of Standards and Technologies) spectral library, and relatively quantified based on internal standards (25 µg/mL

ribitol for polar phase, 10 μg/mL non-polar standard of docosanol). Secondary metabolites were separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH C18 column (100 mm x 2.1 mm particle size of 1 μm). 7 μm) maintained at 55 °C and a 20 min acetonitrile aqueous gradient at a flow rate of 250 μl/min. Solvent A was HPLC grade water with 0.1% formic acid and solvent B was HPLC grade acetonitrile with 0.1% formic acid. The initial condition was 97% A and 3% B, increasing to 45% B at 10 min, 75% B at 12 min where it was maintained at 75% B until 17.5 min before returning to the initial conditions. The eluate was transferred to a 5600 (QTOF) TripleTOF using a font and Duospray™ ions (all AB Sciex, Framingham, MA). The capillary voltage was set at 5.5 kV in positive ion mode and 4.5 kV in negative ion mode, with a breakdown potential of 80V. The mass spectrometer was operated in IDA (Information Dependent Acquisition) mode with a 100 ms search sweep from 100 to 1200 m/z and up to 20 MS/MS product ion scans (100 ms) per duty cycle using an energy collision of 50V with a propagation of 20V. For untargeted analysis, raw LC-MS files were loaded into online XCMS (Tautenhahn et al. 2012) for retention time alignment, automatic integration, and feature detection using default parameters. For the targeted analysis, we identified and quantified five secondary metabolites from the leaf tissue of coffee plants: caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), trigonelline (N-methylnicotinic acid) and chlorogenic acid (3-caffeoylquinic acid). The final concentration of metabolites was quantified on known concentrations of the standards in the MassHunter software. Furthermore, the amounts of metabolites were normalized by the dry weight of the leaf tissue sample.

The data obtained from the amount of phytohormones, and secondary metabolites (targeted analysis) were tested for normality and homoscedasticity. When not met, the data was transformed into a logarithmic scale and later analyzed using LME. If even after transforming they did not meet the assumption, the data were analyzed by GLMM. In all models, the explanatory variable treatment was considered as a fixed effect and the variables block (referring to two days of sample preparation) and extraction block (referring to the days of extraction of phytohormones and metabolites) were considered as random effects. To evaluate the difference in the amounts of phytohormones and secondary metabolites between treatments, an analysis of variance was performed followed by the post-hoc Tukey test. Data obtained from primary and secondary metabolism (untargeted analysis) of coffee plants were analyzed using multivariate analysis. A discriminant analysis of principal components (DAPC) was used,

which applies discriminant analysis to the principal components to find the features (the combination of mass - mz – and retention time – rt - of each metabolite found) that most contribute to the separation of groups (Jombart et al., 2010). In addition to the previous analysis, we performed PERMANOVA, using the Bray-Curtis method with 999 permutations. Subsequently, a classification analysis was conducted through Random Forest algorithm to classify the main features that could better distinguish the pairs of treatments (ntree=10000). This method is recommended for analyzing data sets whose number of variables is considerably greater than the number of samples (Ranganathan & Borges, 2010). And finally, analyzes were carried out using linear models to identify the features that were expressed in different ways between each pair of treatments. The packages 'lme4', 'hnp', 'fitdistrplus', 'Car', 'multcomp' 'vegan', 'dplyr', 'adegenet', 'varSelRF' and 'limma' were used in the statistical software R (version 4.2.2).

3. Results

3.1 Host selection and performance of *L. coffeella*

In the dual choice tests, the *L. coffeella* females did not discriminate between non-infested plants and leaf miner-infested plants ($F_{1,22}$ = 0.06; P = 0.802; n = 13; Fig. 1A) neither between non-infested plants and mite-infested ($F_{1,24}$ = 4.12; P = 0.053; n = 14, Fig. 1B) laying a similar mean number of eggs in both treatments. However, when exposed to non-infested and mite-infested plants, *L. coffeella* laid 1.75x more eggs in mite-infested plants than in non-infested ones.

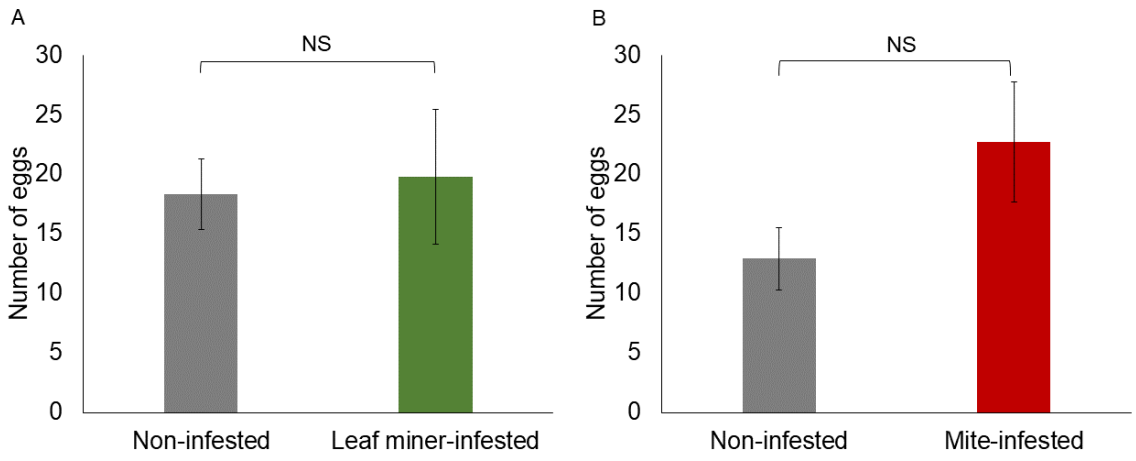

Figure 1 – Host selection for oviposition of the coffee leaf miner (*Leucoptera coffeella*) in dual choice

tests comparing: non-infested plants *vs.* leaf miner-infested plants (A); non-infested plants *vs.* mite-infested plants (*Oligonychus ilicis*) (B). NS: non significant difference.

The results of *L. coffeella* oviposition in the non-choice test were similar to those of the dual-choice tests. Females laid a similar number of eggs on the non-infested plants and leaf miner-infested plants ($\chi 2 = 1.31$, $P = 0.251$, n = 11, Fig. 2A). Embryonic, larval and pupal development times were also not significantly different between *L. coffeella* fed on leaf miner-infested *versus* non-infested plants (Table 1). However, the egg-to-adulthood period, which considers the entire immature stage, was shorter in leaf miner-infested relative to non-infested plants (Table 1). No significant differences were found in food consumption by *L. coffeella*, assessed by the size of the leaf mines, between the two treatments ($\chi 2 = 0.01$, $P = 0.744$, n = 11, Fig 2B).

In the performance tests, mite infestation did not affect neither the number of eggs ($\chi 2 = 2.38$, $P = 0.122$, n = 11, Fig. 2C) nor the egg-to-adulthood period of *L. coffeella* (Table 2). However, the leaf area consumed by the larvae of *L. coffeella* was smaller in the mite-infested plant than in the non-infested plant ($\chi 2 = 14.62$, $P < 0.001$, n = 11, Fig. 2D).

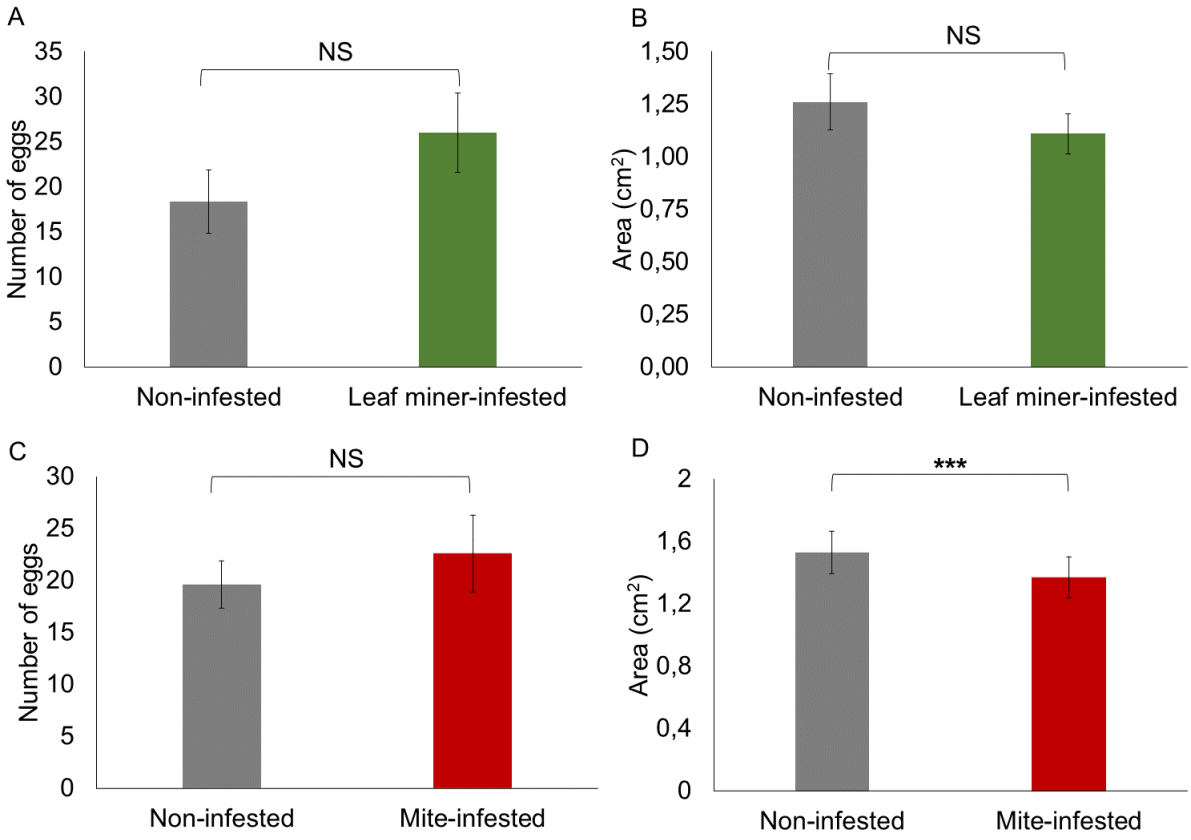

Figure 2 – Number of eggs of the leaf miner *Leucoptera coffeella* in the performance test on non-infested plants *vs.* leaf miner-infested plants (A); and mean mine area (cm^2 ± standard error) resulting from *L. coffeella* larvae feeding on non-infested plants *vs.* leaf miner-infested plants (B). Number of *L. coffeella* eggs in the performance test on non-infested plants *vs.* mite-infested plants (*Oligonychus ilicis*) (C); and mean mine area (cm^2± standard error) resulting from *L. coffeella* larvae feeding on non-infested plants *vs.* mite-infested plants (D). NS: non significant difference; *** significant $P < 0.001$.

Table 1- Duration of developmental stages of the leaf miner *Leucoptera coffeella* in non-infested *versus* leaf miner-infested plants assessed by the performance experiment.

Stage	Non-infested	Leaf miner-infested	*P value*	Chisq
Embryonic	7.3 ± 0.4	6.36 ± 0.3	0.564	0.33
Larval	15.6 ± 1.1	14.1 ± 1.0	0.370	0.80
Pupal	7.6 ± 0.3	6.64 ± 0.5	0.234	1.41
Egg-Adult	31.4 ± 1.7	28.1 ± 1.7	0.0354 *	44.25

* Significant difference ($P < 0.05$) between treatments according to LME on total performance (egg-to-adulthood) of *Leucoptera coffeella*.

Table 2- Duration of developmental stages of *Leucoptera coffeella* in non-infested versus mite-infested plants assessed by the performance experiment.

Stage	Non-infested	Mite-infested	*P value*	Chisq
Embryonic	5.9 ± 0.1	5.8 ± 0.1	0.884	0.02
Larval	17.9 ± 0.8	16.8 ± 0.5	0.588	0.29
Pupal	8.0 ± 0.3	8.4 ± 0.3	0.068	3.31
Egg-Adult	32.8 ± 1.1	32.0 ± 0.7	0.651	0.20

3.2 Host selection and performance of *O. ilicis*

When mites were tested in dual choice tests, leaf miner-infested plants were more attractive than non-infested plants over time ($\chi2 = 4.71$, $P < 0.05$, n = 11, Fig. 3). However, *O. ilicis* females deposited a lower number of eggs in leaf miner-infested plants ($\chi2 = 15.68$, $P < 0.001$, Fig. 4) compared to non-infested plants. Furthermore, there was no significant difference in the number of eggs oviposited by *O. ilicis* between leaves with or without mines of leaf miner-infested plants (local *vs.* systemic effect: $P = 0.864$), that is, the presence of *L. coffeella* in the plant was a determining factor for a lower number of mite eggs.

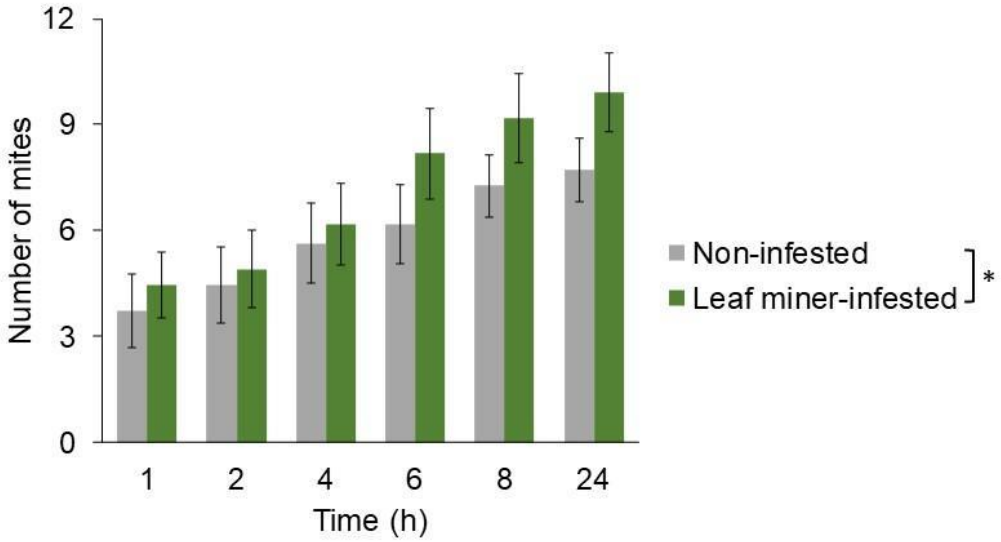

Figure 3 – Host selection of the southern red mite (*Oligonychus ilicis*) between non-infested plants *vs.* leaf miner-infested (*Leucoptera coffeella*) plants assessed in a dual test over time.

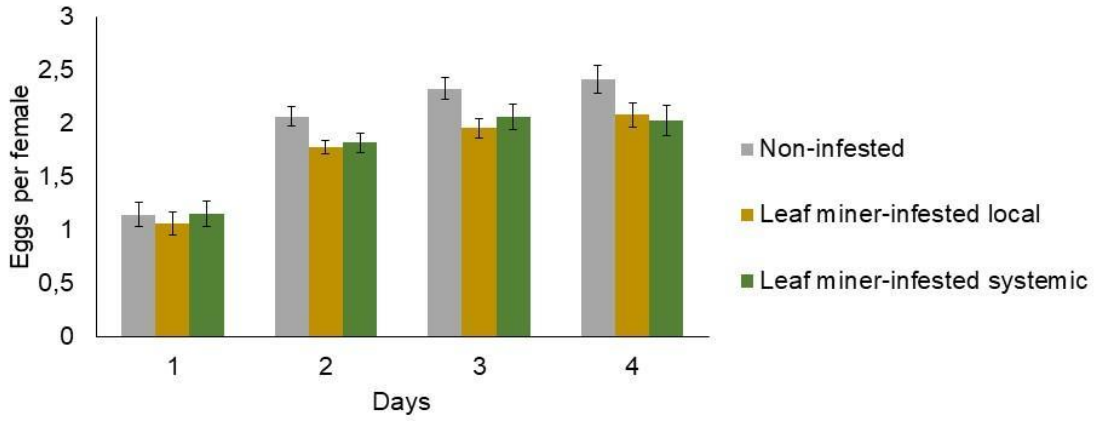

Figure 4 – Performance of the southern red mite (*Oligonychus ilicis*) assessed in terms of number of eggs per female over time on non-infested plants, mined leaves of leaf miner-infested (*Leucoptera coffeella*) plants (leaf miner-infested local) and leaves without mines of leaf miner-infested plants (leaf miner-infested systemic).

3.4 Metabolomics

We observed differences in SA ($\chi 2 = 11.67$, $P < 0.01$) and OPDA ($\chi 2 = 10.53$, $P < 0.05$) concentrations between treatments. Leaf miner-infested and mite-infested plants showed higher levels of SA than non-infested plants (leaf miner-infested *vs.* non-infested: $P < 0.01$; mite-infested *vs.* non-infested: $P < 0.05$, Fig. 5A). However, multiple-infested plants showed

concentrations of this phytohormone similar to those of non-infested and single-infested plants (multiple-infested *vs.* non-infested: $P = 0.115$; multiple-infested *vs.* mite-infested: $P = 0.856$; multiple-infested *vs.* leaf miner-infested: $P = 0.410$, Fig. 5A).

Although the OPDA levels of non-infested plants were similar to those of single-infested or multiple-infested plants (leaf miner-infested *vs.* non-infested: $P = 0.966$; mite-infested *vs.* non-infested: $P = 0.157$; multiple-infested *vs.* non-infested: $P = 0.222$, Fig. 5B), plants infested by *O. ilicis*, either alone or simultaneously with leaf miner, showed higher levels of this precursor than leaf miner-infested plants (mite-infested *vs.* leaf miner-infested: $P < 0.05$; multiple-infested *vs.* leaf miner-infested: $P < 0.05$, Fig. 5B). Interestingly, the leaves mined by the *L. coffeella* larvae from multiple-infested plants showed higher levels of OPDA than the mined leaves from leaf miner-infested plants ($\chi2 = 6.81$, $P < 0.01$, Fig. 5B).

Concentrations of JA and its active form, JA-Ile, did not differed between non-infested, leaf miner-infested, mite-infested and, multiple-infested plants (JA: $\chi2 = 3.52$; $P = 0.317$, Fig. 6C; JA-Ile: $\chi2 = 1.06$, $P = 0.786$, Fig. 5D). However, leaves infested by the mite from multiple-infested plants showed a lower concentration of JA when compared to leaves of mite-infested plants ($\chi2 = 6.59$, $P < 0.05$, Fig. 5C). This trend was not observed for its active form JA Ile ($\chi2 = 1.40$, $P = 0.235$, Fig. 5D).

Although not significant, leaf miner-infested plants had 24% more ABA in their leaf tissues than non-infested plants and 22% more than mite-infested plants (leaf miner-infested *vs.* non-infested: $P = 0.059$; leaf miner-infested *vs.* mite-infested: $P = 0.093$, Fig. 5E). In contrast, the concentrations of this phytohormone were similar between leaf miner-infested plants and multiple-infested plants ($\chi2 = 1.18$, $P = 0.647$, Fig. 5E).

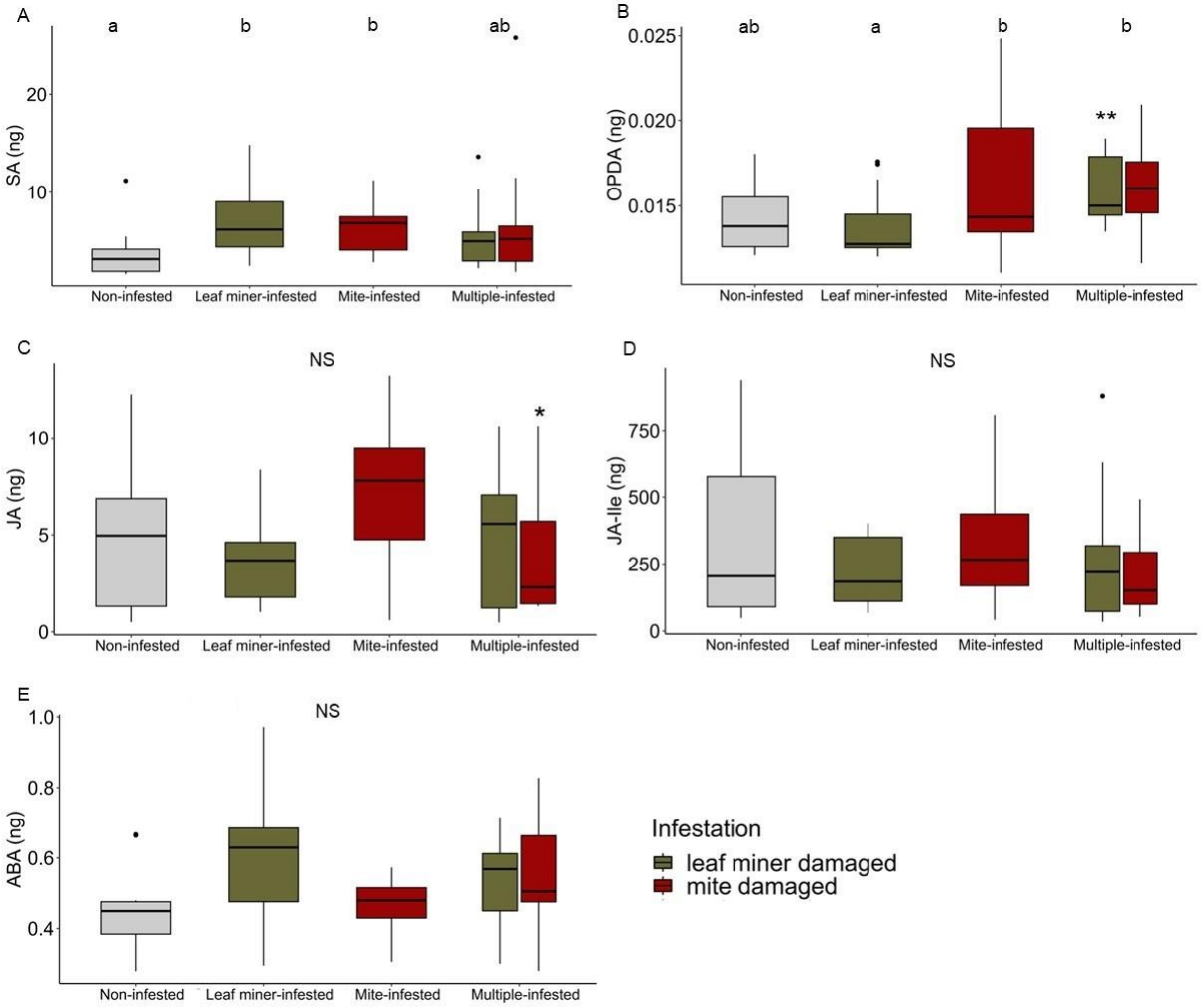

Figure 5 – Amount of phytohormones (ng/mg of dry tissue) in *Coffea arabica* leaves of non-infested plants (n = 12), leaf miner-infested (*Leucoptera coffeella*) (n =13), mite-infested (*Oligonychus ilicis*) (n = 12) and multiple-infested plants (leaf miner + mite) (n = 13), identified and quantified by analysis in liquid chromatography coupled to mass spectrometry (LC-MS). Salicylic acid (SA) (A). 12-Oxophytodienoic Acid (OPDA) (B). Jasmonic acid (JA) (C). Jasmonic acid-Isoleucine (JA-Ile) (D). Abscisic acid (ABA) (E). Bars with different letters indicate differences between treatments at the 5% level according to the Tukey test, NS = non significant difference, ** $P < 0.01$, * $P < 0.05$ according to anova indicate differences between infestations of multiple-infested plants and simple-infested plants.

3.5 Targeted analysis of secondary metabolites

Simple-infested plants and multiple-infested plants had higher concentrations of caffeine than non-infested plants (treatment: $\chi2 = 13.41$; $P < 0.01$; leaf miner-infested *vs.* non-infested: $P < 0.05$; mite-infested *vs.* non-infested: $P < 0.05$; multiple-infested *vs.* non-infested: $P < 0.01$; Fig. 6A). The caffeine level of leaves infested by *O. ilicis* of the multiple-infested plants did not differ from that of mite-infested plants ($\chi2 = 0.69$, $P = 0.402$), and the same pattern was repeated for leaves infested with the leaf miner comparing the plants under multiple and single infestation ($\chi2 = 0.37$, $P = 0.542$).

Theophylline concentrations were higher in leaf miner-infested and mite-infested plants when compared to non-infested plants (treatment: $\chi 2 = 14.34$, $P < 0.01$; leaf miner-infested *vs.* non-infested: $P < 0.05$; mite-infested *vs.* non-infested: $P < 0.01$, Fig. 6C). However, multiple-infested plants had similar levels of this compound to those observed in non-infested plants or under infestation only with the mite and only with the leaf miner (multiple-infested *vs.* non-infested: $P = 0.387$; multiple-infested *vs.* mite- infested: $P = 0.139$; multiple-infested *vs.* leaf miner-infested: $P = 0.212$; Fig. 6C). Interestingly, the mined leaves by *L. coffeella* larvae of multiple-infested plants showed a significant reduction in theophylline content compared to the mined leaves of leaf miner-infested plants ($\chi 2 = 3.88$; $P < 0.05$; Fig. 6C).

The leaves infested by *O. ilicis* from the mite-infested plants showed an average level of chlorogenic acid 1.4 times lower than that observed in the leaves infested by the mite from multiple-infested plants, while the levels of theobromine, trigonelline and chlorogenic acid were similar.

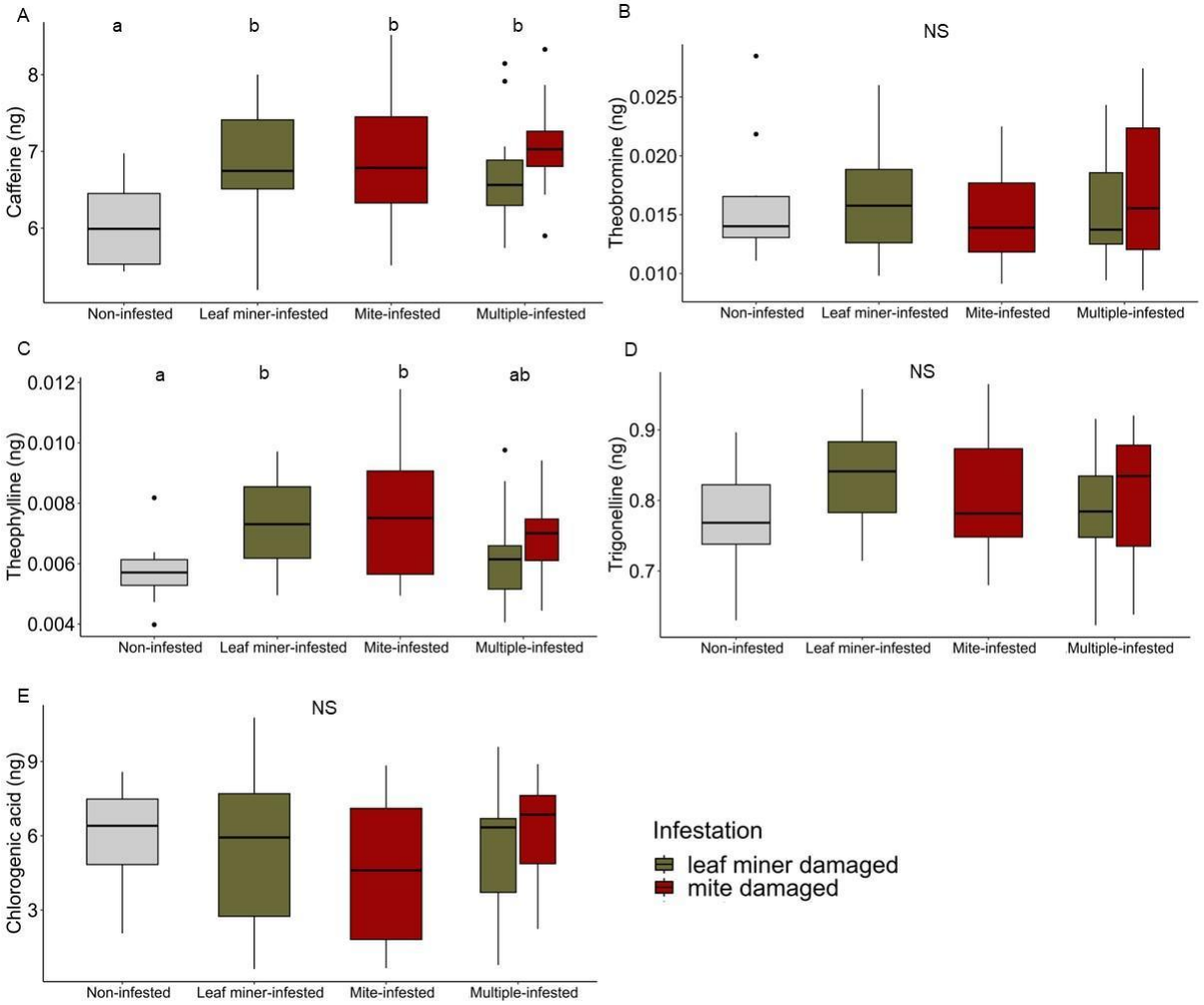

Figure 6 – Amounts of secondary metabolites (ng/mg of dry tissue) in leaves of coffee plants (*Coffea arabica*) from non-infested plants (n = 12), leaf miner-infested (*Leucoptera coffeella*) plants (n = 13), mite-infested (*Oligonychus ilicis*) plants (n = 12), and multiple-infested plants (leaf miner + mite) (n = 13), identified and quantified by analysis in liquid chromatography coupled to mass spectrometry (LC-MS). Caffeine (A). Theobromine (B). Theophylline (C). Trigonelline (D). Chlorogenic acid (E). Bars with different letters indicate differences between treatments at the 5% level according to the Tukey test, NS = non significant.

3.6 Untargeted analysis of primary and secondary metabolites

In total, 4.910 features, which are unidentified metabolites of the primary and secondary metabolism of coffee plants, were detected using LC-MSMS and GC-MS. Discriminant analysis of principal components (DAPC) showed a clear separation between treatments and infestations (Fig. 7A). However, the PERMANOVA analysis did not show statistical significance in metabolic composition between treatments (F = 1.31, *P* = 0.143) and between infestations (*F* = 1.52, P = 0.084).

The Random Forest analysis classified 362 features that best predict the pairs of treatments separation (Table S1). Then, PERMANOVA showed a significant difference in metabolic composition between treatments (F = 2.16, $P < 0.05$,) and between infestations (F = 3.09, $P < 0.05$). Therefore, the classification analysis of predictor features was effective in separating the treatments.

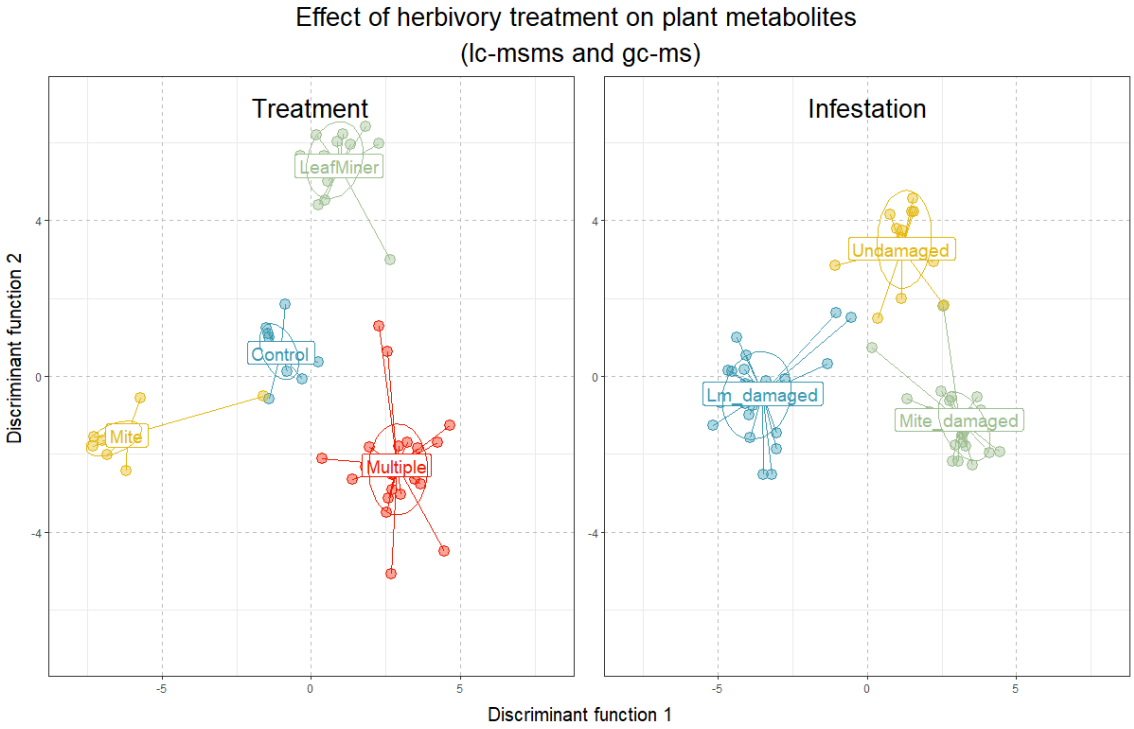

Figure 7 – Discriminant analysis of principal components (DAPC) showing the effect of non-infested coffee plants (*Coffea arabica*) (n = 12), leaf miner-infested (*Leucoptera coffeella*) plants (n = 13), mite-infested (*Oligonychus ilicis*) plants (n = 12), and multiple-infested plants (leaf miner + southern mite) (n = 13), and the infestations by leaf miner (Lm_damaged), by the mite (Mite_damaged) and without infestation (Undamaged) on the metabolomic composition of *C. arabica*.

Our results showed that leaf miner-infested plants or mite-infested plants showed most features under higher concentrations than non-infested plants (Table 3, Fig. 8). However, when comparing leaf miner-infested and mite-infested plant only 15 features showed a significant difference, and the vast majority of them (13 features) showed higher concentrations in plants infested by *O. ilicis* than in plants infested by *L. coffeella*.

The comparisons between simple-infestation and multiple-infested plants showed suppression of metabolites in the treatment of multiple infestation. In all comparisons, multiple-infested plants showed a greater number of features with reduced than with increased concentrations. We highlight the comparison between multiple-infested plants and mite-

infested plants, in which 107 features had significantly different concentrations, and 89 features of them were at lower concentrations in multiple-infested plants and 18 showed higher concentrations in multiple-infested plants (Table 3, Fig. 8).

When analyzing only leaves infested by mites from multiple-infested plants and mite-infested, 28 features were at different concentrations, among which the majority (17) were up-regulated in multiple-infested plants. On the other hand, the mined leaves from multiple-infested plants and leaf miner-infested plants showed 21 features with different concentrations with the majority (14) being down-regulated in multiple-infested plants.

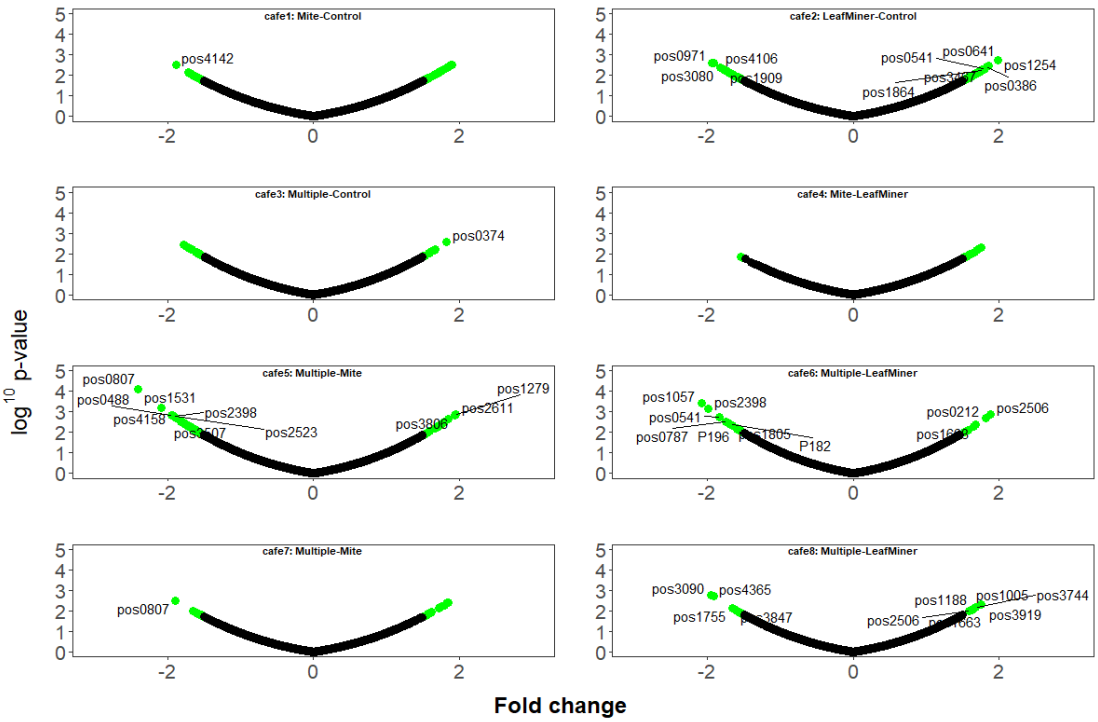

Figure 8– Volcano plot showing individual changes in feature concentration between pairs of treatments and infestations. Features that showed significantly different concentrations (upregulated or downregulated) ($P < 0.05$) between pairwise comparisons are shown in green.

Table– 3 - Number of features found in the untargeted analysis of primary and secondary metabolism of *Coffea arabica* plants that showed significantly different concentrations ($P < 0.05$) between pairwise comparisons of treatments and infestations.

	Pairwise Comparison	Significant *features*	Downregulated	Upregulated
Treatment	Coffee1: Mite *vs.* Control	93	34	59
	Coffee2: Leaf Miner *vs.* Control	74	27	47
	Coffee3: Multiple *vs.* Control	36	25	11
	Coffee4: Mite *vs.* Leaf Miner	15	2	13
	Coffee5: Multiple *vs.* Mite	107	89	18
	Coffee6: Multiple *vs.* LeafMiner	25	18	7
Leaf Infestation	Coffee7: Multiple *vs.* Mite	28	11	17
	Coffee8: Multiple *vs.* Leaf Miner	21	14	7

4. Discussion

Arthropod herbivores need plants for food, shelter, and oviposition to complete their life cycle. Thus, the nutritional conditions and defense levels of the host plant are of great importance for its survival and development (Awmack & Leather, 2002; Mithöfer & Boland, 2012). In this study, we observe that the herbivory by the leaf miner *L. coffeella* altered the metabolic profile of the coffee plant, which is likely associated with favorable changes to the conspecifics, but unfavorable to the phytophagous southern red-spider mite, *O. ilicis*. Similarly, *O. ilicis* negatively influenced the behavior and biology of *L. coffeella*. These interactions result from species-specific alterations induced by arthropod herbivory in the primary, secondary, and phytohormonal metabolic profiles of the coffee plant, which are perceived differently by conspecifics and heterospecifics.

Host selection and performance of L. coffeella *in plants infested by conspecifics and heterospecifics*

Leucoptera coffeella females did not differentiate between non-infested plants and plants infested by their conspecifics but preferred to lay more eggs on plants infested by the

heterospecific, *O. ilicis*, than on non-infested plants. The development of *L. coffeella* offspring was faster in conspecific-infested plants, while it did not differ between mite-infested plants and non-infested, although the pupal stage was slightly longer in plants infested by the heterospecific. Contrary to the prediction of the preference-performance hypothesis (Levins & MacArthur, 1969; Thompson, 1988), *L. coffeella* did not select coffee plants that were better hosts for the performance of its offspring, as found by Santiago-Salazar et al. (2021) with susceptible and resistant coffee genotypes to the fungus *Hemileia vastatrix* (Basidiomycota, Pucciniales). Targeted analysis revealed a similar profile for the phenolic compound chlorogenic acid and alkaloids from leaf-miner infested or mite-infested plants. Studies suggest that chlorogenic acid has no role in the resistance of coffee plants against *L. coffeella* (Ramiro et al., 2006). Caffeine, on the other hand, is an alkaloid known to stimulate *L. coffeella* oviposition (Magalhães et al., 2008), but it must not have been responsible for the preference of *L. coffeella* for plants infested by *O. ilicis*, since the increase in this compound was similar to that of leaf miner-infested plants.

However, the untargeted analysis revealed that the metabolomic composition of the treatments leaf miner-infested and mite-infested plants is different due to differences in amounts of 15 features (unidentified metabolites). This differential metabolic regulation could be the result of the induction of different defense pathways by herbivores modulated by phytohormones. Leaf miner infestation increased the level of SA and ABA, while it did not change the levels of OPDA, JA, and JA-Ile. Although mite infestation also induced accumulation of SA, it increased the concentration of OPDA (precursor of JA), and there was a trend towards an increase in JA and its derivative JA-Ile. These results suggest that herbivory by *L. coffeella* triggers an antagonistic interaction between JA-SA, which is a well-known suppression mechanism of plant induced defense reported for phloem feeder insects (Zarate et al., 2007; Giordanengo et al., 2010; Zhang et al., 2015), but not reported for leaf miners. Few studies have shown that herbivory by leaf miners induces the accumulation of both SA and JA in host plants (Zhang et al., 2016; Yang et al., 2021), like phytophagous mites do (Arena et al., 2018; Schimmel et al., 2018; Zhang et al., 2020). This differentiation in the accumulation of phytohormones can have effects on subsequent ecological interactions, facilitating or hindering the development, survival, and reproduction of species (Stam et al., 2018). Possibly, suppression of the JA pathway through negative crosstalk could be responsible for shortening the *L. coffeella* development cycle in plants infested with conspecifics. On the other hand, the accumulation of OPDA in plants infested by heterospecific species could have caused

alterations in primary metabolism that reflected in the less feeding of *L. coffeella* the with consequences to the development.

Host selection and performance of O. ilicis *in plants infested by* L. coffeella

Leaf miner-infested plants were more attractive to *O. ilicis* than non-infested plants, however they had a negative effect on their oviposition rate. In another study, *O. ilicis* was unable to discriminate between hosts and its performance was also negatively affected in plants infested by another herbivore (Peñaflor et al., 2019). However, plants infested by conspecifics were more attractive to *O. ilicis* and promoted a higher rate of oviposition over time than non-infested plants (Andrade et al., unpublished data). The authors suggest that herbivory by *O. ilicis* suppresses the defenses of the coffee plant, but in a different way from that reported for other phytophagous mites (Glas et al., 2014; Alba et al., 2015), which may have led to its greater attractiveness.

As discussed previously, *L. coffeella* infestation induced SA accumulation, which may have caused the suppression of JA-mediated defenses through negative crosstalk. Furthermore, *L. coffeella* also induced ABA accumulation in plants, which can interact with the JA and SA pathways and affect host defense responses (Erb et al., 2012). This phytohormonal profile may have triggered a blend of herbivory-induced plant volatiles (HIPVs) that was more attractive to the phytophagous mite than the volatiles emitted by non-infested plants, since in addition to providing important information to natural enemies, HIPVs can also be exploited by herbivores searching for suitable hosts (Dicke & Baldwin, 2010). However, the greater attractiveness of mites to heterospecific infested plants did not reflect the host quality, which was detrimental to their performance.

Interestingly, infestation by leaf miner *L. coffeella* elicited local metabolic changes (mined leaf) and systemic (non-mined leaf), different from other studies with leaf miners that identified changes only in mined areas (Zhang et al., 2016) or in mined leaves (Body et al., 2013). Among the secondary metabolites investigated in the targeted analysis, chlorogenic acid is an important phenolic compound in the plant defense against mites (Hoffland et al. 2000; Lattanzio et al., 2006; Hata et al. 2019). Although no significant differences were observed between treatments, this phenolic compound increased its concentration by 19% in leaf miner-infested plants and 24% in multiple-infested plants, as in another system where high levels of phenolic compounds were related to the presence of leaf miner herbivores (Sinclair & Hughes, 2008). In the untargeted analyzes, in addition to the 15 features that differed in concentrations

between simple-infested plants, 79 features showed different concentrations between plants infested by *L. coffeella* and non-infested plants. These results show that the leaf miner of *L. coffeella*, as other leaf miner insects, seems to modulate its host metabolism. However, such an effect is expressed both locally and systemically in the coffee plant. Furthermore, our results also do not support the preference-performance hypothesis, and, from an ecological perspective, this strategy may favor the leaf miner by avoiding competition for resources with other herbivores.

Multiple-infested plants differ from single-infested plants and showed a suppression of features concentration

In plants under multiple infestations, the species identity and arrival sequence in the plant are important factors in determining changes in the chemical profile of the host plant (Voelckel & Baldwin, 2004; Poelman et al., 2008; Erb et al., 2011). In this study, our objective was to investigate the metabolic changes that result from the feeding of both herbivores on coffee plants and, therefore, we sought to standardize the simultaneous feeding time (seven days). To date, no studies have been found in the literature showing the effect of simultaneous infestation by leaf miner larvae and phytophagous mites on the primary and secondary metabolic profile of hosts. Studies that investigated simultaneous infestation by herbivores from different feeding guilds showed the effects on the expression of marker genes, on the composition of volatiles induced by herbivory and the attractiveness to natural enemies (Rodriguez-Saona et al., 2003; Moayeri et al., 2007; Zhang et al., 2009; Rodriguez-Saona et al., 2010; Oliveira and Pareja, 2014; Kroes et al., 2015; Kiełkiewicz et al., 2019), while the profile of the host plant including primary and secondary metabolism has received little attention (Errard et al., 2016).

The simultaneous feeding by the leaf miner and mite in the coffee plants did not generate an additive effect on the amounts of phytohormones and secondary metabolites (targeted analysis). Except for the OPDA levels, which differed between leaf-miner infested plants and mite-infested or multiple-infested plants, all phytohormones and secondary metabolites investigated in plants under multiple infestation showed intermediate amounts between those observed in simple-infested plants and in non-infested plants. However, it is possible to notice that in all combinations of treatments involving plants under multiple infestation, most of the significant features have their concentrations decreased compared to the other treatments. And the multivariate analysis showed that the treatments were separated into distinct groups.

Together, these results show that the infestation of the two herbivores simultaneously triggered a “new” metabolic profile in the coffee plant characterized by suppression of metabolites.

In addition to what has already been discussed, we highlight that herbivores were able to change the metabolic profile of the leaf on which the other was feeding in different ways. The presence of the leaf miner in the multiple-infested plant caused 17 features to increase their concentrations on leaves infested by *O. ilicis*, compared to leaves infested with the mite of simple-infested plants. On the other hand, in the presence of the mite, leaves infested with *L. coffeella* of multiple-infested plants showed 14 features with lower concentrations than those observed in leaf miner-infested plants. These results could be associated with the interaction between the different signaling pathways triggered by both herbivores on the coffee plant. Although both herbivores appeared to suppress coffee plant defenses, since their simple infestation favors conspecifics (Andrade FM, manuscript in preparation) but not heterospecifics, molecular assays are necessary to understand how this suppression occurs and how these mechanisms interfere in each other. Another important aspect to study is whether the order of infestation of multiple-infested plants causes different coffee plant responses.

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SUPPLEMENTARY INFORMATION

Figures

Figure S1 - Cages made with transparent plastic cups and voile for infestation of leaf miner-infested plants (*Leucoptera coffeella*) and multiple-infested plants (treatments). These cages were also used in the *L. coffeella* performance experiment on non-infested plants *vs.* plants infested by conspecifics and non-infested plants *vs.* plants infested by the heterospecific, the southern red mite, *Oligonychus ilicis*.

Figure S2 - T-shaped wooden structure positioned in a Petri dish (20 cm diameter) containing 20 mites, *Oligonychus ilicis*, connecting a leaf of a non-infested plant to the corresponding one of the leaf miner-infested plant, *Leucoptera coffeella*.

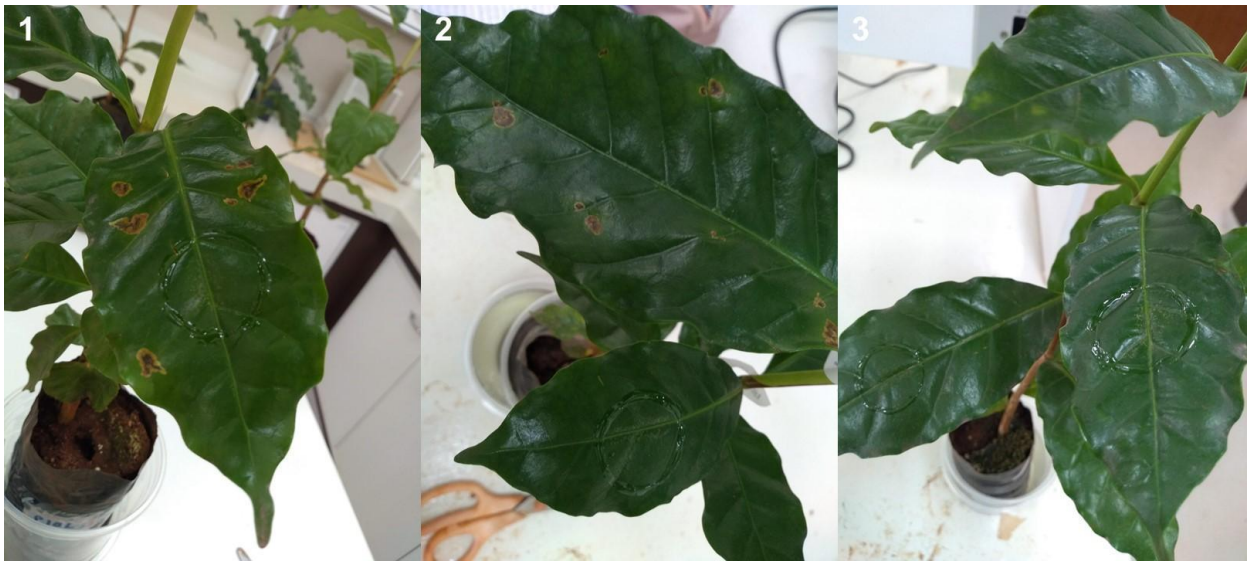

Figure S3 - Circular areas (2 cm diameter) delimited with a mixture of glue+lanolin on mined leaves (local effect) (1) and non-mined leaves (systemic effect); (2) of leaf miner-infested plants (*Leucoptera coffeella*); and on leaves of non-infested plants (3).

Tables

Tabela S1 – The main predictor features for pairwise comparisons of treatments and infestations using the Random Forest algorithm.

	Treatment						**Leaf infestation**	
	Mite *vs.* Non-infested	**Leaf Miner *vs.* Non-infested ***	**Multiple *vs.* Non-infested**	**Mite *vs.* Leaf Miner***	**Multiple *vs.* Mite**	**Multiple *vs.* Leaf Miner***	**Multiple *vs.* Mite***	**Multiple *vs.* Leaf Miner***
Specificity	1.000	10.000	0.7143	0.6667	1.000	1.0	0.6667	1.000
Accuracy	0.6667	0.8333	0.6	0.6667	0.8	0.7	0.6667	0.8333
OOB estimate of error rate	5.56%	5.26%	7.14%	5.26%	3.57%	6.9%	5.26%	10%
	pos4370	pos1504	pos0093	pos0426	pos0784	P172	pos0428	pos4365
	pos1531	pos1917	pos3010	pos4006	pos0807	P182	pos1163	pos2611
	pos0741	pos2726		pos0189	pos2398	pos0069	pos2821	pos1193
	pos4212	pos0386		pos4338	pos2523	pos0090	pos1057	pos1117
	pos2146	pos3586		pos1577	pos3586	pos0124	pos3740	pos0316
	pos1174	pos1174		pos3972	pos4196	pos0142	pos1920	pos3845
		pos1665		pos2721		pos0212	pos3748	pos2016
		pos4370		pos1441		pos0807	pos0830	pos1005
		pos4190		pos0216		pos1057	P98	pos1384
		pos2734		pos0386		pos1550	pos2538	pos3744

*****Pairwise comparisons that have more than 10 significant features classified as important predictors.

Tabela S2 – The significant features that have its concentration upregulated or downregulated according to different infestations on coffee plants.

Treatment	**Leaf infestation**

Mite *vs.* Non-infested*	**Leaf Miner *vs.* Non-infested***	**Multiple *vs.* Non-infested***	**Mite *vs.* Leaf miner**	**Multiple *vs.* Mite***	**Multiple *vs.* Leaf miner**	**Multiple *vs.* Mite**	**Multiple *vs.* Leaf miner**
pos3467	pos0641	pos0374	pos1577	pos0807	pos1057	pos0807	pos3090
pos1864	pos0971	pos3795	pos0488	pos1531	pos2398	pos2619	pos4365
pos4142	pos4106	neg067	pos0648	pos1279	pos2506	pos2931	pos1005
pos2450	pos1254	pos0093	pos3797	pos2611	pos0541	pos2611	pos3744
pos3362	pos0386	neg169	pos3889	pos3806	pos0212	pos1913	pos3919
pos1174	pos3080	NP27	pos3870	pos0488	pos0787	pos3806	pos1755
pos1531	pos0541	neg048	pos0460	pos4158	P196	pos2390	pos1188
pos2607	pos3467	P15	pos1720	pos2398	P182	pos1279	pos3847
pos4098	pos1909	neg050	pos1531	pos2523	pos1663	pos3589	pos1663
pos4212	pos1864	neg046	pos0510	pos3507	pos1805	pos4371	pos2506
pos1850	pos0366	pos2351	pos1441	pos1254	pos1005	P185	pos0807
pos1230	pos3100	pos0480	pos0426	pos2146	pos1183	pos4034	pos1057
pos2164	pos2512	neg084	pos1153	pos3559	pos1172	pos2821	pos2398
pos2721	pos0029	pos4359	pos2390	pos0299	NP20	pos1837	pos0745
pos1834	pos1098	pos0216	pos3444	pos1913	pos3459	pos3210	pos1895
pos3501	pos2250	pos0820		pos1428	pos3247	pos0220	pos3459
P106	pos1417	neg146		pos2824	pos1715	pos3559	pos0491
pos2250	pos1558	neg144		pos2181	pos0807	pos2506	pos0787
pos0807	pos1172	pos1376		pos3506	pos3919	pos1153	pos3837
pos1708	pos3151	neg026		pos3872	pos0745	pos4079	NP20
pos0454	pos3152	neg063		pos2619	pos2973	pos4196	pos1474
pos1913	pos3786	pos3321		pos1445	pos3995	pos1531	
pos1577	pos1834	pos2920		pos0784	pos4374	pos2523	
pos3263	pos3252	neg129		pos1188	P61	pos3433	
pos3806	pos4278	pos1864		pos1343	P77	pos2968	
pos3641	pos2894	neg103		pos2767		pos1057	
neg009	pos1797	neg014		pos2968		pos1188	
pos0398	pos3547	neg168		pos3922		pos1065	

*Pairwise comparisons with more than 30 significant features with different concentrations.