UNIVERSIDADE FEDERAL DE LAVRAS

SOFIA PIMENTA DE OLIVEIRA

PLANT RESPONSE TO EARLY HERBIVORY CUES: HOW DOES A PLANT PERCEIVE AN IMMINENT ATTACK?

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 em Ecologia e Conservação de Recursos em Paisagens Fragmentadas e Agrossistemas, para a obtenção do título de Doutor.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Oliveira, Sofia Pimenta de.
Plant response to early herbivory cues: How does a plant perceive an imminent attack? / Sofia Pimenta de Oliveira. - 2024.
115 p.: il.

Orientador(a): Maria Fernanda Gomes Vilalba Penaflor.
Coorientador(a): Renato Chaves de Macedo-Rego.
Tese (doutorado) - Universidade Federal de Lavras, 2024.
Bibliografia.

1. Plant priming. 2. Airborne cues. 3. Insect volatile. I. Gomes Vilalba Penaflor, Maria Fernanda. II. Macedo-Rego, Renato Chaves

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RESPOSTAS DAS PLANTAS A PISTAS INICIAIS DE HERBIVORIA: COMO AS PLANTAS PERCEBEM UM ATAQUE IMINENTE?

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LAVRAS - MG
2024

“Não sou nada.
Nunca serei nada.
Não posso querer ser nada.
À parte isso, tenho em mim todos os sonhos do mundo.”
(Álvaro de Campos – Fernando Pessoa)

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001. Agradeço a concessão da bolsa de estudos.

À Universidade Federal de Lavras pelo apoio técnico.

Ao Programa de Pós-graduação em Ecologia Aplicada, pela oportunidade, suporte e conhecimento concedido a mim por todos os professores, funcionários e alunos.

Agradeço a todos os membros que estiveram no Laboratório de Ecologia Química das Interações Inseto-Planta (LEQIIP) durante meus anos de doutorado, em especial a Fernanda Moreira e Livia Souza que me deram grande suporte, ajuda técnica e conhecimento nos meus anos iniciais.

Agradeço a minha orientadora, professora Maria Fernanda, mais conhecida como chefa. Obrigada por ser um exemplo de profissional a ser seguido, obrigada por todo o apoio, por acreditar em mim, por me ajudar e me guiar sempre que foi preciso, por toda a compreensão, carinho e empatia nos momentos difíceis. Agradeço imensamente todo o ensinamento que você me passou. Guardarei para sempre um carinho especial por você.

Agradeço imensamente ao meu coorientador Renato Chaves. Obrigada Renato por sempre ter sido extremamente paciente e presente ao longo do meu doutorado. Obrigada por me guiar e me ensinar com tanta calma e sabedoria. Levarei para sempre seus ensinamentos comigo.

A special thanks to Dr. Helms from Texas A&M University for accepting me as her student for 10 months. I truly appreciated your guidance and support throughout my time in your lab. Thank you very much! A genuine thanks to all the members of Dr. Helms Lab, especially Natalie Aguirre and John Grunseich, for the invaluable conversations, lessons, friendship, support, laughter, and experiences we shared. You all are truly the best, and I am forever grateful for having you in my life!

Agradeço aos grandes amigos que fiz durante meu período de doutorado-sanduiche, em especial a Andresa, Mari, Dani e Caroline. Obrigada meninas! Vocês fizeram esta experiencia mais leve, engraçada e divertida. Eu amo vocês!

As meninas que compartilhei moradia em Lavras e estiveram comigo durante toda a minha jornada no doutorado, Brenda, Nayara e Carol, faltam palavras para explicar o tanto que vocês são importantes na minha vida. Amizade rara, verdadeira, que levarei para a vida toda! Meu muito obrigado por TUDO. Eu amo muito, muito vocês!

Agradeço imensamente as minhas amigas de Belo Horizonte, Giovanna, Laura, Livia, Tati e Renata por sempre estarem comigo durante quase duas décadas. Agradeço por nunca terem soltado a minha mão, por sempre terem me apoiado e me encorajado. Vocês são a família que eu escolhi e continuarei sempre escolhendo. Obrigada, obrigada e obrigada. Amo vocês!

Aos meus amigos de Viçosa, Keminy, Eduardo e Morgana. Pessoas que compartilharam momentos extremamente importantes comigo e que fizeram e fazem parte da pessoa que sou hoje. Amo vocês! Saudades.

Agradeço ao meu companheiro, Gustavo, que compartilha comigo o meu dia a dia sempre me ajudando, conversando e incentivando a ser uma pessoa melhor. Obrigada gatinho, eu aprendo todos os dias com você! Te amo!

Por fim, agradeço a toda a minha família por toda a ajuda que me foi dada durante estes quatro anos. Eu não teria chegado aqui se não fosse por vocês. Em especial a Dandan, Tia Carminha, Tio Neia e Tio Do pelo suporte inegável de sempre.

Dedico esta tese a minha mãe, Beatriz da Cunha Pimenta. Obrigada mãe, sei que olha por mim ai de cima e que está sempre presente nos meus passos. Te amo. Saudades eternas!

RESUMO

Plantas e herbívoros têm uma relação ecológica complexa. Plantas podem reagir a sinais de herbivoria que são indicativos de ataques futuros e ativarem o estado de "priming", e são capazes de reconhecer a herbivoria, induzindo a síntese de defesas. O "priming" de defesa refere-se a um estado de alerta elevado em plantas desencadeado por estímulos externos como sinais de estresse causados por herbívoros. Em vez de ativar imediatamente uma defesa induzida, as plantas em estado de "priming" respondem mais rapidamente e de forma mais eficaz quando confrontadas com o ataque real. Desta forma, esta pesquisa integra estudos sobre o "priming" de defesa das plantas através da detecção de pistas iniciais de herbivoria. No capítulo 1, foi encontrado que o feromônio sexual do minador das folhas do café *Leucoptera coffeella* desencadeia um aumento de defesa em plantas de café, reduzindo a postura de ovos e danos pela larva. Adicionalmente, a exposição ao feromônio sintético 5,9-dimetilpentadecano aumentou os níveis de compostos defensivos presentes nas folhas de café. No capítulo 2, encontramos que a domesticação de *Cucurbita pepo* alterou as respostas de defesa "priming" em plantas de *C. pepo* quando expostas ao feromônios de agregação do besouro-listrado, *Acalymma vittatum*. As variedades domesticadas mostraram um aumento no mecanismo de defesa em comparação com variedades selvagens. O capítulo 3 consistiu em uma meta-análise sobre as defesas "priming" em plantas a pistas voláteis de herbivoria. Foi demonstrado que a ativação da defesa "priming" em plantas é comum que tanto plantas anuais e perenes ativam suas defesas "priming" assim como, plantas selvagens e domesticadas. Adicionalmente, vimos que a duração da exposição a pista volátil, as condições experimentais e a natureza do composto volátil modificam as respostas de defesa "priming" em plantas. Essas descobertas sublinham coletivamente a importância da comunicação planta-inseto por meio de pistas químicas, oferecendo insights sobre o manejo sustentável de pragas, o impacto evolutivo da domesticação na defesa de plantas e novas perspectivas para estudos futuros.

Palavras-chave: defesa "priming"; pistas de herbivoria; domesticação de plantas; meta-análise; feromônio de inseto.

ABSTRACT

Plants and insect herbivores have a complex ecological relationship. Plants can react to direct signals from herbivores that indicate future attacks. When faced with herbivory damage or cues from insects, they frequently induce or prime their defenses. "Priming" of defense refers to a heightened state of alertness in plants triggered by external stimuli such as signals of stress caused by herbivores. Instead of immediately activating an induced defense, plants in a "priming" state respond more quickly and effectively when confronted with the actual attack. This research integrates studies on plant defense priming through detection of insect delivered cues. For instance, the sex pheromone of the coffee leaf miner *Leucoptera coffeella* triggers defense priming in coffee plants, reducing egg-laying and damage. Exposure to the synthetic pheromone 5,9-dimethylpentadecane altered the levels of defensive compounds, suggesting intricate chemical signaling in plant-insect interactions. Similarly, the domestication of *Cucurbita pepo* has altered its varieties' responses to insect pheromones like vittatalactone from *Acalymma vittatum*, with domesticated types showing an increase in defense mechanism compared to wild types. Furthermore, a meta-analysis highlights the broader phenomenon of plant priming of defense in response to herbivore stress. It has been shown that the activation of "priming" defense in plants is common and that both annual and perennial plants activate their "priming" defenses, as well as wild and domesticated plants. Additionally, we have seen that the duration of exposure to volatile cues, the experimental conditions, and the nature of the volatile compound modify the "priming" defense responses in plants. These findings collectively underscore the significance of plant-insect communication through chemical cues, offering insights into sustainable pest management, the evolutionary impact of domestication on plant defense, and new perspectives for future studies.

Keywords: plant priming; herbivory cues; plant domestication; meta-analysis; insect pheromone.

INDICADORES DE IMPACTOS

Impactos sociais, tecnológicos, econômicos e culturais

O objetivo principal de nossa tese foi investigar o conceito de estimular as defesas das plantas, provocando investigações adicionais e propondo direções para estudos futuros. Esperamos que nossa pesquisa melhore a compreensão da sustentabilidade, proteção e manejo de culturas. Ao aprofundar nas complexas relações entre as respostas de defesa das plantas e os sistemas ecológicos, visamos iluminar a interligação das práticas agrícolas com as dinâmicas ecológicas mais amplas. Esta investigação não só avança o conhecimento na agricultura de culturas sustentáveis, mas também destaca a necessidade crucial de conservação da biodiversidade para fortalecer os ecossistemas agrícolas resilientes.

Social, technological, economic and cultural impacts

The primary goal of our thesis was to investigate the concept of priming plant defenses, prompting additional inquiries and proposing directions for future study. We expect that our research will enhance comprehension of crop sustainability, protection, and management. By delving into the intricate relationships between plant defense responses and ecological systems, we aim to illuminate the intertwining of agricultural practices with broader ecological dynamics. This inquiry not only advances knowledge in sustainable crop farming but also underscores the crucial need for biodiversity conservation to bolster resilient agricultural ecosystems.

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PRIMEIRA PARTE

1. GENERAL INTRODUCTION

Chemical communication plays a crucial role in facilitating interactions among organisms in ecosystems, serving as a key component in establishing inter and intra relationships (ZU et al., 2023). It involves the transmission of chemical signals by an organism to convey information, which is then perceived and responded to by a recipient, resulting in benefits for both parties (SEARCY; NOWICKI, 2005). This exchange of information is believed to be essential for the stability of communication (HEIL; KARBAN, 2010). A classic example of chemical communication in nature is from the interactions between plants and insects (KARBAN et al., 2014). Plants have developed an array of mechanisms to detect herbivores, including herbivory-associated compounds, like saliva, oviposition, excretion, pheromones, and other substances from herbivores that may signal a potential attack (BANDOLY et al., 2016; PEÑAFLOR et al., 2011; REDDY; GUERRERO, 2004; REYMOND, 2013; RIVERA-VEGA et al., 2017). This communication is effective because both plants and insects can recognize and respond to each other's signals (HOUGEN-EITZMAN; RAUSHERT, 1994). Plants can activate defense mechanisms to protect themselves when they detect signals of herbivory or are being consumed by herbivores (MITHÖFER; BOLAND, 2012). Conversely, many insects have evolved to tolerate, detoxify, and even exploit certain defensive strategies of plants, leveraging them to their advantage (BERAN; PETSCHENKA, 2022; KARBAN; AGRAWAL, 2002).

Plants, limited by finite resources like all living beings, must use their energy effectively under herbivores attack (ERB, 2018). This involves deploying the most suitable defense mechanisms and allocating the necessary resources when necessary (SCHIMMEL, 2016). Plants have developed two strategies of defenses against herbivores to optimize energy allocation in defenses: (*i*) constitutive defenses, which is constantly present in plants tissue; and (*ii*) induced defenses, which are synthesized in response to herbivory and/or herbivory-associated compounds. These defenses can be further classified into direct and indirect defenses if they directly or indirectly target herbivores. Direct defenses include physical barriers like trichomes and spines, as well as the production of chemical compounds that deter or reduce herbivore’s survival through their repellence, unpalatability, or toxicity (e.g. caffeine and cucurbitacin) (KARBAN; MYERS, 2003). Indirect defenses involve the release of herbivore-induced plant volatiles (HIPVs) that recruit parasitoids and predators of herbivores (GEBREZIHER, 2018). For example, when an herbivore is detected by a plant, it initiates specific molecular signaling pathways involving the entry of calcium into the cytosol, increased generation of reactive oxygen species (ROS), the release of nitric oxide (NO), and other processes that eventually lead to the production of phytohormones like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (CAMPOS et al., 2014). Consequently, transcriptional activation regulated by the phytohormones induces alterations in the plant's metabolic profile, encompassing both non-volatile and volatile secondary metabolites that are used by plants to protect themselves (ERB; REYMOND, 2019).

It has been argued that antiherbivore defenses, while effective in reducing damage from insect herbivores, may impose significant fitness cost on plants given their high energetic costs (KARBAN, 2011). These fitness costs arise primarily from the unpredictability of herbivore presence, which is mitigated by the plant’s ability to detect herbivory and induce synthesis of novel defenses only when needed (BRODY; KARBAN, 1992). For example, a recent meta-analysis conducted by GARCIA et al., (2021) confirmed that induced defenses are costly to plants in both short-term and long-term herbivory infestation. In short-term infestations, a negative impact on photosynthesis and reproduction was detected, while longer exposures to herbivory stress led to larger negative impacts on plant fitness. Nevertheless, it is important to point out that the costs associated with induced defenses are extremely complex and vary according to different parameters and variables mediating the plant-herbivore interaction.

Plants have constantly evolved and thus adapted to manage the costs associated with their defense mechanisms (CIPOLLINI et al., 2017). One approach plant uses to offset these costs is through priming defenses (CONRATH et al., 2006). Plant priming is a process where prior exposure to stimuli, such as herbivore-associated cues, plants undergo a heightened state of alertness (HILKER; SCHMÜLLING, 2019). Therefore, instead of immediately activating an induced defense, plants in a "priming" state respond more quickly and effectively when confronted with the actual attack, while imposing lower fitness costs compared to induced defenses (MARTINEZ-MEDINA et al., 2016). For example, plants can undergo priming by exposure to HIPVs emitted by neighboring plants experiencing herbivore attacks (MARKOVIC et al., 2019; PAUDEL; TIMILSENA et al., 2020). Consequently, these HIPVs function as a warning signal to a nearby plant, indicating a potential threat (KARBAN et al., 2000). The HIPVs prompt plants to activate defense mechanisms, enabling a faster and/or stronger defense response upon subsequent attack, resulting in increased resistance and/or stress tolerance in contrast to unprepared plants (FROST et al., 2008, BECKERS; CONRATH, 2007).

To date, many stimuli have been identified as priming agents of plant defenses, under various conditions. Priming could be achieved through plant communication within the same or different species (KESSLER et al., 2006; MICHEREFF et al., 2021), as well as through natural or synthetic volatile compounds indicative of future harm (FROST et al., 2008b; HEIL et al., 2007; LLORENS et al., 2016). Only recently, it has been observed that plants can be primed by insect volatile such as sex and aggregation pheromone (HELMS et al., 2013; MAGALHÃES et al., 2019; YIP et al., 2017). Plants previously exposed to insect volatiles exhibit reduced damage, increased production of phytohormones, and higher levels of HIPVs compared to plants without prior exposure.

Limited research has explored the priming effect of insect derived compounds on plant defense mechanisms, leaving many questions unanswered about which plants can detect and respond to these signals, and how different types of exposure impact plants with varying life cycles and genotypes. To address these gaps, we investigated the effects of both natural and synthetic sex or aggregation pheromones on agronomically important plants with perennial and annual life cycles: coffee and squash, respectively (CHOMICKI *et al.*, 2020; GÓNGORA *et al.*, 2023; HANCOCK, 2012; PESSARAKLI, 2002). Furthermore, we performed a meta-analysis assessing how plants enhance their defense mechanisms through exposure to airborne signals, providing valuable insights and future perspectives in this field.

In the first chapter of this thesis, we aimed to explore whether the sex pheromone emitted by a coffee-specialist herbivore, *Leucoptera coffeella*, a significant threat to coffee plantations, can serve as a potential indicator of future damage for coffee plants. Our investigation into coffee plant defenses was based on assessing leaf consumption by *L. coffeella* larvae and the egg-laying behavior on plants that had been previously exposed to the natural sex pheromone compared to those that have not. Furthermore, we investigated how exposure to the synthetic major component of the *L. coffeella* sex pheromone regulates the production of constitutive and herbivore-induced secondary metabolites. In the second chapter, our focus was on examining how exposure of various cultivars of squash plants to *Acalymma vittatum* aggregation pheromone changes their defense levels. We studied how this pheromone affects the consumption of leaf area in different squash plant varieties, as well as the release of both constitutive and inducible volatile organic compounds (VOCs) and phytohormone production in plants exposed to the pheromone compared to those that were not. Additionally, we investigated how the synthetic vittatalactone, *A. vittatum* aggregation pheromone, affects plant leaf area damage and phytohormone production. In the third chapter, we present a meta-analysis that examines the literature on plant priming via airborne cues. This analysis evaluates the effect of key factors on the magnitude of priming due to exposure to airborne signals, aiming to enhance comprehension of studies conducted in this topic over the past decade.

The primary goal of this thesis was to investigate the concept of priming plant defenses, prompting additional inquiries and proposing directions for future studies. We expect that our research will enhance the comprehension of crop sustainability, protection, and management. By delving into the intricate relationships between plant defense responses and ecological systems, we aim to highlight the connections between agricultural practices and broader ecological dynamics. This inquiry not only advances knowledge in sustainable crop farming but also underscores the crucial need for biodiversity conservation to bolster resilient agricultural ecosystems.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - Coffee plant defensive responses to the coffee leaf miner sex pheromone

Artigo elaborado de acordo com as normas do periódico "*Journal of Chemical Ecology*".

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Abstract

Plants and insect herbivores share a fundamental ecological relationship, influenced not only by interactions during the active feeding stages of insects but also by herbivory cues that anticipate the attack. Detection of sex pheromones from insect herbivores by plants can trigger defense priming, enhancing their responses once the plant is damaged. This research aimed to explore how the sex pheromone of the coffee leaf miner *Leucoptera coffeella*, a primary pest of coffee plantations and a specialized herbivore, influences the antiherbivore defenses of coffee plants. Initially, we assessed the resistance of coffee plants exposed to the natural sex pheromone gland extract of *L. coffeella* through traditional preference and performance tests with the herbivore. Our results showed that plants exposed to the pheromone were less favored for egg-laying by females and experienced less damage from leaf miner larvae compared to non-exposed plants. Subsequently, we investigated whether exposure to synthetic 5,9-dimethylpentadecane, a key component of the *L. coffeella* sex pheromone, affected the constitutive or herbivore-induced levels of specific defensive secondary metabolites. We observed that exposure to the synthetic pheromone led to a decrease in the constitutive levels of hexanol and an increase in the induced production of citronellyl valerate, along with an overall rise in the total quantity of induced compounds. These findings shed light on the intricate interplay between coffee plants and their specialist herbivore, providing insights for additional sustainable pest management strategies in coffee crops.

Key Words: Defense priming. Insect sex pheromone. Coffee plants. Coffee leaf miner. 5,9-dimethylpentadecane.

Introduction

Plants live in a dynamic and heterogeneous environment, being constantly under selection pressure from either biotic or abiotic stressors (Dolferus, 2014). Insect herbivores impose substantial biotic pressure on plants such that plants need to precisely detect herbivory cues and respond effectively deploying an array of defensive mechanisms (Schoonhoven et al., 2005, Farmer and Ryan 1990). Plant defenses can be categorized into two types: constitutive, which are continuously present in the plant tissues, and inducible defenses, which are triggered in response to an external stimulus, such as herbivory damage (Dicke and Baldwin 2010). As part of herbivore-induced defenses, plants emit a distinct blend of volatiles (herbivore-induced plant volatiles, HIPVs), which are well-known to serve as cues for natural enemies of herbivores in prey finding (Heil 2008). These signals also play an important ecological role in plant-plant communication (van Doan et al. 2020; Grunseich et al. 2020; Brosset et al. 2021; Aguirre et al. 2023). The detection of HIPVs by neighboring plants often triggers defense priming (Mauch-Mani et al., 2017), enabling receiver plants to prepare for future attacks with minimal metabolic cost (Conrath et al., 2006). Once primed plants are damaged by herbivores, they exhibit a faster and more robust defensive response compared to plants that were not previously exposed to HIPVs (Heil et al., 2007b).

In addition to HIPVs, plants can detect various indirect cues associated with imminent attack by herbivorous insects, including intraspecific airborne signals from insect herbivores such as sex pheromone (Helms et al., 2013, 2014, 2017) or aggregation pheromone (D. M. Magalhães et al., 2019). To date, there are few recent reports in the literature showing that plants respond to pheromone of their insect herbivores. For example, Bittner et al. (2019) has shown that exposure to the sex pheromone of a specialist sawfly enhances Scots pine defense against insect eggs. This response involves changes in the expression of defense-related genes and chemical compounds that are harmful for egg hatching. Magalhães et al. (2019) found that the detection of a pheromone from a specialist herbivore and pest of cotton plants trigger indirect defenses, increasing the recruitment of natural enemies. Also, the sex pheromone of a specialist gall-making fly primes plant responses, indirectly enhancing plant fitness by inhibiting gall formation and, female flies of this species avoid ovipositing into pheromone-exposed plants (Yip et al., 2021).

Sex pheromones of insect pests serve as highly effective tools for their management in agricultural settings (M. C. Larsson, 2016). Over the past three decades, the advancement of large-scale chemical synthesis has facilitated the exploitation of insect pheromones in environmentally friendly tactics for monitoring and controlling of insect pests in crops (Larsson and Svensson 2009). Among the pheromone-based tactics, mating disruption stands out as the most promising method for controlling moth pests (Horner et al., 2020; Ortiz et al., 2021; Ricciardi et al., 2021; Savoldelli et al., 2023). This tactic involves the release of large quantities of a single or a few main pheromone components into the field through spraying or dispensers, effectively hindering male moths from locating their mates (Carde & Minks, 1995). Considering the substantial quantities of insect pheromone released into the environment and the potential for plants to detect sex pheromones of their herbivores, mating disruption can offer an additional benefit to crop protection against insect pests (Hendrichs et al., 2020).

Coffee is among the most extensively consumed beverages and one of the most traded commodities globally (Pancsira, 2022). Originally from Africa, *Coffea arabica* plants are now cultivated across several geographic regions, where they are attacked by a diverse array of herbivores, including leaf miners from the genus *Leucoptera* (Barrera, 2017). In Brazil, coffee plants were introduced in the 18th century, along with a key pest species, *Leucoptera coffeella* (Lepidoptera: Lyonetiidae) in the early 19th century (Ukers, 1935). Since then, *L. coffeella* has adapted to the Brazilian coffee varieties and growing environment becoming the primary coffee pest in Brazil (Góngora et al., 2023). Furthermore, *L. coffeella* has evolved mechanisms to tolerate elevated caffeine concentrations found within leaf tissues (Guerreiro-Filho & Mazzafera, 2000). Such adaptation underscores a robust coevolving system that traces back to the origin of coffee plants and their relationship with the genus *Leucoptera* (Reis and Souza 1996; Pantoja-Gomez et al. 2019; Infante et al. 2022).

L. coffeella is a monophagous leafminer specialized in the genus *Coffea* (Ramiro et al. 2006). The larva of *L. coffeella* inflicts damage by consuming the leaf epidermis, penetrating the mesophyll, and causing tissue necrosis (Albuquerque et al., 2020). This process results in decreased leaf photosynthesis and significant plant defoliation (Jaramillo et al. 2019). Females of *L. coffeella* produce a sex pheromone composed of a secondary compound (5,9-di-methylhexadecane) and the main compound, 5,9-dimethylpentadecane (5,9-DMPD) (Francke et al., 1988; Arn et al., 2000). The synthetic pheromone 5,9-DMPD of *L. coffeella* has displayed biological effectiveness in the field in tactics of mass trapping, mating disruption, and attract-and-kill strategies (Arn et al., 2000; Dantas et al., 2021; Góngora et al., 2023; Malo et al., 2009). While it has been employed in coffee crops to monitor and control pest populations, it is unknown whether the *L. coffeella* sex pheromone, synthetic or naturally released, influence the antiherbivore defenses of coffee plants.

In this study, we examined how exposure of *C. arabica* plants to the sex pheromone of *L. coffeella* influences plant antiherbivore defenses and explored the potential application of the main sex pheromone component to enhance plant resistance against the pest. We specifically addressed the following questions: i) does exposing coffee plants to the natural *L. coffeella* sex pheromone dimmish leaf feeding and oviposition rate by the herbivore? ii) does applying the synthetic main component of *L. coffeella* sex pheromone change constitutive or herbivore-induced levels of plant chemical defenses? We hypothesized that exposing *C. arabica* plants to natural sex pheromone of its specialist herbivore would increase plant resistance, reducing colonization and damage by *L. coffeella*; and that exposure of *C. arabica* to the synthetic main component of *L. coffeella* sex pheromone would increase levels of plant chemical defenses either before or after the herbivore attack. To test these hypotheses, we conducted laboratory experiments exposing coffee plants to *L. coffeella* sex pheromones and evaluating coffee chemical defenses, leaf damage, and *L. coffeella* oviposition rate.

Material and methods

Plants and insects

Seeds of *C. arabica* cv. Mundo Novo were sown in plastic bags (14 × 18 cm) containing commercial substrate (TropStrato HT®, Vida Verde) and sand (3:1:1). Two seeds were sown in each seedling bag to ensure the germination of at least one seed. The seedlings were maintained in an insect-free greenhouse under natural oscillations of temperature and light incidence. When coffee plants reached their cotyledonary stage, plants were fertilized with calcium nitrate every 15 days until they were 6-8 months old or had one pair of fully expanded leaves.

The colonies of *L. coffeella* were initiated from individuals collected in coffee crops in Lavras, Minas Gerais, Brazil. Adults were kept in cages containing *C. arabica* plants serving as oviposition sites and a 10% honey solution as a food source. After eclosion, larvae penetrated leaves of coffee plants, where they fed on the mesophyll cells until reaching the pupal stage. The colonies were kept in a rearing room under controlled conditions (25 ± 2°C and 12 h photophase).

Sex pheromone exposure

We conducted an initial experiment to assess how exposing coffee plants to extracts of female *L. coffeella* sex pheromone gland affects plant resistance. To obtain the sex pheromone extract, 100 pheromone glands were dissected from one-to-three-day old virgin *L. coffeella* females during calling behavior (10:00- 13:00) (Lima et al., 2008; Michereff et al., 2007). These glands were then placed in a glass vial containing 1 mL of n-hexane (Sigma-Aldrich®, St Louis, USA). The extraction involved removing the pheromone gland from the terminal abdomen segment using microdissection forceps under a stereomicroscope. The pheromone glands of *L. coffeella* contains 5,9-DMPD, as a major component, and 5,9-di-methylhexadecane, as a minor component (Malo et al., 2009). The resulting hexane extract was at concentration of 0.1 gland female^{-1}. µL^{-1}, or 0.16 ng µL^{-1} as an estimate based on previous reports that a single *L. coffeella* pheromone gland contains 1.6 ng of 5,9-DMPD (Lima et al. 2008).

We created pheromone dispensers consisting of a cotton roll embedded with 100 µL of a solution of 70% *L. coffeella* pheromone gland hexane extract in paraffin oil (Sigma-Aldrich®, St Louis, USA) to slow down the volatile release rate (Shields & Hildebrand, 2001). Therefore, each dispenser contained the equivalent of 7 sex pheromone glands, and each coffee plant (named pheromone-exposed plant) received 3 dispensers, placed on the stem of one basal, one middle, and one apical leaf. Subsequently, each plant was enclosed in an individual perforated polyethylene bag for 48 h (Figure 1). The control plant (non-exposed plant) received a similar procedure, but the dispenser contained 70% hexane in paraffin oil (n=12 plants for each treatment).

In a separate experiment, we assessed changes in coffee plant chemical defenses following exposure to the synthetic racemic mixture of the major compound in *L. coffeella* sex pheromone blend, 5,9-DMPD (Arn et al., 1986; Kuwahara et al., 2000). Briefly, the synthesis of a racemic mixture of 5,9-dimethylpentadecane involved Grignard reactions for chain elongation, along with bromination, acetylation, dehydration, and hydrogenation reactions (Giló et al. data not published). The identity of the synthesized compound was confirmed by the analysis of the ^{13}C NMR mass spectrum (Figure S1). Previous field studies have reported effective capture of *L. coffeella* males using solutions at concentrations ranging from 3 μg/μL to 5 μg/μL of the sex pheromone blend (Michereff et al. 2007; Malo et al. 2009). Therefore, to achieve a similar concentration, we mixed our racemic mixture of 5,9-DMPD with 70% of hexane (Sigma-Aldrich®) and 30% of paraffin oil, resulting in a solution with a concentration of 2.5 μg/μL of *L. coffeella* sex pheromone. A 100 μL aliquot was applied to the dispenser, as described earlier, and placed on the stem of the apical fully expanded leaf of a coffee plant (pheromone-exposed plant), which was enclosed in a polyethylene bag for 48 h (Figure 2). As a result, the plant was exposed to 250 μg of 5,9-DMPD, which closely approximates the total amount of synthetic sex pheromone per dispenser used in the field for male capture (Michereff et al. 2007; Malo et al. 2009). The control plant (non-exposed plant) received a dispenser embedded with the same amount of the solvents (70% hexane and 30% paraffin oil). After the exposure treatment, half of the plants of both treatments (pheromone-exposed and non-exposed) were individually bagged with three females for 24h to obtain L. *coffeella* eggs on plants. After 24 h, eggs were removed leaving a single egg per plant. Six days after larval eclosion, leaf tissue was collected from the four treatments (pheromone-exposed, non-exposed, pheromone-exposed+damage, non-exposed+damage) for chemical analysis, as described below (Figure 2).

Oviposition behavior of *L. coffeella*

A no-choice experiment was performed to investigate whether the exposure of coffee plants to extracts of the *L. coffella* sex pheromone gland influences egg laying by the moth. Male and female moths were separated by sex, following the methods described in Alves (2006). Each mating pair was placed in a microtube for a 24-hour mating period. Subsequently, three mated females were confined on either apical or middle plant sections (more eggs deposited on these leaf sections) of pheromone-exposed or non-exposed plants, resulting in a total of six females per plant. As leaves of the basal plant section had few or no eggs in preliminary assays, this section was excluded in the experiment. The cage used to enclose *L. coffeella* females on each plant section was made of a plastic cup (300 ml capacity) positioned around individual leaves and sealed with foam around the leaf stem. The bottom of the plastic cup was replaced with a fine-mesh fabric cover to facilitate air circulation. After a 48-hour period, the number of eggs present in each plant section were recorded (Figure 1). The experiment was carried out with 12 plants of pheromone-exposed and non-exposed treatment maintained in a room under controlled conditions (25 ± 2°C and a 12-hour photophase).

***L. coffeella* leaf consumption**

To assess the potential impact of exposing coffee plants to extracts from the *L. coffella* sex pheromone gland on plant resistance to larval herbivory, the necrotic leaf area resulting from larval feeding was recorded over a time course. The same set of non-exposed and pheromone-exposed plants from the above-mentioned assay were used to evaluate the leaf consumption of the coffee leaf miner. Eggs on each leaf of non-exposed and pheromone-exposed plants were carefully harvested using a fine brush, leaving only a single egg on each individual apical and middle leaf (Figure 1). The basal leaves were excluded from this assay as females laid only few eggs on them. Once the *L. coffeella* larva penetrated the leaf tissue, leaf damage was assessed at three-day intervals over a total period of 30 days by capturing images of the leaves. Leaf consumption was determined by measuring the area of necrotic leaf tissue resulting from larval feeding on the mesophyll using ImageJ software (Maryland, USA) (Schneider et al., 2012). The experiment included 10 pheromone-exposed plants and 10 non-exposed plants. Environmental conditions were the same as previously described.

Plant chemical defenses

We analyzed the amounts of caffeine, hexanol, palmitate, a-ionone, citronellyl-valerate and palmitic acid as proxies of plant chemical defences in the leaf tissue of seven pheromone-exposed, non-exposed, pheromone-exposed+damage, non-exposed+damage plants respectively, by a modified protocol based on Elida et al. (2001). Moreover, these compounds were selectively analyzed as they were consistent and abundant across analyses of the chromatograms. Approximately 100 mg of the youngest fully expanded and non-damaged leaf from each treatment were flash-frozen in liquid nitrogen and ground into powder (Bullet Blender Storm 24, New York, USA). Then, 750 μL of hexane was added to each tube, heated in the microwave for 30 sec at 800W, and placed in a water bath to cool for approximately 60 sec. The tubes were centrifuged (Hermle Labor Technik GmbH – Z 233 M-2, Wehingen, Germany) at 1400 rpm for 3 min, and 150 μL of the supernatant was transferred to a glass vial. Subsequently, 5 μL of nonyl acetate (80 ng/μL) was added to the vial as an internal standard. The samples were analysed using an Agilent 7890B gas chromatograph coupled with a 5977B mass spectrometer (Agilent, Santa Clara, USA), equipped with a spitless injector maintained at 250°C, and Helium as the carrier gas. A 1-μL aliquot was injected into a HP-5MS column (30m× 0.250 mm-ID, 0.25 μm film thickness; Agilent Technologies), initially held at 40°C for 5 minutes before the temperature was ramped up at a rate of 12°C/min until it reached 250°C. Compounds were ionized by electron impact at 70 eV, and mass spectra were acquired by scanning from 40 to 300 m/z at a rate of 5.30 scans/sec. Tentative compound identification was based on the comparison with mass spectral libraries (NIST 17 and Adams2 [Allured Publishing Corporation]). The quantification of identified compounds was carried out relative to the concentration of the nonyl acetate and expressed as ng $g^{-1}$of dry leaf mass. The experiment included 8 pheromone-exposed plants, 8 non-exposed plants, 8 non-exposed+damage plants and 6 pheromone-exposed+damage plants.

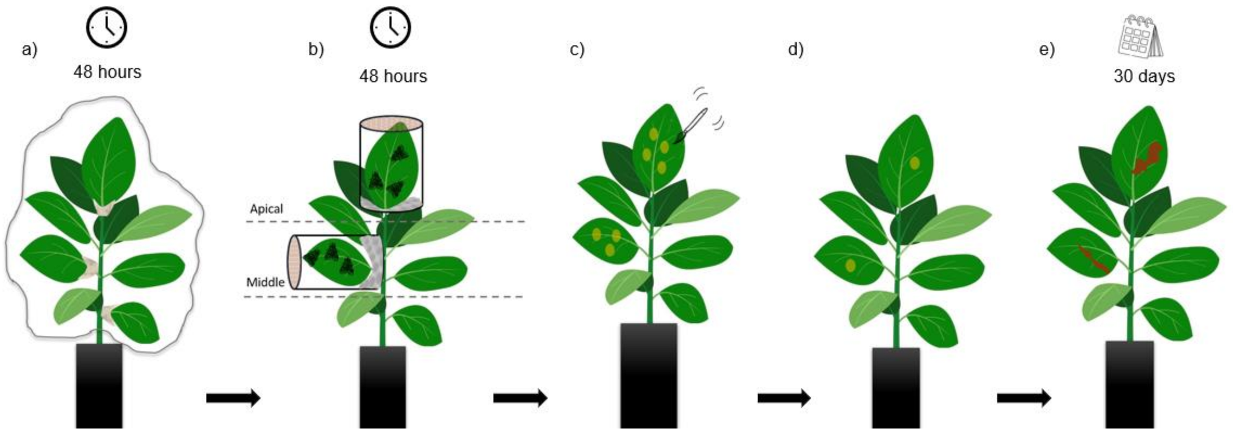

Figure 1 Schematic representation of the behavioral assays of coffee plants exposed to extracts of female *Leucoptera coffeella* sex pheromone glands. a) Coffee plants were exposed to *L. coffeella* sex pheromone gland extract or solvent only released from dispensers positioned around the stems of leaves in apical, middle, and basal sections and enclosed in polyethylene bags. b) In the oviposition experiment, three mated *L. coffeella* females were confined to a leaf in the apical and middle sections of coffee plants for 48 hours. c) Eggs (represented by yellow dots) laid on the leaves were counted. d) Eggs were removed using a fine brush leaving a single egg each on one apical and middle leaf. e) The necrotic leaf area (depicted as irregular brown shapes) was assessed at three-day intervals over a total period of 30 days by capturing images of the leaves to estimate leaf consumption by the larva.

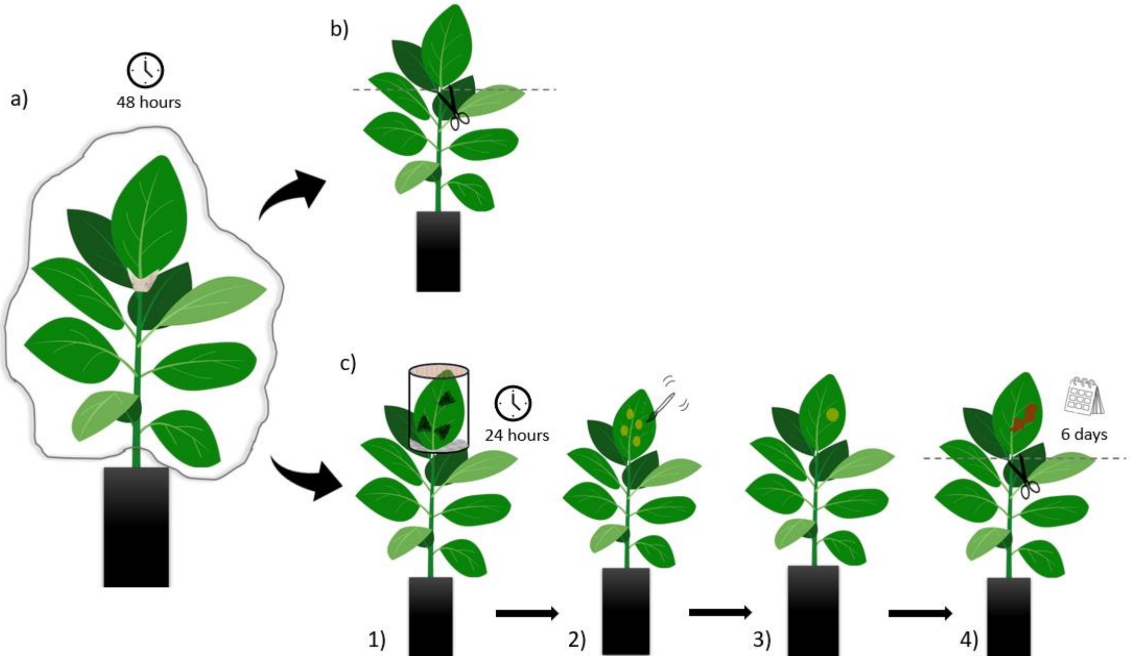

Figure 2 Schematic representation of experiment exposing coffee plants to synthetic 5,9- DMPD and quantifying defence metabolites with and without herbivory by *Leucoptera coffeella*. a) Coffee plants were enclosed in a polyethylene bag for 48 hours with a dispenser containing either 250 µg of the 5,9-DMPD (pheromone-exposed) or solvent only (non-exposed) positioned around the apical leaf stem. b) Leaf tissue was collected from half of the pheromone-exposed and non-exposed plants for chemical analysis to quantify constitutive levels of secondary metabolites. c) Half of the plants from the pheromone-exposed treatment and non-exposed treatment were subject to herbivory damage. Plants were infested with mated *L. coffeella* females on the apical leaf for 24 hours. 1-4) After 24 hours, eggs (represented by yellow dots) were carefully removed, leaving a single egg on the leaf. 4) Following egg hatching, herbivore damage by the larva (represented by irregular brown shape) was inflicted for 6 days until leaf tissue collection for chemical analysis of the treatment's pheromone-exposed+damage and non-exposed+damage.

Statistical analyses

All statistical analyses were performed in R software (R Development Team, 2022) or GraphPad Prism version 10.0.0. Data were assessed for normality using D'Agostino-Pearson omnibus (K2), Anderson-Darling (A2*), Shapiro-Wilk (W), and Kolmogorov-Smirnov (distance) tests. When necessary, data were transformed using a square root transformation to meet normality assumptions. Figures present untransformed data for ease of interpretation, while statistical analyses were conducted on transformed data where applicable. Oviposition data was done by two-way ANOVA with pheromone exposure and tissue location as predictors, including their interaction. Šídák's multiple comparison test was used for post-hoc comparisons between pheromone-exposed and non-exposed treatments within each tissue type. Leaf area consumed was done by two-way ANOVA with pheromone exposure and time as predictors, including their interaction. Šídák's multiple comparison test was used for post-hoc comparisons between pheromone-exposed and non-exposed treatments within each time point. Plant Chemical Defenses was done by identification of influential compounds using Random Forest algorithm. Effects of herbivory and pheromone exposure were assessed by two-way ANOVA with herbivory and pheromone exposure as predictors, including their interaction. Tukey's Honestly Significant Difference (HSD) post-hoc test was used for comparisons between constitutive and herbivore-induced levels within each treatment.

Results

L. coffeella laid fewer eggs on coffee plants exposed to sex pheromone gland extract regardless of leaf section.

In the no-choice oviposition experiment, we compared the number of eggs laid by *L. coffeella* on leaves of the apical or middle sections of coffee plants exposed to *L. coffeella* sex pheromone gland extract (pheromone-exposed treatment) or non-exposed plants (Figure 3). Overall, a significant difference was found in the total number of eggs laid per female between the pheromone-exposed and non-exposed treatments (one-way ANOVA, F = 4.19, p-value= 0.03), with females exhibiting reduced egg laying on pheromone-exposed plants. Female moths laid similar mean numbers of eggs on the apical or middle sections of coffee plants, although *L. coffeella* typically prefers apical leaves as demonstrated previously (Guerreiro-Filho, 2006).

Figure 3 Number of eggs laid by *Leucoptera coffeella* on coffee plants exposed to sex pheromone gland extract (n=12) or non-exposed (n=12), across two distinct sections on coffee plants (apical and middle). Black bars represent pheromone-exposed treatment and grey bars represent non-exposed treatment (control). Asterisks denote significant differences: *p-value 0.03 by one-way analyses of variance (ANOVA) followed by Šídák's multiple comparison test. Bars indicate means ± SEM.

Coffee plants exposed to female sex pheromone extract were more resistant to *L. coffeella* herbivory

We assessed leaf consumption by *L. coffeella* larvae across both pheromone-exposed and non-exposed coffee plants, measured in terms of necrotic leaf area (Figure 4). The necrotic leaf area due to larval feeding was smaller in pheromone-exposed plants than non-exposed plants along the time course (two-way ANOVA, F = 2.3, p-value = 0.01), with the greatest reductions observed on day 27 (Tukey HSD p-value= 0.001) and day 30 (Tukey HSD p-value= <0.0001).

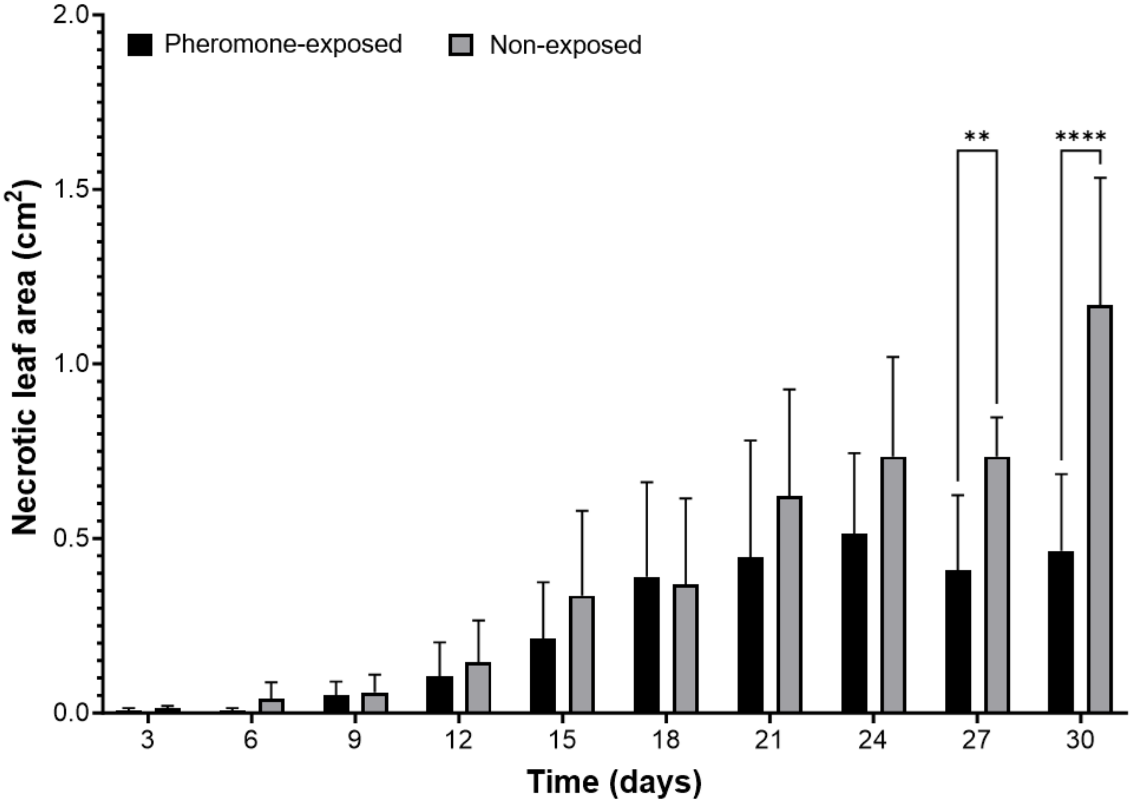

Figure 4 Necrotic leaf area due to *Leucoptera coffeella* feeding on coffee plants exposed, or not (non-exposed), to sex pheromone gland extract (pheromone-exposed) over a 30-day period. Black bars represent pheromone-exposed treatment (n=10), and grey bars represent non-exposed control (n=10). Error bars indicate means ± SD. Asterisks denote significant differences: **p-value ≤ 0.01 by two-way analyses of variance (ANOVA) followed by pairwise comparisons.

Coffee plants exposed to the primary *Leucoptera coffeella* sex pheromone component induced higher levels of chemical defenses following herbivory

Coffee plants exposed to the racemic mixture of 5,9-DMPD, the main component of *L. coffeella* sex pheromone blend, had quantitative changes of secondary plant metabolites (Table 1). The constitutive amounts of the individual compounds octadecane, a-Ionone, caffeine, methyl palmitate, hexadecenoic acid and citronellyl valerate did not change in response to the pheromone exposure, neither the total amount of compounds (Table 1). However, there was a downregulation on the constitutive amount of hexanol on plants under pheromone exposure (one-way ANOVA, F= 12.26, p-value= 0.003). When we combined pheromone exposure and herbivory damage, we observed an increase of compounds compared to non-exposed+damage plants (Table 1). Specifically, pheromone-exposed+damage plants had greater amounts of citronellyl valerate (two-way ANOVA, F= 10.34, p-value = 0.003; Tukey`s p-value < 0.05) and showed elevated total amount of compounds compared to non-exposed+damage plants (two-way ANOVA, F= 4.77, p-value = 0.03; Tukey`s p-value < 0.05) (Table 1). Compounds highlighted in bold refer to the Random Forest variable importance values. These findings imply that the exposure of coffee plants to the racemic mixture of 5,9-DMPD followed by herbivory damage upregulates the production of defensive compounds, suggesting a potential priming effect.

Table 1 Relative amounts of secondary plant metabolites (mean ± SEM ng/g) found on the apical leaf of *Coffea arabica* plants under pheromone-exposed, non-exposed, pheromone-exposed+damage and non-exposed+damage treatments to the racemic mixture of 5,9-DMPD.

Compounds	Treatments			
	Pheromone-exposed	Non-exposed	Pheromone-exposed+damage	Non-exposed+damage
Alcohol				
Hexanol	11.8 ± 2a	29.83 ± 4.4b	9.64 ± 1.3A	3.27 ± 2.5A
Alkane				
Octadecane	119.58 ± 65.9a	127.46 ± 8a	287.3 ± 65.1A	256.5 ± 50.7A
Monoterpene				
a-Ionone	26.48 ± 8.6a	25.36 ± 8.21a	49.26 ± 4.4A	42.9 ± 3.3A
Alkaloids				
Caffeine	192 ± 24.7a	164.48 ± 24.3a	561.7 ± 149.6A	324 ± 98.8A
Ester				
Citronellyl valerate	174.17 ± 29.4a	123.48 ± 33.7a	588.5 ± 73.2A	315.5 ± 56.8B
Fatty acid derivates				
Methyl palmitate	64.71 ± 22.6a	48 ± 23.4a	241.92 ± 128.1A	78.9 ± 26.19A
Hexadecanoic acid	154.25 ± 12.8a	170.57 ± 26a	201 ± 20.7A	167 ± 25.1A
Total	742.99 ± 27.3a	689 ± 23.7a	1939 ± 85.7A	1188 ± 49.9B

Quantification was based on the peak area relative to the internal standard (nonyl acetate).
*Different letters in the same row indicate significant differences among the treatments according to ANOVA followed by the Tukey’s test (P < 0.05).
Lower case letters indicate differences between Pheromone-exposed and non-exposed treatment according to ANOVA followed by the Tukey’s test (P < 0.05).
Upper case letters indicate differences between Pheromone-exposed+damage and non-exposed+damage treatment according to ANOVA followed by the Tukey’s test (P < 0.05).
Compounds highlighted in bold refers to the Random Forest variable importance values.

Discussion

Plants detection of insect herbivore pheromones is predicted to occur most commonly in tightly co-evolved plant–insect relationships (Helms et al. 2013). The close association between *L. coffeella* and coffee plants has been documented since the 18th century, and it has been largely attributed to the monophagous behavior of *L. coffeella*, which feeds exclusively on coffee plants (Pantoja-Gomez et al., 2019; Dantas et al., 2021). Our findings demonstrate that coffee plants exposed to the female *L. coffeella* sex pheromone exhibited reduced damage over time compared to unexposed plants, and *L. coffeella* females laid fewer eggs on previously exposed plants. Also, plants' exposure to the main component of the *L. coffeella* pheromone (racemic mixture of 5,9-DMPD) influenced production of plant defense compounds. This resulted in a significant reduction in the constitutive levels of hexanol and an increase in the induced levels of citronellyl valerate and the total quantity of induced secondary compounds in coffee leaves. Overall, these findings suggest that both natural and synthetic *L. coffella* sex pheromone compounds can be detected by *C. arabica*, potentially serving as a signal for future herbivore attacks based on the observed behavioral and chemical changes. Therefore, the results presented here corroborate our hypotheses in that *L. coffeella* sex pheromone is perceived by coffee plants as a signal of potential herbivore attack, and that the major component of the sex pheromone increases the levels of plant chemical defenses.

The host selection by *L. coffeella* females for egg deposition plays a crucial role in their fitness, as the larvae lack the ability to move from the leaf where the egg was deposited (Dantas et al., 2021; Guerreiro-Filho, Silvarolla, & Eskes, 1999). *L. coffeella* females exploit coffee plant chemicals to select a suitable host for the offspring. For instance, *L. coffeella* preference for oviposition seems to be influenced by high concentrations of the monoterpene p-cymene, but low concentrations of beta-cymene (Magalhães 2005; Magalhães et al. 2008). In the present study, we found that exposure of coffee plants to sex pheromone gland extract caused a reduction in the egg-laying activity of *L. coffeella* females, regardless of whether the leaves were located at the apical or middle third. Our results agree with those found in a different system, in which *Solidago altissima* plants exposed to the male sex pheromone of a specialist herbivore had lower ovipunctures relative to non-exposed plants (Helms et al., 2013, Yip et al., 2019). The observed effect on herbivore host selection may be associated with the greater resistance of pheromone-exposed plant to offspring, given that larvae in this treatment consumed less, potentially indicating lower host quality. This aligns with the preference–performance hypothesis, which posits that natural selection favours females capable of discriminating among hosts of varying suitability to lay eggs on which offspring perform better (Jaenikjz, 1978; J. Thompson & Pellmyr, 1991).

We also found that exposure of coffee plants to the major compound of *L. coffella* sex pheromone 5,9-DMPD caused reduction of the constitutive levels of hexanol present in coffee leaves. Hexanol is a known compound found in the composition of both arabica coffee leaves and coffee beans (Patil et al., 2022; Quijano-Célis et al., 2015; Zhang et al., 2019). The suppression in its level in pheromone-exposed plants may be attributed to its role as a precursor for synthesizing other metabolites not measured in our study, which could potentially contribute to the reduction in the egg deposition by *L. coffeella* on these plants. It is also possible that the reduction in hexanol of coffee leaves may be less stimulant to egg-laying females given that hexanol act as oviposition stimulant for other insect herbivores (Bingjun et al., 2023; Solé et al., 2010). It is important to note that other plant chemicals, particularly volatile organic compounds (VOCs) emitted by pheromone-exposed coffee plants, or even the absorption and emission of *L. coffeella* sex pheromone by coffee leaves, may be involved in females' response to pheromone-exposed plant. Therefore, future studies should investigate how exposure to *L. coffeella* sex pheromone affects the constitutive volatile profile of coffee plants, and whether leaves have residues of sex pheromone that influence interactions with ovipositing females on the plant.

After egg eclosion, the *L. coffeella* caterpillars penetrate the leaf tissue through the lower part of the egg and immediately starts feeding (Michereff et al., 2007). In our study, we found that pheromone exposure of coffee plants reduced the feeding damage over time by *L. coffeella* larvae. The pheromone extracts likely activated chemical defense pathways in coffee plants, rendering them less palatable to *L. coffeella*. This deleterious effect became detectable at the end of larval development, specifically 27 and 30 days after hatching. Our results corroborate with the findings of previous studies (Helms et al. 2013, 2014, 2017), in which an insect-airborne signal triggers plant defense mechanisms, resulting in reduced subsequent feeding damage by herbivores. Most of these studies link such responses to the elevated levels of jasmonic acid (JA) as well as priming of herbivore-induced plant defenses modulated by this phytohormone. Previous studies examining the priming effect of pheromone exposure on plants, attributed such effect to the production of phytohormones, and changes in gene expression related to plant defense mechanisms (Helms et al., 2013, 2014; Magalhães et al., 2019; Yip et al., 2019, 2021). Although we found a more vigorous herbivore-induced response of pheromone-exposed coffee plants than non-exposed plants with the racemic mixture of 5,9-DMPD, we cannot conclusively assert that the plant was primed by exposure to 5,9-DMPD, as we did not measure phytohormone levels or expression of plant-defense.

Resistance of coffee plants to *L. coffeella* feeding has not been associated with physical aspects or phenological traits (Filho et al., 1999; Guerreiro Filho & Mazzafera, 2000; Leroy et al., 2000), but rather to the composition of secondary metabolites of coffee plants (Guerreiro-Filho, 2006). Larval herbivory by *L. coffeella* caused an overall increase of several metabolites measured in our study, suggesting their involvement in coffee plant defense against herbivores (Mazzafera et al. 1991; Magalhães et al. 2010). However, exposure to the major compound of *L. coffeella* sex pheromone 5,9-DMPD promoted a stronger plant response induced by larval feeding, as pheromone-exposed, herbivore-damaged plants contained 1.6-fold more in the total amount of secondary metabolites relative to non-exposed, herbivore-damaged plants. Regarding the individual compounds, while exposure of coffee plants to 5,9-DMPD led to a reduction in the constitutive level of hexanol, it triggered a 1.8-fold increase in the herbivore-induced level of the terpene citronellyl valerate. Known for its insecticidal and repellent properties, citronellyl valerate is commonly found in essential oils from various plants, including coffee plants (Borges Pereira et al., 2012; Guo et al., 2016; Kadir et al., 2023), and may potentially contribute to the observed resistance in pheromone-exposed plants to *L. coffeella*.

Conclusion

Our study demonstrated that exposure to *L. coffeella* sex pheromone increases the plant's ability to mount defense against *L. coffeella*. The observed reduction in feeding damage and egg-laying by *L. coffeella* females, together with the alterations in plant secondary metabolites on pheromone-exposed plants, highlights the significance of the impact of insect sex pheromones on plant resilience. Moreover, the effectiveness of both natural and synthetic forms of *L. coffeella* sex pheromone in triggering plant defenses indicates the significance of these signals in both ecological and agricultural contexts. Since the synthetic pheromone of *L. coffeella* is commercially available, future studies should explore how exposure to the commercial synthetic lures might enhance defenses of coffee plants to this major pest in field conditions. Our findings not only contribute to the understanding of plant-insect interactions but also opens new perspectives for exploring novel sustainable strategies in crop protection for management of *L. coffeella* in coffee plantations.

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Supplementary materials

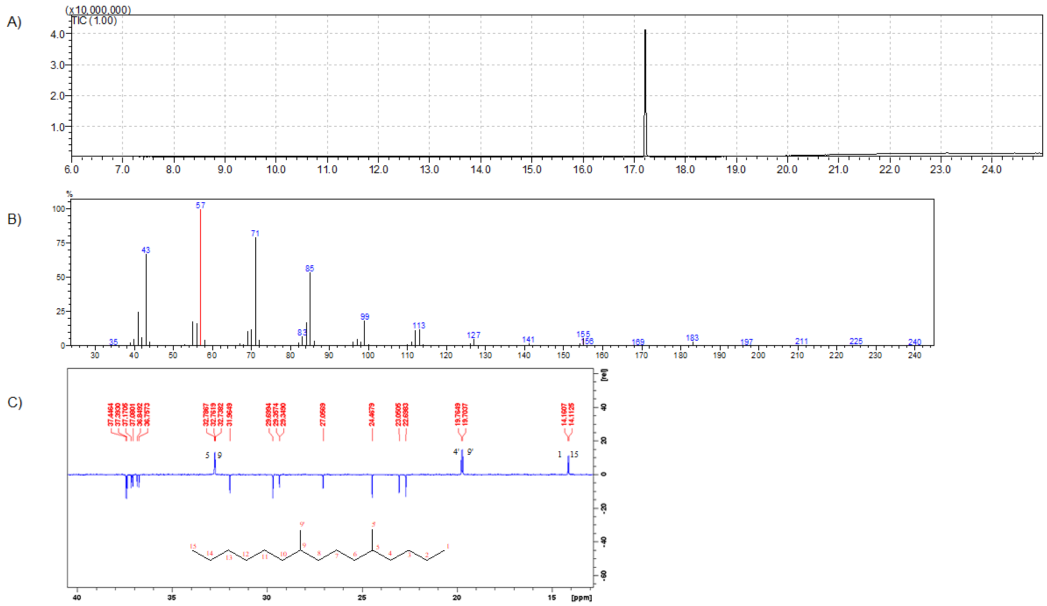

Figure S1: A) Chromatogram of 5,9-dimethylpentadecane (**10**); B) Mass spectrum of 5,9-dimethylpentadecane (**10**); C) ^{13}C NMR spectrum of 5,9-dimethylpentadecane (**10**).

ARTIGO 2 - Wild and cultivated *Cucurbita pepo* varieties exhibit elevated defence responses to herbivore-associated volatile cues.

Artigo elaborado de acordo com as normas do periódico "*Plant, Cell and Environment*".

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Abstract

Plants can detect a variety of early warning cues associated with insect herbivory and respond by activating or preparing their defenses. Crop domestication has commonly resulted in reduced plant investment in defense-related traits, selecting instead for increased growth and yield. An outstanding question is whether domestication has also affected plants' ability to respond to herbivore-associated cues. The origin and consequences of domestication are well studied among plants in the family Cucurbitaceae, several of which are economically important crop plants. In the current study, we evaluated the role of domestication in shaping plant responses to two types of herbivory-associated cues: an insect herbivore aggregation pheromone (AGP) and herbivore-induced plant volatiles (HIPV). We exposed three closely related varieties of *Cucurbita pepo* (two domesticated–zucchini squash and yellow squash and one wild–Texas gourd) to striped cucumber beetle (*Acalymma vittatum*) aggregation pheromone (AGP) or HIPV and assessed plant resistance and expression of chemical defenses against beetle herbivory. We predicted that domestication has negatively impacted plants' ability to respond to herbivore-associated cues and that the wild plant variety would exhibit the strongest defense response. Unexpectedly, domesticated *C. pepo* varieties demonstrated greater resistance to herbivore damage than their wild counterparts. This increased resistance was reflected in the reduced feeding damage and higher phytohormone levels found in the domesticated varieties. Notably, there was some diversity in the volatile organic compounds (VOCs) produced among the domesticated varieties. Additionally, in the yellow squash variety, exposure to elevated doses of AGP was associated with increased resistance to *A. vittatum* feeding and higher levels of defense phytohormones, especially jasmonic acid (JA) and salicylic acid (SA).

Key Words: Aggregation pheromone; *Curcubita pepo*; Defense priming; Herbivore-induced plant volatiles; Plant domestication.

Introduction

Plants can recognize various signals linked to insect herbivory, enabling them to activate an effective defense mechanism (Heil & Karban, 2010). These signals include early pre-herbivory cues that alert plants to the presence of herbivores and the likelihood of impending damage (Ninkovic et al., 2021). For instance, plants can detect herbivorous insect saliva, egg-laying, physical contact, excretions, and insect-derived volatiles such as pheromones (Acevedo et al., 2019; Bittner et al., 2019; A. Helms et al., 2017; Keefover-Ring, 2013; Musser et al., 2012; Peñaflor et al., 2011; Reymond, 2013). When a plant detects an herbivory cue, it activates molecular signaling pathways, resulting in changes to the plant's metabolite profile and triggering induced defenses and/or defense priming (Erb & Kliebenstein, 2020; Van Loon et al., 2006). Defense priming prompt plants to better handle future challenges. When exposed to stress or signaling molecules, plants improve the activation of their inducible defenses during subsequent herbivory attacks (Martinez-Medina et al., 2016; C. R. Rodriguez-Saona et al., 2009). This enables plants to respond more quickly and efficiently to future threats while conserving metabolic resources (Conrath et al., 2015). Most studies assessing plant defense priming have concentrated on plant responses to HIPVs released by damaged neighboring plants, which signal potential future damage and thereby prime the plants for upcoming herbivory attacks (Ida et al., 2018; Karban et al., 2000; Paudel Timilsena et al., 2020). The use of HIPV as priming agents has been widely documented across various species, nevertheless there is increasing evidence that different plant species also respond to insect herbivore pheromones (A. Helms et al., 2017; D. M. Magalhães et al., 2019; Yip et al., 2019, 2021). Studying various systems and signals that trigger plant defense priming is essential for understanding plant defense mechanisms. Additionally, there has been an increasing effort to investigate plant priming in different ecological contexts, such as under abiotic stress conditions (Aguirre et al., 2023), in association with pathogens (Hönig et al., 2023), or beneficial microbes (Woo et al., 2023), among others. For instance, priming has been observed in both cultivated and wild plant species (Liao et al., 2021; van Doan et al., 2020). However, a critical question persists: has domestication affected plants' ability to detect and respond to early warning cues associated with insect herbivory?

Plant domestication is the process by which wild plants are cultivated and genetically altered over time to express traits that are favorable for human use, such as improved yield or taste

(Ladizinsky, 1998). However, domestication has broadly resulted in reduced resistance and lower expression of defense-related traits across diverse plant species (Benrey et al., 1998; Turcotte et al., 2014; Whitehead et al., 2017), possibly due to trade-offs between growth and defense mechanisms aimed at enhancing yield (Rosenthal & Dirzo, 1997; Kempel et al., 2011). Consequently, the production of a wide range of specialized plant metabolites may be limited to certain plant populations or lineages because of domestication, potentially changing the way plants protect themselves (Smith, 2006). Plant domestication has led to a reduction in production of a variety of specialized metabolites including constitutively produced metabolites and metabolites that are induced or synthesized in response to a stressor such as herbivory. For example, the cabbage domestication for leaf consumption has significantly reduced the inducibility of glucosinolates – an important anti-herbivore defensive mechanism of plants in the family Brassicaceae (Moreira et al., 2018). Another study found that cranberry plants bred for increased productivity and fruit characteristics hindered the production of defense-related phytohormones and emission of volatile organic compounds, impacting the plants' ability to resist insects (Rodriguez-Saona et al. 2011). Although there is strong evidence that domestication has negatively influenced plant production of specialized metabolites, there is a lack of studies investigating how domestication may affect plant defense priming.

The origin and consequences of domestication have been extensively studied for plants in the family Cucurbitaceae. Many species in this family are economically important crops, including cucumbers, melons, pumpkins, and squashes. The domestication of *Cucurbita pepo* represents a fascinating aspect of agricultural history (Gong et al., 2012). *C. pepo* is native to the Americas, and its domestication likely began around 8,000 to 10,000 years ago, involving human-driven selection for traits like larger fruit size, enhanced taste, and modified growth patterns (Paris, 1989). Through successive generations of artificial selection, cultivated varieties of *C. pepo* emerged, displaying significant differences from their wild predecessors(Kates et al., 2017). Many studies have found support of difference in traits related to plant defenses being lost during domesticated varieties of *C. pepo* (Theis et al., 2014). Cucurbitacin content is the most known defensive compound that has been lost during domestication of the genus *Cucubita* (Pickersgill, 2018). Nevertheless, research has found no evidence suggesting that the loss of cucurbitacin has adversely impacted herbivores (Bruno et al., 2023). For instance, recent studies comparing wild type *Cucurbita* plants with high-cucurbitacin levels to domesticated varieties with low-cucurbitacin levels demonstrated that larvae of the specialist herbivore (*Acalymma vittatum*) and the generalist herbivore (*Diabrotica balteata*) were more attracted to the bitter roots of wild plants than those of domesticated plants(Jaccard et al., 2023). Additionally, another study discovered that *D. balteata* larvae feeding on high-cucurbitacin roots experienced higher predation rates compared to larvae that fed on domesticated plants with lower-cucurbitacin content(Jaccard et al., 2022). These findings have introduced new insights into plant chemical defenses under domestication by exploring below-ground interactions. Nevertheless, plant priming of defenses through herbivory cues of *C. pepo* plants under domestication has not yet been explored.

Cucurbit plants face damage from several species of specialist herbivores, including the striped cucumber beetle (*Acalymma vittatum*) (Brewer, et al. 1987). These species share a co-evolutionary history in the Americas, where *A. vittatum* has evolved to sequester cucurbitacin's, for its own defense against predators (Jolivet et al., 1994). Adult *A. vittatum* beetles inflict foliar and fruit damage (Hoffmann et al., 2003), and serve as a vector for bacterial wilt (*Erwinia tracheiphila*). This pathogen is introduced into the plant's vascular system during *A. vittatum* feeding, facilitating disease spread (Haber et al., 2021). The cumulative impact, especially when coupled with bacterial wilt transmission, can markedly reduce crop yields (Ayyappath et al., 2001). A relatively recent discovery is that male *A. vittatum* beetles produce an aggregation pheromone, containing the primary component vittatalactone (Morris et al. 2005; Yadav et al. 2011). The aggregation pheromone plays a crucial role in the social behavior and communication of this insect species by attracting conspecifics and coordinating group activities, such as feeding or mating (Gardner et al., 2015; Haber et al. 2021). There is growing interest in developing semiochemical control methods using vittatalactone, with several studies reporting it as an effective lure under field conditions (Haber et al., 2023; Weber et al., 2023, 2022).

The Cucurbitaceae family and the major specialist beetle provide an excellent system to dissect the relationship between plant domestication and plant priming defense. The goal of this study was to examine responses of three varieties of *C. pepo* to two volatile cues associated with cucumber beetle herbivory: AGP and HIPV. We exposed two domesticated *C. pepo* cultivars, zucchini squash (*C. pepo* subsp. *pepo* var. Raven) and yellow squash (*C. pepo* subsp. *ovifera* [syn. texana] var. yellow crookneck) and a wild variety, Texas gourd (*C. pepo* subsp. *ovifera* [syn. texana] var. texana), to male beetle aggregation pheromone (AGP) or HIPVs emitted by beetle-damaged conspecific plants. We then assessed plant resistance and expression of chemical defenses against beetle herbivory. We also examined whether yellow squash plants respond to different doses of synthetic AGP. We hypothesized that exposing all varieties of *C. pepo* plants to the naturally produced beetle aggregation pheromone would enhance plant resistance, leading to reduced damage and higher expression of defenses compared to controls. We predicted that domestication has negatively impacted *C. pepo's* ability to respond to herbivore-associated cues and that the wild Texas gourd plants would exhibit a stronger increase in resistance compared to the two domesticated cultivars. We also predicted that yellow squash would respond to synthetic AGP in a dose-dependent manner. Overall, this study offers insights into the effects of domestication on plant defense priming, revealing intricate aspects of *C. pepo* plant domestication and how it has influenced these plants' ability to perceive and respond to insect cues.

Material and Methods

Plant and insects:

Three varieties of *Cucurbita pepo* from two distinct subspecies were obtained from the following sources: zucchini squash (*C. pepo* ssp. *pepo* cv. Raven) from West Coast Seeds®, yellow squash (*C. pepo* ssp. *ovifera* syn. ssp. *texana* cv. yellow crookneck) from Johnny's Selected Seeds®, and Texas gourd (*C. pepo* subsp. *ovifera* syn. ssp. *texana* var texana), from the Stephenson lab at The Pennsylvania State University. All plants were grown from seed and used in experiments after 3–4 weeks of growth. Plants were grown in individual pots in topsoil mix (Hyponex Corporation) with 3g Osmocote® fertilizer (15-9-12 N-P-K; Scotts) and were kept in an insect-free, climate-controlled growth room with supplemental lighting (16 hr light: 8 hr dark; 22°C: 29°C; 56% RH, Fluence). Striped cucumber beetles *A. vittatum* were maintained in a laboratory colony on cultivated squash *C. pepo* subs. *ovifera* cv. yellow squash and cucumber (*Cucumis sativus* cv. Max Pack) under controlled conditions (16 hr light: 8 hr dark; 22°C: 29°C; 56% RH).

Collection and analysis of *A. vittatum* aggregation pheromone on different *C. pepo* varieties

To confirm that male *A. vittatum* beetles emit detectable quantities of vittatalactone under our experimental conditions, we collected and quantified vittatalactone emissions from beetles feeding on each of the three *C. pepo* varieties. We collected VOCs from groups of 25 male *A. vittatum* beetles, aerated in 0.4-L glass chambers for 4 hours. Four glass chambers, each containing 25 beetles, were used per variety. Filtered air was pushed into the chambers at 0.5 L min−1, and VOCs were trapped on adsorbent filters containing 60 mg of HaySep®Q (Hayes Separations, Inc) at 0.5 L min−1 (A. Helms et al., 2017). Vittatalactone quantification was measured as the mean (ng) from pooled data of 100 beetles per variety, quantified relative to the internal standard concentrations of nonyl acetate.

Synthetic vittatalactone preparation

Synthesis of the vittatalactone mixture was carried out through a contract between USDA ARS and Chemveda Life Sciences in San Diego, CA, following the procedure outlined in Chauhan and Paraselli (2017) and generously supplied by Dr. Donald C. Weber. The synthetic vittalactone was enclosed within a grey rubber septum (18 mm length × 9 mm maximum diameter). Each septum was loaded with a 1mg/septum vittalactone mixture and 0.6 ml of hexane/septum with a release rate of 14.7 ng of natural (2R,3R,4S,6S,8S) vittalactone per hour (Weber et al., 2023).

Plant exposure to herbivore-associated volatile cues.

In the first experiment, we examined whether wild and domesticated varieties of *C. pepo* respond to volatile, herbivory-associated cues. Plants from the three *C. pepo* varieties were exposed to either HIPV, AGP, or a control. Individual plants were enclosed within 4 L glass domes and exposed to one of three volatile treatments for 24 hours and into each glass dome clean air was pushed at 1.5 L min^{-1} and air containing VOCs was pulled out at 0.5 L min−1 with VOCs collected on an adsorbent filter trap containing 60 mg of HaySep®Q (Hayes Separations, Inc). For the HIPV treatment, female beetles (n = 25) that do not produce aggregation pheromone, were enclosed in polyester mesh bags (8cm x 10cm), each containing one detached leaf as a food source. For the AGP treatment, male beetles (n = 25) that produce aggregation pheromone, were enclosed in polyester mesh bags, each containing one detached leaf as a food source. The control treatment consisted of mesh bags containing one detached leaf. All detached leaves used matched the plant variety being exposed. The polyester bags were positioned beside the respective plants for 24 hours, ensuring that the beetles within each bag did not come into direct contact with the plants (Figure 1). Following 24 hours, we removed all the polyester bags containing the prior volatile sources from each glass dome. The sample size for each plant variety is as follows: yellow squash (n = 24 plants; AGP = 10, HIPV = 9, Control = 5); zucchini (n = 19 plants; AGP = 8, HIPV = 6, Control = 5); Texas gourd (n = 19 plants; AGP = 7, HIPVs = 7, Control = 5).

In a second experiment, we examine whether yellow squash plants respond to different doses of synthetic *A. vittatum* aggregation pheromone. Yellow squash plants were exposed to two different doses of synthetic AGP. In a large mesh cage (40.6cm x 40.6cm x 61cm) we added five yellow squash along with 10 septa (10mg total – 1mg/septum) of the vittatalactone mixture (low dose) and another five plants in a separate cage containing 10 septa with 0.6ml of hexane each serving as controls. For the high dose experiment, the same procedure was carried out but this time, instead of using 10 rubber septa, we employed 50 rubber septa (50mg total – 1mg/septum) either loaded with the vittatalactone mixture or hexane. After 24 hours, plants and septa were removed from the cages. The sample size for each dose is as follows: Low dose: Synthetic AGP = 10 plants, Control = 10 plants; High dose: Synthetic AGP = 10 plants, Control = 10 plants.

All experiments were conducted under laboratory conditions in a climate-controlled, insect-free growth room under artificial full-spectrum growing lights (24°C:21°C, 50% Relative Humidity, 14:10-h light:dark cycle), photosynthetic photon flux density (PPFD) 450 µmol $^{m-2\ s-1}$ (lights were from Fluence).

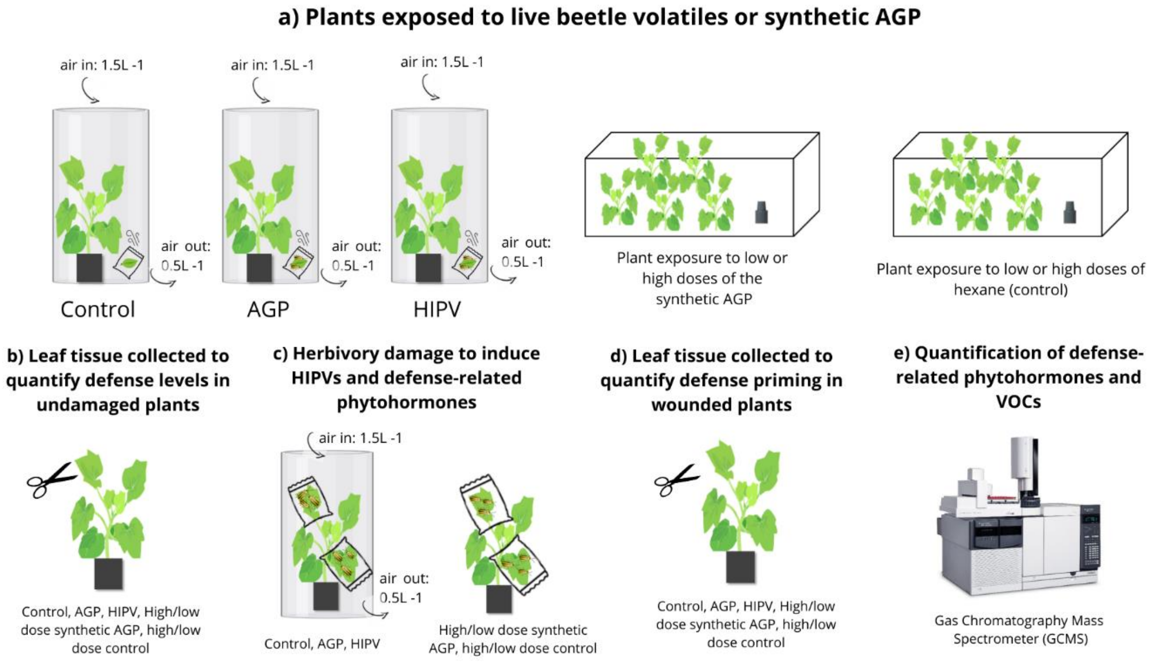

Figure 1 Experimental design for evaluating defense priming of plant exposure to herbivore-associated volatile cues. (a) Squash plants were exposed to different volatiles cues (Control, AGP, HIPV, low and high dose synthetic AGP) for 24 hours; collection of constitutive VOCs was carried out on Control, AGP and HIPV treatment during exposure using an adsorbent filter trap containing 60 mg of HaySepQ® (Hayes Separations, Inc) for 8 hours. (b) An initial leaf tissue sample was collected to quantify defense levels in undamaged plants immediately following volatile exposure. (c) All plants were subjected to *A. vittatum* herbivory damage for 24 hours; collection of HIPVs was carried out on Control, AGP and HIPV treatment during herbivory damage using an adsorbent filter trap containing 60 mg of HaySepQ® (Hayes Separations, Inc) for 8 hours. (d) Two additional leaf samples for all damaged plants were collected to determine whether defense priming occurred by looking at levels of jasmonic acid (JA) and salicylic acid (SA). (e) Both constitutive VOCs and HIPVs were measured, and defense-related hormones, including jasmonic acid (JA) and salicylic acid (SA), were extracted from leaf tissue samples and quantified using gas chromatography-mass spectrometry (GC-MS).

Cucumber beetle herbivory bioassays

To investigate whether *C. pepo* plants from three varieties exhibit elevated resistance to herbivory damage following exposure to herbivore-associated cues, we conducted herbivory bioassays where we challenged plants with *A. vittatum* herbivory and assessed levels of feeding damage. Following the exposure treatments described above, we selected two leaves from each exposed plant and enclosed them in separate polyester mesh bags. Inside each bag, we introduced three *A. vitattum* females that had been starved overnight for 10 hours. Herbivory damage lasted for 24 hours. During herbivory damage, plants exposed to live beetle volatiles (Control, AGP and HIPV treatments) were enclosed within a 4L glass dome for VOC collection. Into each glass dome clean air was pushed at 1.5 L min^{-1} and air containing VOCs was pulled out at 0.5 L min−1 with VOCs collected on an adsorbent filter trap containing 60 mg of HaySepQ® (Hayes Separations, Inc) (Figure 1c). Bioassays were performed on separate days for each cultivar, and the sample size for each is as follows: yellow squash (n = 24 plants; AGP = 10, HIPVs = 9, Control = 5); zucchini (n = 19 plants; AGP = 8, HIPVs = 6, Control = 5); Texas gourd (n = 19 plants; AGP = 7, HIPVs = 7, Control = 5); yellow squash plants exposed to synthetic AGP (high-dose = 10; low-dose = 10) All experiments were conducted under the same laboratory condition as previously described. Damage data was analyzed with ImageJ® software (Maryland, USA).

Collection and analysis of plant volatiles

To understand how herbivory-associated cues affect the emission of volatile organic compounds (VOCs) by plants from the different varieties we quantified plant volatile emissions as a measure of plant chemical defenses. Therefore, dynamic headspace sampling was used to collect VOCs emitted by plants previously exposed to live beetle volatiles before and after damage. Plants were individually enclosed within 4 L glass domes and into each glass dome clean air was pushed at 1.5 L min^{-1} and air containing VOCs was pulled out at 0.5 L min^{-1} with VOCs collected on an adsorbent filter trap containing 60 mg of HaySep®Q (Hayes Separations, Inc) for 8 hours (8AM – 4PM). Then, aboveground biomass was harvest and dried. Compounds from all the VOCs collection were eluted from filter traps using 150 µl dichloromethane. A 5 µl aliquot of standard solution containing nonyl-acetate (80 ng/µl) was added to each sample. Samples were analyzed using an Agilent 7890B gas chromatograph and a 5977B mass spectrometer with a splitless injector held at 250°C and helium as the carrier gas. After sample injection (1 µL), the column (HP-5MS 30m× 0.250 mm-ID, 0.25 µm film thickness; Agilent Technologies) was held at 40°C for 5 min before the temperature was increased at 12°C min−1 to 250°C. Compounds were ionized by electron impact ionization at 70 eV and mass spectra were acquired by scanning from 40 to 300m/z at 5.30 scans/s. Compounds were identified by comparison with mass spectral libraries (NIST 17 and Adams2 [Allured Publishing Corporation]), and structure assignments were confirmed where possible by comparison of mass spectra and retention times with authentic standards (Grunseich et al., 2020). Compounds identified were quantified relative to the nonyl acetate internal standard concentrations and were calculated as ng/g dried leaf mass.

Quantification of plant defense hormones

To further assess the plant defense status, we measured the levels of defense-related hormones in leaf tissues from plants exposed to volatiles emitted by live beetles and to the synthetic AGP mixture, both prior to and following damage. After collecting tissue samples from each plant (~ 100mg) the samples were immediately flash frozen in liquid nitrogen and stored at −80°C until analysis of the defense-related phytohormones: jasmonic acid (JA) and salicylic acid (SA). For phytohormone quantification, endogenous JA and SA were extracted from leaf tissue and derivatized to methyl esters and then isolated using vapor phase extraction. The phytohormones were then analyzed by chemical ionization-gas chromatography/mass spectrometry using selected ion monitoring (Schmelz et al., 2004). For analysis, filter traps containing 60 mg of HaySep®Q were eluted into a sample vial with 150 ul of dichloromethane and analyzed by GC/MS. Phytohormones were verified by comparing their retention times and spectra with known standards. JA and SA quantification involved the addition of 100 ng of an isotopically labeled internal standard for each phytohormone, followed by normalization based on the mass of leaf tissue.

Statistical analysis

All statistical analyses were conducted using R software (R Development Team, 2022). Prior to analysis, data were assessed for normality using the Shapiro-Wilk test (W) and transformed when necessary to meet assumptions of normality. Transformations included square root for damage, total VOC, α-Pinene, and β-Ocimene datasets, and log transformation for JA and SA datasets. Figures present untransformed data for ease of interpretation. Herbivory damage was done by comparison among plant cultivars and treatments using one-way ANOVA with damage as the response variable and treatment exposure as a predictor, including their interaction, Tukey's (HSD) post-hoc test was used for pairwise comparisons. Identification of influential compounds was done with Random Forest algorithm and volatile analysis was done by two-way ANOVA with total VOC, α-Pinene, β-Ocimene or heneicosane abundance as the response variable, and condition (constitutive and induced) and treatment exposure as predictors, including their interaction, Tukey's HSD post-hoc test was used for pairwise comparisons. Leaf area damage with synthetic lures was done by two-way ANOVA with damage as the response variable and treatment and concentration as predictors, including their interaction, Tukey's HSD post-hoc test was used for pairwise comparisons. Phytohormone analysis was done by two-way ANOVA with JA or SA abundance as the response variable, and condition (constitutive and induced) and treatment exposure as predictors, including their interaction, Tukey's HSD post-hoc test was used for pairwise comparisons.

Results

Exposing domesticated squash varieties to volatile herbivory cues increased plant resistance to cucumber beetle herbivory.

The three *C. pepo* varieties exhibited varying responses in herbivory damage following exposure to different volatile cues (Figure 2). The domesticated variety yellow squash experienced significantly less leaf damage after exposure to either AGP (one-way ANOVA, F= 5.30, p= 0.01) and HIPV (one-way ANOVA, F= 5.30, p= 0.02) compared to control plants and no difference in damage was observed between AGP and HIPV treatments (Figure 2a). Domesticated zucchini plants also had less damage after exposure to either AGP (one-way ANOVA, F= 7.89, $p < 0.01$) and HIPV (one-way ANOVA, F= 7.89, p= 0.01) compared to control plants, without a difference between AGP and HIPV (Figure 2b). The wild-type Texas gourd variety did not exhibit significant differences in herbivory damage across the three treatments exposure (Figure 2c). Across all varieties *A. vittatum* consumed significantly more leaf tissue from zucchini plants compared to both yellow squash (one-way ANOVA, F= 16.10, p= 0.04) and Texas gourd (one-way ANOVA, F= 16.10, $p < 0.01$). Yellow squash experienced significantly more herbivory damage compared to Texas gourd (one-way ANOVA, F= 16.10, $p < 0.01$) (Figure 2d).

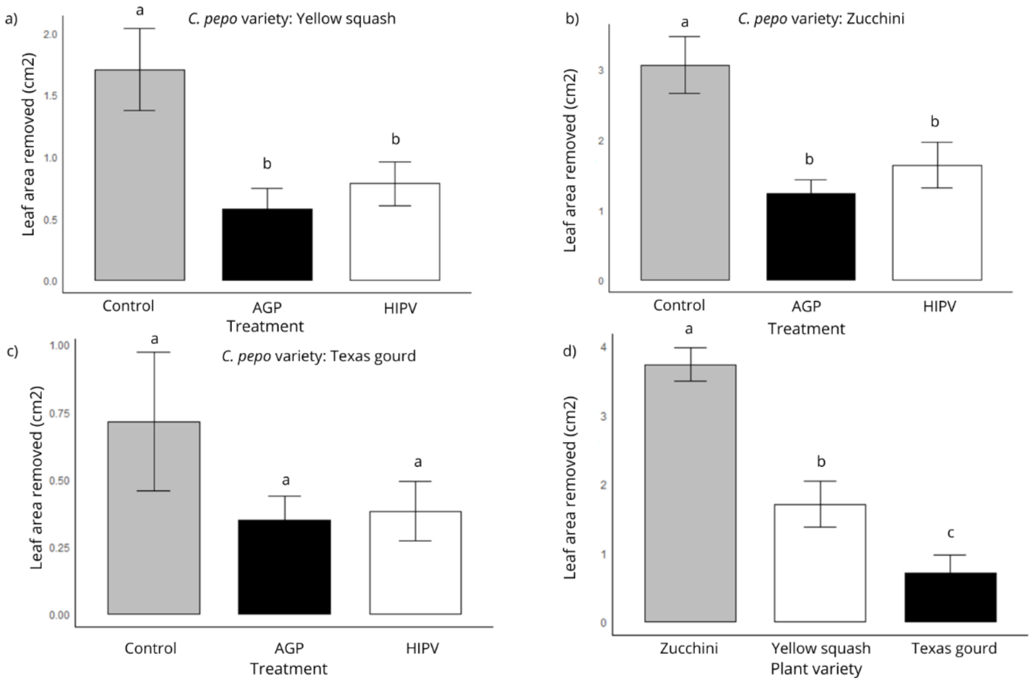

Figure 2 *Acalymma vittatum* damage on the varieties of *Curcubita pepo* plants previously exposed to AGP, HIPV and Control. a) yellow squash plants exposed to Control, AGP and HIPV treatments followed by *A. vittatum* damage. b) zucchini plants exposed to Control, AGP and HIPV treatments followed by *A. vittatum* damage. c) Texas gourd plants exposed to Control, AGP and HIPV treatments followed by *A. vittatum* damage. d) differences in feeding damage by *A. vittatum* per plant variety. The displayed values represent Means ± SEM. Different letters denote significant differences by one-way analyses of variance (ANOVA) followed by Tukey HSD post-hoc comparisons with a statistically significant difference at $p < 0.05$.

A. vitattum aggregation pheromone production is higher on domesticated *C. pepo* varieties.

Across three *C. pepo* varieties (yellow squash, zucchini, and Texas gourd), *A. vittatum* beetles emitted varying levels of vittatalactone. The highest emissions were observed on yellow squash (23.3 ng/beetle), followed by zucchini (16 ng/beetle) and Texas gourd (4.8 ng/beetle), resulting in an average emission of 14.7 ng/beetle across all varieties (Table 1).

Table 1- Quantification of vittalactone production by *Acalymma vittatum* in different *Cucurbita pepo* varieties.

C. pepo variety	*C. pepo* plants state of domestication	Mean (ng) vittatalactone release
Yellow Squash	Domesticated	23.3
Zucchini	Domesticated	16.0
Texas gourd	Wild type	4.8
Total		14.7

VOCs collection from groups of 25 male *Acalymma vittatum* beetles. For each *Curcubita pepo* variety, four glass chambers, each containing 25 beetles, were used. Vittatalactone quantification was measured as the mean (ng) from pooled data of 100 beetles per variety and quantified relative to the internal standard concentrations of nonyl acetate.

C. pepo plant exposure to herbivory associated volatile cues enhances herbivore-induced volatile emissions in yellow squash variety.

We investigated the influence of treatment (AGP, HIPV, control) and condition (constitutive, induced) on VOC emissions across the three *C. pepo* varieties (Figure 4). Random forest analysis identified α-pinene and β-ocimene as the most relevant compounds in the domesticated zucchini and yellow squash plants, while heneicosane and α-pinene were the most relevant in the wild-type Texas gourd variety. Yellow squash exhibited significant treatment (two-way ANOVA, F= 11.16, $p < 0.01$) and condition (two-way ANOVA, F= 73.52, $p < 0.01$) effects for both individual compounds and total VOC emissions. Induced α-pinene levels were significantly higher in AGP-exposed plants compared to both HIPV (Tukey HSD, $p = 0.04$) and control (Tukey HSD, $p < 0.01$) treatments (Figure 4d). Similarly, induced β-ocimene abundance was significantly higher in AGP-exposed plants compared to controls (Tukey HSD, $p = 0.01$) (Figure 4e). Total VOC emissions were significantly higher in induced compared to constitutive conditions across all treatments, and AGP-exposed plants released significantly higher levels of induced VOCs compared to controls (Tukey HSD, $p < 0.01$) (Figure 4f). Zucchini showed significant differences in α-pinene abundance between constitutive and induced conditions (two-way ANOVA, F = 30.5, $p < 0.01$) but no significant treatment effect (Figure 4a). Neither β-ocimene nor total VOC emissions displayed significant treatment or condition effects (Figure 4b,c). Texas gourd exhibited significant differences in α-pinene emissions between constitutive and induced conditions (two-way ANOVA, F = 53.01, $p < 0.01$), and AGP-exposed plants released significantly higher amounts of induced α-pinene compared to controls (Tukey HSD, $p < 0.01$) (Figure 4g). Similarly, induced heneicosane emissions were significantly higher in AGP-exposed plants compared to controls (Tukey HSD, $p = 0.03$) (Figure 3h). No significant differences were observed in total VOC abundance between treatments or conditions (Figure 4i).

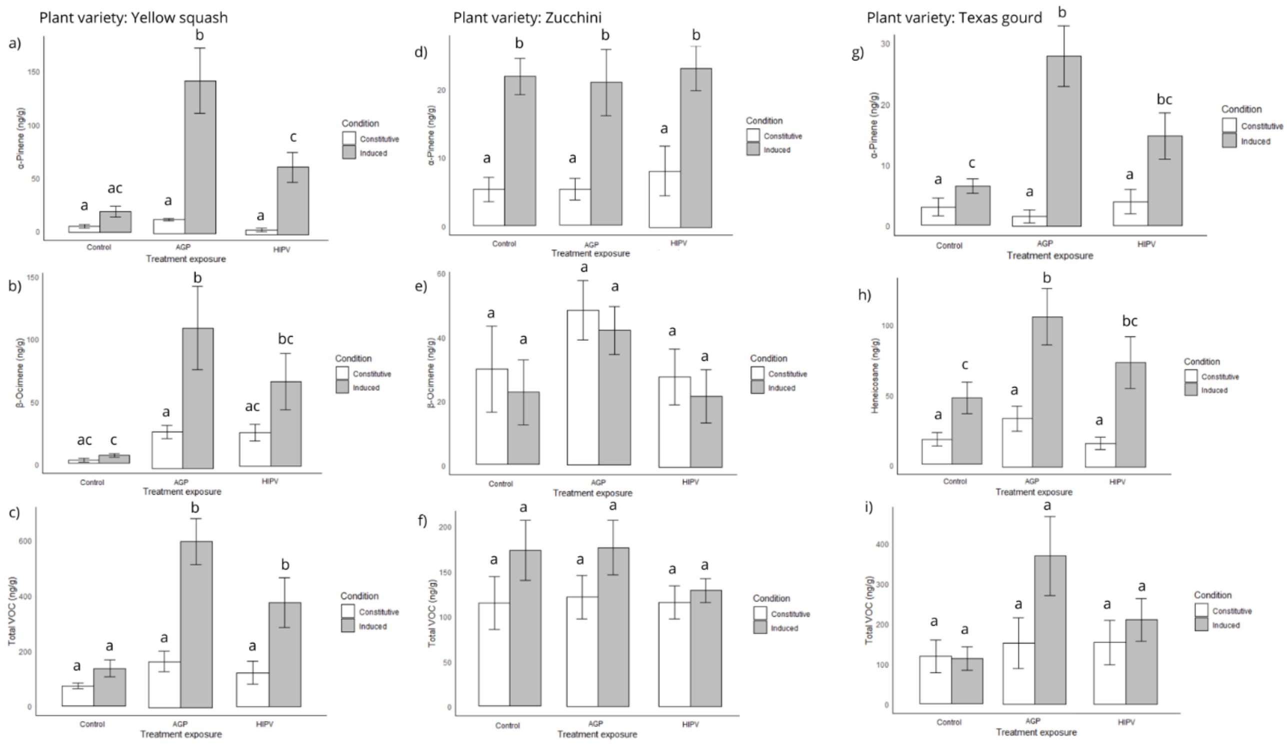

Figure 4 Constitutive and induced volatile abundance of *Curcubita pepo* cultivars under different exposure treatments. a) Yellow squash plants emission of constitutive and induced α-pinene under AGP, HIPV and control treatment exposure b) Yellow squash plants emission of constitutive and induced β-ocimene under AGP, HIPV and control treatment exposure c) Yellow squash plants emission of constitutive and induced total VOC under AGP, HIPV and control treatment exposure d) Zucchini plants emission of constitutive and induced α-pinene under AGP, HIPV and control treatment exposure e) Zucchini plants emission of constitutive and induced β-ocimene under AGP, HIPV and control treatment exposure f) Zucchini plants emission of constitutive and induced total VOC under AGP, HIPV and control treatment exposure g) Texas gourd plants emission of constitutive and induced α-pinene under AGP, HIPV and control treatment exposure h) Texas gourd plants emission of constitutive and induced β-ocimene under AGP, HIPV and control treatment exposure i) Texas gourd plants emission of constitutive and induced total VOC under AGP, HIPV and control treatment exposure The displayed values represent Means ± SEM. Different letters denote significant differences by two-way analyses of variance (ANOVA) followed by Tukey HSD post-hoc comparisons with a statistically significant difference at $p < 0.05$.

Domesticated plants perceive herbivore-associated cues and can prime their defenses upon herbivory.

We quantified levels of jasmonic acid (JA) and salicylic acid (SA) under both constitutive and induced conditions in plants under AGP, HIPV, and control treatments across the three *C. pepo* varieties (Figure 5). JA levels were higher in yellow squash plants exposed to HIPV both before (two-way ANOVA, F= 6.16, p-value < 0.01) and after (two-way ANOVA, F= 6.16, p-value < 0.01) herbivory treatment compared to non-exposed control plants. Plants exposed to AGP had an intermediate JA level (Figure 5a). There was no difference in SA levels for yellow squash across the exposure treatments (Figure 5b). Zucchini plants exposed to AGP (two-way ANOVA, F= 8.95, p = 0.01) had higher induced levels of JA compared to controls and a trend toward higher constitutive JA levels in AGP-exposed plants (Figure 5c). Zucchini plants exposed to AGP (two-way ANOVA, F= 7.87, p < 0.01) also had elevated levels of SA (two-way ANOVA, F= 7.87, p = 0.02) compared to control plants and intermediate levels of SA were observed for HIPV-exposed zucchini plants (Figure 5d). Constitutive and induced JA and SA levels were not different for Texas gourd plants exposed to AGP, HIPV or non-exposed controls (Figure 5e,f).

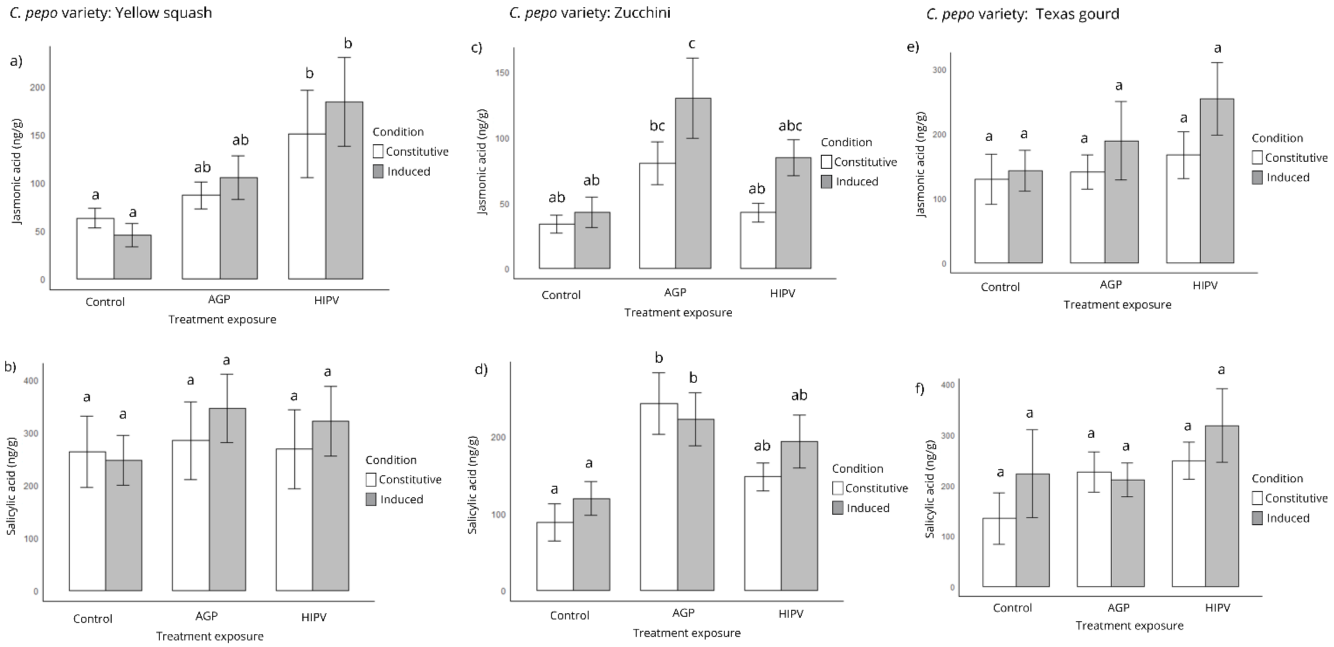

Figure 5. Quantitative analysis of JA and SA phytohormones of *Curcubita pepo* varieties under different exposure treatments. a) Constitutive and herbivore-induced JA levels in yellow squash plants exposed to AGP, HIPV or non-exposed controls. b) Constitutive and herbivore-induced SA levels in yellow squash plants exposed to AGP, HIPV or non-exposed controls. c) Constitutive and herbivore-induced JA levels in zucchini plants exposed to AGP, HIPV or non-exposed controls. d) Constitutive and herbivore-induced SA levels in zucchini plants exposed to AGP, HIPV or non-exposed controls. e) Constitutive and herbivore-induced JA levels in Texas gourd plants exposed to AGP, HIPV or non-exposed controls. f) Constitutive and herbivore-induced SA levels in Texas gourd plants exposed to AGP, HIPV or non-exposed controls. The displayed values represent Means ± SEM. Different letters denote significant differences by two-way analyses of variance (ANOVA) followed by Tukey HSD post-hoc comparisons with a statistically significant difference at $p < 0.05$.

Exposure to synthetic AGP increases yellow squash plant resistance to cucumber beetle herbivory in a dose-dependent manner.

We found that yellow squash plants exposed to volatile herbivory cues from live cucumber beetles had enhanced resistance against cucumber beetle herbivory. Plant exposure to either AGP or HIPV with live beetle herbivory resulted in reduced herbivore feeding damage compared to non-exposed control plants. To discern the specific impact of the aggregation pheromone (AGP), irrespective of HIPVs, we conducted a subsequent experiment exposing yellow squash plants to two different concentrations of synthetic vittatalactone, the primary compound in *A. vittatum* aggregation pheromone. We found that yellow squash plants exposed to high dose (50 mg total – 1 mg/septum) of the synthetic AGP showed a decrease in leaf area consumed compared to the control treatment under high dose exposure with hexane (two-way ANOVA, F= 6.85, p value = 0.02) (Figure 6). However, we did not see a reduction in feeding damage for yellow squash plants exposed to the lower dose of AGP.

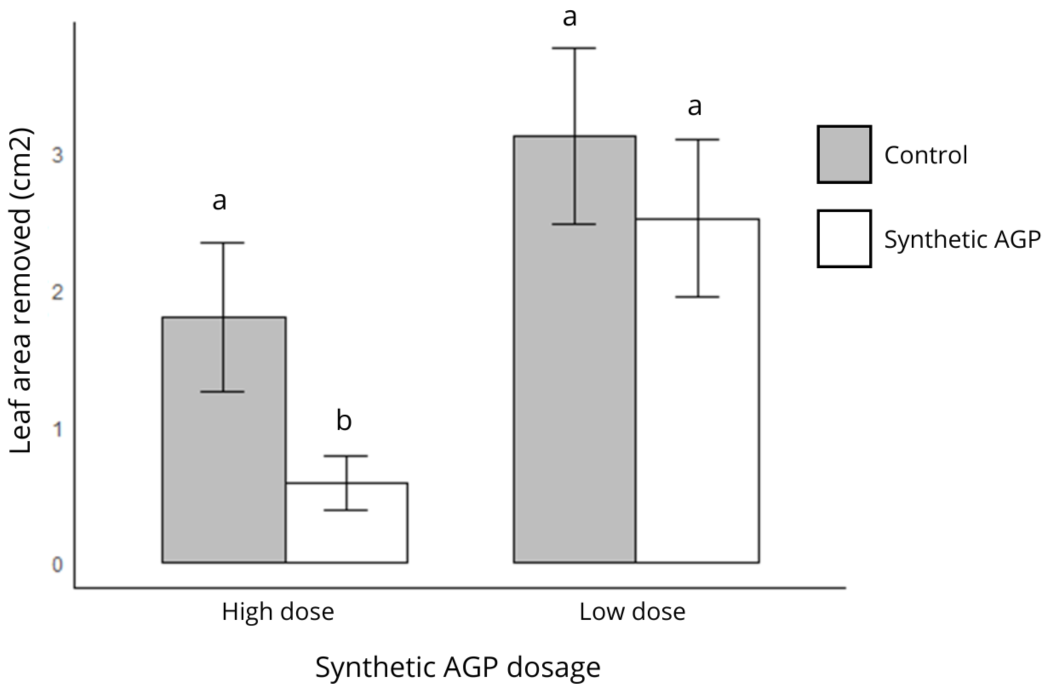

Figure 6 Herbivory damage on *Cucurbita pepo* Yellow Squash plants previously exposed to high and low doses of the synthetic vittatalactone for 24 hours. The displayed values represent Means ± SEM. Different letters denote significant differences by two-way analyses of variance (ANOVA) followed by Tukey HSD post-hoc comparisons with a statistically significant difference at $p < 0.05$.

Yellow squash plants exposed to a high dose of AGP induce higher levels of JA and SA.

We quantified levels of the defense-related phytohormones JA and SA in yellow squash plants exposed to different doses of synthetic vitttatalactone AGP before and after cucumber beetle herbivory. We found that exposure to the lower dose of AGP (10 mg total – 1 mg/septum) did not affect constitutive or herbivore-induced levels of JA or SA in yellow squash plants compared to controls. However, plants exposed to the higher dose showed an increase on induced JA between high dose control and high dose synthetic vittatalactone exposure (two-way ANOVA, F= 6.54, p-value= 0.01) (Figure 7a) with higher exposure to synthetic AGP enhancing the amount of induced JA. For the abundance of SA, we have also seen an increase on the induction of SA between high dose control and high dose synthetic vittatalactone exposure (one-way ANOVA, F=7.32, p-value=0.01) with higher exposure to synthetic AGP enhancing the amount of induced SA (Figure 7b). For the low dosage concentration, we have not seen any difference either between or within treatments for both JA and SA levels (Figure7c,d).

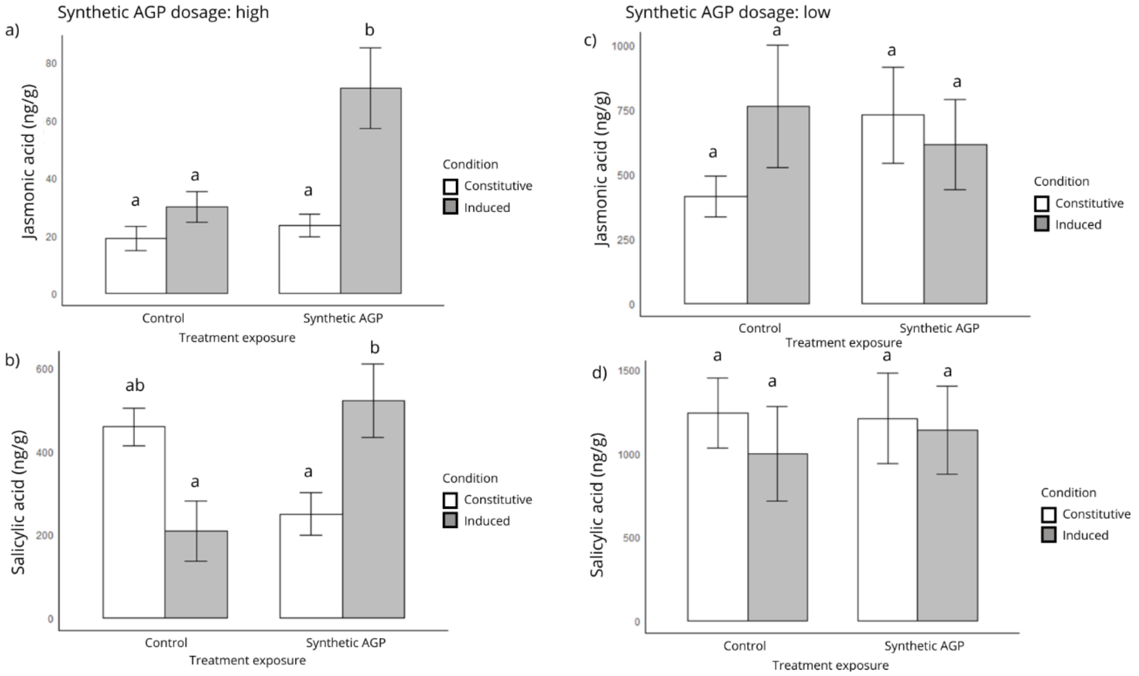

Figure 7 Quantitative analysis of JA and SA phytohormones of *C. pepo* yellow squash to *Acalymma vittatum* synthetic AGP. a) JA abundance of yellow squash plants exposed to high doses of synthetic AGP. b) SA abundance of yellow squash plants exposed to high doses of synthetic AGP. c) JA abundance of yellow squash plants exposed to low doses of synthetic AGP. d) SA abundance of yellow squash plants exposed to low doses of synthetic AGP. The displayed values represent Means ± SEM. Different letters denote significant differences by two-way analyses of variance (ANOVA) followed by Tukey HSD post-hoc comparisons with a statistically significant difference at $p < 0.05$.

Discussion

Plants can detect environmental cues associated with insect herbivory and respond by mounting their defenses as a strategic response to reduce potential harm (Schoonhoven et al., 2005). In this study, we examined plant responses to two herbivory-associated cues, HIPV and AGP, and evaluated whether these cues enhanced plant resistance to herbivory. Our research reveals that different varieties of *C. pepo* exhibit distinct defense mechanisms in response to volatile cues associated with beetle herbivory. Domesticated *C. pepo* varieties previously exposed to herbivory cues exhibited greater resistance to herbivore damage compared to their wild relatives. This enhanced resistance was evident in the reduced feeding damage and elevated phytohormone levels along with elevated VOC emission observed on domesticated varieties. Interestingly, that was an increase in total VOC emissions and a few key compounds from plants exposed to AGP or HIPV. Furthermore, within a specific variety (yellow squash), exposure to elevated doses of AGP correlated with heightened resistance to *A. vittatum* feeding and elevated levels of defense phytohormones, particularly JA and SA.

There is growing evidence that HIPVs play important roles in *C. pepo* anti-herbivore defense. For example, Brzozowski et al., (2019) found that *C. pepo* volatiles induced by active *Trichoplusia ni* damage had a deterrent effect on *T. ni* growth. Work from our own lab has reported that systemic HIPVs repel cucumber beetles and squash bugs (M. N. Thompson et al., 2022). Recent studies have also documented evidence of volatile-mediated signaling in *C. pepo*. It was previously found that systemic HIPVs from *C. pepo* plants with root herbivory enhance resistance in neighboring plants (M. N. Thompson et al., 2024) while HIPVs from plants infested with saltmarsh caterpillars (*Erwinia acrea*) resulted in defense suppression (Marmolejo et al., 2021). In this study, we observed an upregulation of total VOC, α-pinene and β-ocimene in the domesticated yellow squash plant that were previously exposed to the aggregation pheromone and HIPVs. Similar upregulation of α-pinene has been observed in cotton plants exposed to the aggregation pheromone of *Anthonomus grandis*, along with an overall increase in VOC emissions. This upregulation played a crucial role in attracting natural enemies of *A. grandis* to plants previously exposed to the pheromone (Magalhães et al., 2019). For zucchini squash plants, there was no observed increase in HIPV emissions after exposure to either AGP or HIPV when compared to control plants. In Texas gourd plants, we did not observe an overall increase in total VOC emissions; however, there was an increase in the emission of α-pinene and heneicosane following exposure to either AGP or HIPV compared to control plants. Together, these findings suggest that yellow squash plants exposed to herbivory-associated cues, including HIPV and AGP, have elevated volatile defenses that could play important roles in repelling herbivores or attracting natural enemies. Several natural enemies of cucumber beetles and squash bugs have been identified including parasitoids and nematodes (Coco et al., 2020). Future work should examine the role of HIPVs in natural enemy attraction and squash indirect defense.

Nevertheless, our study noted elevated phytohormone levels in both domesticated varieties when exposed to herbivore volatiles. Although the domesticated zucchini did not exhibit increased VOC levels, the relationship between phytohormones and VOC production could be related to the timing of sampling. Some research indicates that higher phytohormone levels do not always directly result in increased VOC production (Ponzio et al., 2013). We have also seen that the wild-type plant exposed to AGP and HIPV exhibited increased release of induced α-pinene and heneicosane compared to control plants but showed no differences in phytohormone levels. Previous studies have indicated that plants exposed to insect volatiles demonstrated a decrease in feeding damage and an enhancement in defense related compounds compared to non-exposed plants (A. M. Helms et al., 2013; Yip et al., 2019). This could be explained by the movement of gases in and out of leaf air spaces by diffusion laws, with gases moving from higher to lower concentrations until equilibrium is reached (Fick, 1855). This process is controlled by the opening and closing of stomata, regulated by ion fluxes (e.g., K^+, Cl^-, malate^{2-}) and plant hormones (e.g., jasmonates) (Acharya & Assmann, 2009; Assmann & Jegla, 2016). Therefore, we propose that the observed differences in defense compounds between plants exposed to herbivory associated cues and non-exposed plants are primarily attributable to the plant's mechanistic properties. Our data suggest that different varieties have distinct conditions that regulate the entry of gases and the subsequent induction of their defense mechanisms. While not quantified in this study, future research should measure stomatal conductance in these three different varieties, both in exposed and non-exposed plants.

Taken altogether, domesticated *C. pepo* plants show an increased production of defense-related compounds and a reduction in leaf area consumed by herbivores in response to AGP and HIPV, compared to control plants. In contrast, while the wild Texas gourd variety demonstrated some enhanced defense responses, these did not result in increased resistance to beetle herbivory.

This suggests that domesticated plants have kept this trait despite the domestication process. However, subsequent research should expand on these results to investigate how domestication has influenced plant priming and communication across various species. Interestingly, *A. vittatum* shows a preference for feeding on domesticated varieties over wild types, with a particularly strong preference for the zucchini variety, consistent with previous studies (L. Brzozowski et al., 2016). This find is intriguing because most of the studies evaluating herbivory on domesticated plants tend to infer that the high feeding on domesticated plants is due to loss in plant defensive traits (Gaillard et al., 2018; Hancock, 2012). In *C. pepo* plants, beetle feeding is commonly associated with cucurbitacin content. However, domesticated varieties are known to contain very low concentrations of cucurbitacins (Bruno et al., 2023; Brzozowski et al., 2020; Tallamy and Gorski, 1997). *A. vittatum* produced different amounts of AGP when feeding on each of the three *C. pepo* varieties, with the highest production on yellow squash, followed by zucchini, and the lowest on the wild type (Brzozowski et al., 2020). Also, factors such as leaf nitrogen levels and other measured leaf traits did not predict leaf damage by *A. vittatum* (Theis et al., 2014). Thus, the mechanism driving *A. vittatum* preference towards domesticated *C. pepo* plants remain elusive. We have also observed that the wildtype Texas gourd exhibited high constitutive resistance, demonstrated by significantly less damage compared to domesticated varieties. Plants with high constitutive resistance typically incur minimal damage, making the activation of an additional priming or induced response unnecessarily costly (Kessler, 2015) This is evidenced by the lower phytohormone production and no effects on feeding damage in the Texas gourd when exposed to herbivory-associated cues. Additionally, our observations demonstrated that domesticated varieties, which showed the least resistance to *A. vittatum* feeding, exhibited a higher ability to induce or prime their defenses when exposed to herbivory cues. Contrary to the wildtype variety, this suggests that plants with relatively low constitutive resistance are likely to experience stronger selective pressure to develop inducible resistance (Campbell & Kessler, 2013; Carmona & Fornoni, 2013; Kessler, 2015).

Here we found a dose-dependent relationship in the anti-herbivory defense of domesticated yellow squash plants. We demonstrated that higher doses of synthetic vittatalactone render the plants less palatable to herbivores and increase defense-related phytohormone levels following herbivory. Priming of defense has been shown to be dose-dependent in other studies, with an increase in dose concentration leading to intensification of defenses (A. Helms et al., 2017; Yip et al., 2017). In the presence of herbivores exhibiting aggregation behavior, an increase in the number of beetles gathered leads to a higher release of aggregation pheromones into the environment, attracting more beetles to feed on the host plant (Ellers-Kirk & Fleischer, 2006). Therefore, the level of vittatalactone that plants are exposed to could be a crucial factor in their ability to detect this volatile signal. A study conducted using vittatalactone-baited traps found that squash bugs species are strongly attracted to vittatalactone in field experiments with attraction increasing with vittatalactone doses (L. J. Brzozowski et al., 2022; Weber et al., 2022). Our discoveries, combined with the documented attraction of *A. vittatum* to synthetic vittatalactone in natural settings (Haber et al., 2023; Weber, 2018), are crucial for unraveling the intricacies of this dynamic ecosystem. Deploying synthetic vittatalactone in field settings might enhance plant resistance, with dosage being an important factor. The aggregation pheromone of *A. vittatum* is detected not only by herbivores of the same and different species but also by the host plant itself—yellow squash plants in this instance—triggering a primed defense response in the plant.

In conclusion, we found that domesticated plants demonstrated enhanced resistance and could prime their defenses in response to feeding, unlike wild-type *C. pepo* plants. This difference could be because wild-type plants either do not respond to feeding or the differences in feeding are negligible due to their existing resistance. This finding contradicts our earlier hypothesis that domestication would impair *C. pepo's* ability to prime defenses. It is possible that the ability to prime defenses in *C. pepo* did not persist through domestication because wild plants never had this trait. Nevertheless, future studies should investigate this question more thoroughly. Additionally, consistent with other domestication studies, we observed that domesticated plants suffered more damage compared to wild types. This leads us to speculate whether the evolution of defense priming in *C. pepo* occurred as a protective mechanism for domesticated plants to mitigate damage. Plant priming of defense is an adaptive strategy for coping with environmental stress (Mauch-Mani et al., 2017). However, to our knowledge that isn't studies that have investigated the priming of defenses, particularly in response to insect cues, in wild-type plants compared to domesticated plants. Future research should explore this area further to provide more insights into the priming capacities of *C. pepo* genotypes.

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ARTIGO 3 - Plant defense priming through airborne cues: A meta-analysis.

Artigo elaborado de acordo com as normas do periódico "*Frontiers in Ecology and Evolution*".

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Abstract

Research has shown that plants communicate through airborne signals, especially when threatened by herbivores. When attacked, plants release a mixture of volatile organic compounds (VOCs) that play various ecological roles, such as attracting natural enemies of herbivores and warning nearby plants, prompting them to prepare defenses against potential attack. Consequently, when the herbivore, these plants are better equipped to handle the damage. This process known as priming of defenses enables plants to prepare more effectively for potential threats. Despite substantial evidence that various plants can prime their defenses, it remains unclear whether this is a widespread phenomenon and what are the critical factors driving research in plant defense priming. Therefore, the goal of this meta-analysis was to untangle key factors influencing plant defense priming in studies that assess this phenomenon through airborne cues. Our findings indicate that exposure to volatile compounds can significantly enhance plant resistance to herbivores, with both annual and perennial plants capable of priming their defenses. Moreover, the nature of the exposure plays a role in influencing priming responses, with synthetic compounds producing increased plant response. Overall, priming of defenses appears to be a widespread phenomenon among plants. These findings emphasize the pressing need for further research on plant defense priming across all the aspects outlined, to enhance understanding of this phenomenon and uncover its broader ecological and practical implications.

Plant defense priming through airborne cues: A meta-analysis

Key-Words: airborne chemical, herbivory, insect plant interaction, plant communication, plant performance, priming agent, VOCs.

Introduction

More than 40 years ago, Baldwin and Schultz (1983) and Rhodes (1983) first suggested communication among plants. Initially, this intriguing phenomenon was met with skepticism by many ecologists, who doubted its reproducibility in natural conditions (Dicke and Lawton, 1985; Dicke and Bruin, 2001). However, as research in this field advanced, plants began to be recognized as active participants in communication (Karban et al., 2014). Indeed, effective plant communication requires both an emitter and a receiver to recognize and respond to the signal (Ali et al., 2013; Meents and Mithöfer, 2020). Growing evidence now indicates that plant communication occurs both below (Duc et al., 2022; Sharifi et al., 2022) and aboveground through organic compounds with varying volatility (Massalha et al., 2017, Ninkovic et al., 2021; Zu et al., 2023).

Aboveground plant communication can occur through volatile organic compounds (VOCs), and when plants experience herbivory, they release a distinct volatile blend known as herbivore-induced plant volatiles (HIPVs). HIPVs signal the location of prey for natural enemies of herbivores during foraging and convey information about potential risks of herbivore infestations to neighboring plants (Turlings and Ton, 2006). Upon perceiving HIPVs, receiver plants can prime their defenses, entering a state of heightened alertness. This priming enables a faster and stronger response, characterized by a mild initial defense followed by a more robust reaction upon actual herbivore attack (Conrath et al. 2006; Karban and Agrawal, 2002; Karban, 2008; Martinez-Medina et al., 2016). For example, corn plants previously exposed to green leaf volatiles (GLVs) followed by herbivory damage showed enhanced production of VOCs and increased levels of jasmonic acid compared to non-exposed corn plants followed by herbivory damage (Engelberth et al., 2004). This strategy of priming defenses upon detecting neighboring herbivore-infested plants through HIPVs optimizes the cost-benefit ratio, as fully expressing defenses is costly (Mauch-Mani et al., 2017).

Some questions arise regarding the potential advantage of plants emitting signals about threats to other plants, including across different plant species (Hamilton, 1964; Peñuelas and Llusià, 2004; Karban et al., 2013). The questions assumed that natural selection would not favor emitting cues to inform neighboring competitors about herbivores (Karban et al., 2014). However, plants encounter a variety of environmental cues, and communication has been shown to be an effective tool for plants to protect themselves (Karban, 2008). Therefore, plants may prioritize, ignore, and respond appropriately to relevant signals of potential stresses (Grof-Tisza et al., 2021). In fact, responding to a signal that does not truthfully convey information about environmental stresses may disadvantage the overall fitness of the plant (Wilson et al., 2015). For instance, *Solidago altissima* (Asteraceae) plants showed a decrease in rhizome growth due to an insect-volatile cue that did not convey precise information about existing threats (Yip et al., 2019). Hence, despite plants not intentionally co7mmunicating with nearby plants, it is evident that plants release airborne compounds that can serve as signals for other organisms (Karban et al., 2010).

In addition to HIPVs, chemical airborne signals from the herbivores themselves can also be recognized by plants. Helms et al. (2013) first demonstrated that plants recognize intraspecific volatile cues of insect herbivores (pheromones) as potential warning cues and respond by mounting defenses against the insect herbivores (Helms et al., 2014, 2017). Subsequently, other authors have shown similar findings on plant response to pheromones of insect herbivores (Yip et al., 2017, 2019, 2021; Bittner et al., 2019; Magalhães et al., 2019). Research on plant communication via volatiles of plant or insect origin can be applied in agroecosystems to suppress insect pest populations within a more sustainable approach to integrated pest management (Kansman et al., 2023). Several studies have indicated the potential of activating the priming state of agronomic plants for enhancing foraging of biological control agents (Bobadilla et al., 2023; Devescovi et al., 2024; van Griethuysen et al., 2024), or optimizing push-pull strategies (Afzal et al., 2023; Deganutti et al., 2023; Wellington et al., 2023), and improvements in plant resistance against herbivores (Sobhy et al., 2017; van Doan et al., 2020; Liao et al., 2021; Michereff et al., 2021).

Even though numerous studies have examined airborne signals (HIPVs or insect pheromones) as defense priming agent in a variety of plant species and methodologies, it is unclear whether priming via airborne signals can effectively contribute to pest management in the field. Another overlooked aspect is that most studies on this topic report positive effect of airborne signals, irrespective of their origin (plant or insect), on plant priming, suggesting that plants perceive and respond to any volatile compound at any concentration. However, this may be a publication bias due to the pressure of publish positive results within the scientific community.

Furthermore, several studies have examined priming effects were examined under unrealistic conditions, such as exposing plants to high concentrations of volatiles in closed chambers with no airflow. As a result, little is known about the frequency that defense priming occurs in natural settings or the threshold that a given volatile triggers plant defense priming. Therefore, examining the existing literature on plant priming defenses using airborne signals is important to elucidate and underscore the importance of further fundamental and practical research in this area.

In this scenario, we conducted a meta-analysis of published data on plant communication through airborne signals between plants and insects, specifically focusing on the magnitude of priming responses in plants and on which factors shape prime responses. Our study aimed to address the following questions: 1) Can exposure to airborne signals emitted by plants and insects prime plant defenses? 2) Do plant traits influence priming effects on plant antiherbivore defenses? 3) Do methodologies of exposure and experimental conditions influence priming effects on plant antiherbivore defenses? 4) Is there any indication of publication bias in the reporting of plant priming defenses through airborne signals? To assess the impact of various factors on the priming effect of airborne signals on plants, we examined the influence of plant life cycle (annual or perennial), domestication level (wild or cultivated), type of airborne signal exposure (natural or synthetic), experimental condition (laboratory, field, or greenhouse), and duration of exposure (short, medium, or long).

Materials and methods

The dataset

To collect data for our meta-analysis, we initially performed a bibliometric analysis (Aria and Cuccurullo, 2017) focusing on the quantitative examination of bibliographic information, including publication records, citation patterns, key topics, keywords. This analysis aims to provide valuable insights into the attributes and evolving trends within the scope of scientific publications. We conducted naïve research on Scopus (n = 127 papers) and Web of Science (n = 135 papers) using our prior knowledge of the topic (Figure 1). The terms used in the naïve research were overrepresented by duplicates; therefore, we used Litsearchr® to assemble and deduplicate our results (Grames et al., 2019). Subsequently, the word compilation for this meta-analysis was refined to include the following search terms: ((\"herbivore-induced plant\" OR \"organic compounds"\ OR \"plant communication\" OR \"plant volatile\" OR \"volatile organic\") AND (\"herbivore-induced plant\" OR \"indirect defense\" OR \"induced plant\" OR \"plant defense\" OR \"plant defense\" OR \"defense responses\") AND (\"herbivore-induced plant\" OR \"organic compounds\" OR \"plant volatile\" OR \"volatile organic\") AND ("priming" OR "prime*")). Finally, our search resulted in a total of 73 studies on Web of Science and 23 studies on Scopus.

From the resulting list of documents, we first read titles and abstracts to exclude studies not relevant for our meta-analysis (in this step, 163 studies were excluded). Then, we screened the remaining studies by reading the full text. In this last step, 34 studies were selected (Figure 1), as they provided estimates of plant defense priming through airborne cues indicative of future herbivory damage.

Our selection criteria focused on the impact of plant defense priming through airborne cues from plants or insects. We included studies in the meta-analysis that met the following criteria: (1) plants were subjected to at least two treatments – exposure to a volatile cue either from plants or insects, followed by herbivory damage, and a control followed by herbivory damage; (2) the authors provided means, some measure of variance, and sample sizes for each experimental group; (3) the studies had clear and detailed information about the procedure made on the material and methods sections; (4) the study tested the effect of these treatments on herbivores, plant resistance, plant fitness, and plant chemical defenses (direct and indirect) caused by exposure to volatiles upon herbivory; and (5) experimental studies that included either natural and synthetic forms of volatile exposure. We excluded studies that documented plant exposure to volatiles that did not originate from insects or aboveground parts of plants. We excluded studies that did not mention plant priming defense. The final dataset included 565 effect sizes extracted from 34 studies across 14 plant species, from nine different families (Figure 1).

The effect sizes were first calculated as Hedges' d estimates (Hedges et al., 1988) and then z-transformed following Rosenberg et al. (2013) and Lajeunesse (2013). Accordingly, the sampling variances of each effect size was calculated as 1 divided by the sample size minus 3 (Rosenberg et al., 2013). We extracted data from the main text, tables, figures, and supplementary material. To retrieve the data reported in figures, we used WebPlotDigitizer (https://apps.automeris.io/wpd/). Some studies presented multiple estimates through time or space. We reported the mean values over time and space when these data were presented by the authors or could be easily calculated.

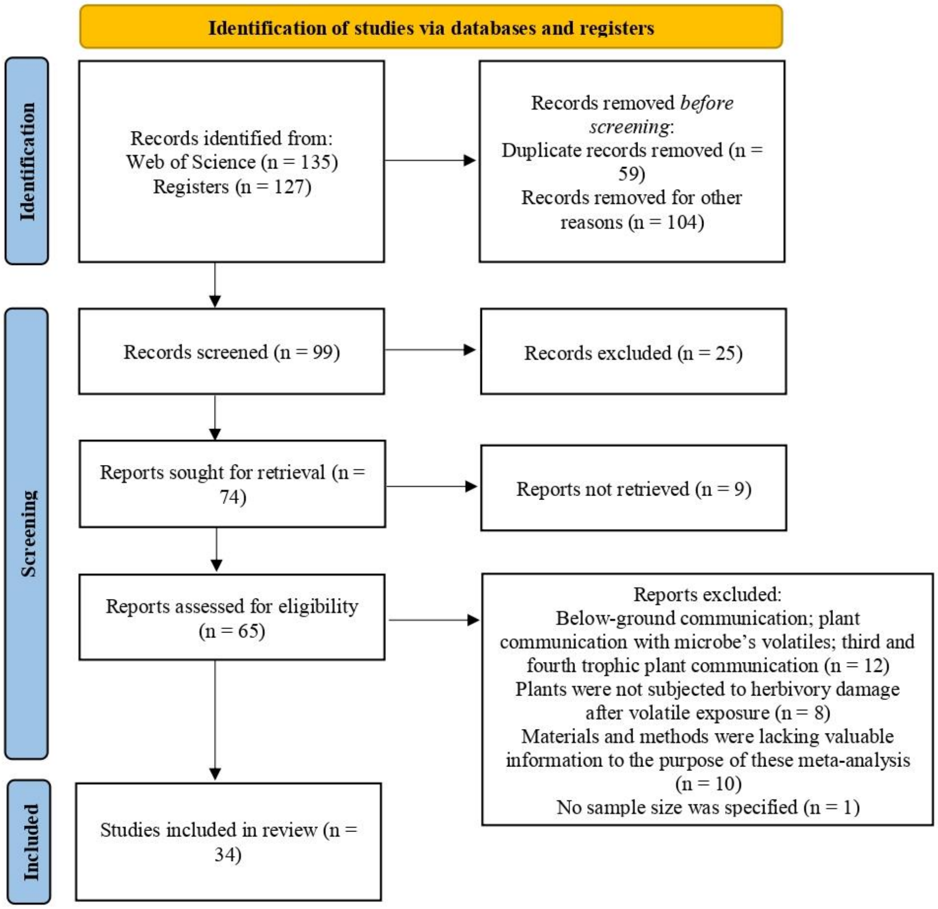

Figure 1. Full output plot from PRISMA 2020 flow diagram (Page et al., 2021; Haddaway et al., 2022).

Predictor variables

From the 34 selected studies, we gathered parameters predicted to modulate plant priming response. The selected parameters were:

1) Plant life cycle, categorized into annual and perennial types. Because annual plants have a short life cycle (Friedman, 2020; Lundgren and Des Marais, 2020), we hypothesize that annual plants prioritize production of defenses more than perennial plants, as a way of maximizing their chances of survival and reproduction within a limited time frame (Lundgren and Des Marais, 2020). Consequently, annual plants are expected to exhibit priming of defenses via airborne signals conveying potential future attack to a greater extent than perennial plants.

2) Genotype, categorized as cultivated or wild type. We hypothesized that wild genotypes present a stronger priming defense than cultivated individuals because most cultivated plants have weakened defense levels due to domestication and genetic breeding (Ladizinsky, 1998; Van Hulten et al., 2006; Cipollini et al., 2017). This reduction in defense levels may render cultivated plants less prompt to perceive and respond (prime defense) to an airborne cue upon insect damage compared to wild plants, which have maintained robust defense mechanisms throughout evolutionary processes (Chaudhary, 2013; Chen et al., 2015a).

3) Experimental conditions, categorized as laboratory, field, or greenhouse settings. We hypothesized that priming effects are weaker under field conditions compared to those examined under laboratory and greenhouse conditions. This is because plants cultivated and tested under controlled conditions, such in greenhouses or laboratories, facilitate the detection of priming responses with greater precision and reliability compared to field set-ups.

4) Duration of exposure, categorized as short (24 h), medium (> 24 h, < 7 d), or long (> 7 d). We hypothesized that priming effects are more pronounced to short and medium exposure periods than longer ones. This is because responding to long periods of stimulation should incur higher fitness expenses than responding to short or moderate stress exposure prior to the threat (Hilker and Schmülling, 2019; Turgut-Kara et al., 2020; Liu et al., 2022). As plants are capable of distinguishing between singular, repeated, and continuous stressors, adjusting the activation of

stress-responsive genes accordingly (Liu et al., 2022), plants tend to cease response as exposures periods are extended.

5) Type of exposure, categorized as natural (plant or insect as the source of airborne signals), semi-natural (extracts obtained from odor collections of insects or plants), or synthetic standards. We hypothesized that exposure to synthetic versions of airborne signals results in a stronger priming effect than exposure to natural or semi-natural signals. This is because synthetic and pure compounds provide a stronger stimulus than natural sources, which release more complex blends that include non-biologically active compounds and are subjected to other factors that affect concentration of compounds in the blend (e.g., light incidence on the production of HIPVs (Becker et al., 2015)) (Mori, 2000; Reddy and Guerrero, 2004).

Statistical analysis

In all meta-analytical models, the response variable was the collection of Zr estimates obtained from comparisons of plant priming defense after stimulus in treatment and control groups. We ran one model with no moderators, to estimate the overall average response of plants. We also ran one model for each of the five predictor variables: plant life cycle, plant genotype, type of exposure, duration of exposure, and experimental conditions. Each of these predictors was included as moderator in analyses. For the model on the influence of exposure duration, long volatile exposure was excluded from analyses due to the small number of effect sizes found (effect sizes:9; species:1).

Because some studies and species in our dataset provided multiple effect sizes and because species are phylogenetically linked, the effect sizes were not independent. Thus, to address data interdependence and incorporate random effects into our analyses, we employed multilevel models (see Bolker et al., 2009; Nakagawa et al., 2017). All models included at least three random effects: study identity, comparison identity and phylogeny. Comparison identity refers to each comparison between a given treatment group and a given control group, as some studies compared experimental groups through time and these estimates are not independent from each other. The phylogeny was obtained from Timetree® (Kumar et al., 2022) and integrated as a correlation matrix to account for species relationships and control species identity, and it was constructed using the package ape (Paradis and Schliep, 2019) in R software® (R Core Team 2020). The model to estimate the overall average plant response contained type of exposure as one additional random variable because this was the only random variable that influenced plant priming response (see Results). All analyses were carried out using the Restricted Maximum Likelihood (REML) methodology, using the function rma.mv from the package metafor (Viechtbauer, 2010).

To assess whether our dataset indicates the existence of publication bias in the literature, we ran an Egger's regression (Egger et al., 1997; Nakagawa and Santos, 2012). Finally, we estimated data heterogeneity by calculating I2 using the function i2_ml from the package orchaRd (Nakagawa et al., 2023).

Results

Overall pattern

Overall, plants present a priming response (Zr = 0.30, 95% CI = 0.002 – 0.61, p = 0.048, effect size: 565, species: 16). We detected no publication bias (Zr intercept = 0.02 ± 0.05, t = 0.35, p = 0.72). Data heterogeneity was high (I2 = 96.30%) and the random variable that better explained data variation was comparison identity (86.16%), while the others little explained data variation (study identity: 6.08%; type of exposure: 4.06%, phylogeny: < 0.01%).

Plant life cycle

Annual and perennial plants respond when facing the risk of herbivory (annual: Zr = 0.26, 95% CI = 0.06 – 0.46, p = 0.01, effect sizes: 388, species: 12; perennial: Zr = 0.52, 95% CI = 0.14 – 0.90, p = 0.01; effect sizes: 177; species: 3), and there was no difference between annual and perennial responses (Zr = 0.26, 95% CI= -0.16 – 0.69, p = 0.23).

Plant genotype

Cultivated and wild-type respond when facing the risk of herbivory (cultivated: Zr = 0.27 95% CI = 0.08 – 0.47, p = 0.01, effect size: 497, species: 12; wild type: Zr = 0.52, 95% CI = 0.16 – 0.88, p = 0.005, effect sizes: 68, species: 5), and there was no difference between cultivated and wild type responses (Zr = 0.24, 95% CI = -0.14 – 0.62, p = 0.21).

Experiment condition

Priming responses were observed in plants exposed to experimental conditions in both laboratory and greenhouse settings (laboratory: Zr = 0.25, 95% CI = 0.05 – 0.45, p = 0.01, effect size: 348, species: 13; greenhouse: Zr = 0.55, 95% CI = 0.19 – 0.90, p = 0.002, effect sizes: 124, species: 5), whereas field experiments did not elicit a priming response in plants (Zr = 0.28, 95% CI = -0.13 – 0.69, p = 0.18, effect sizes: 93, species: 4). However, no differences were found between greenhouse and field experiments (Zr = 0.27, 95% CI = -0.24 – 0.78, p = 0.30), nor between laboratory and field experiments (Zr = -0.03, 95% CI = -0.48 – 0.42, p = 0.91), nor between greenhouse and laboratory experiments (Zr = 0.30, 95% CI = -0.11 – 0.70, p = 0.15).

Duration of exposure

Plants exposed to a volatile signal for a medium to short duration show a priming effect (short: Zr = 0.27, 95% CI = 0.05 – 0.49, p = 0.02, effect sizes: 354, species: 11; medium: Zr = 0.34, 95% CI = 0.05 – 0.64, p = 0.02, effect size: 202, species: 7), with no difference between them (Zr = 0.07, 95% CI = -0.30 – 0.44, p = 0.71).

Type of exposure

Only plants exposed to synthetic volatile cues display a heightened priming response (Zr = 0.63, 95% CI = 0.28 – 0.97, $p < 0.01$, effect size: 166, species: 7), while plants exposed to a natural (Zr = 0.21, 95% CI = -0.08 – 0.51, p = 0.16, effect sizes: 360, species: 10) or semi-natural volatile cues (Zr = -0.05, 95% CI = -0.60 – 0.51, p = 0.87, effect sizes: 39, species: 3) exhibit no directional response (i.e. the confidence intervals overlap zero).

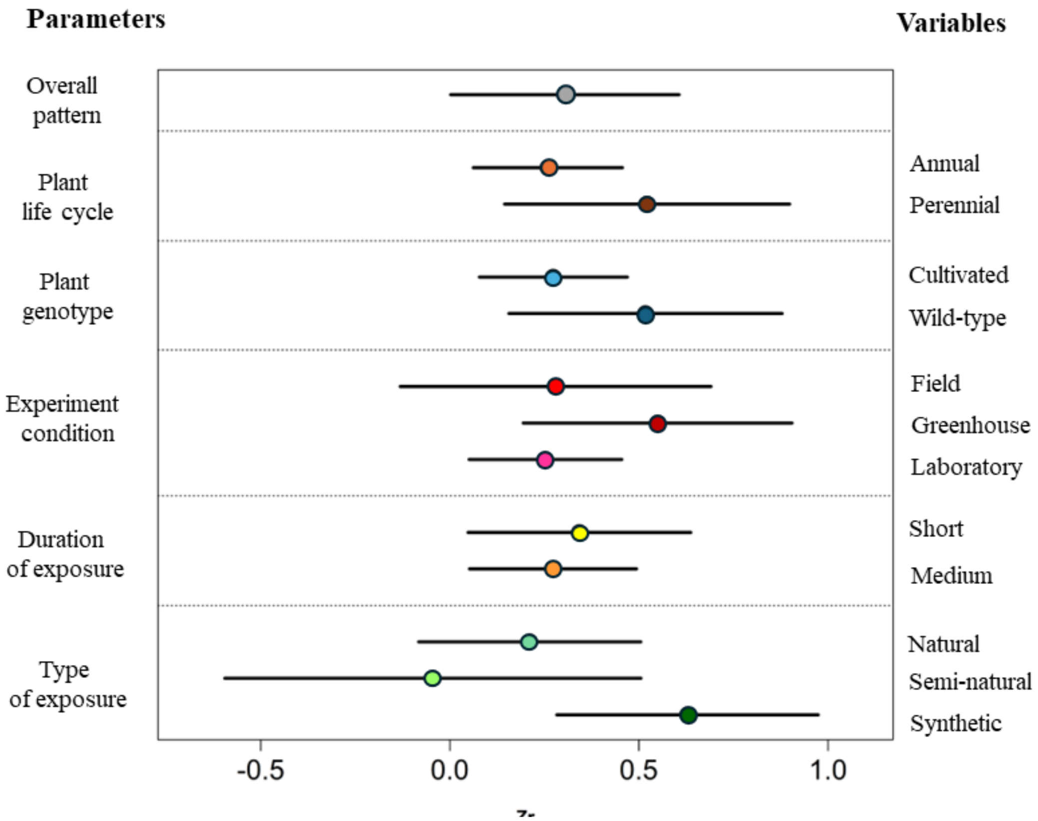

Figure 2. Estimates of plant defense priming via airborne signals (with 95% confidence intervals, CIs) for each parameter (left side) were measured within their corresponding variables (right side). CIs overlapping zero indicate no average prime response (p-value > 0.05; i.e., field studies, natural chemicals, and semi-natural chemicals). Positive CIs not overlapping ($p < 0.05$) zero indicate the occurrence of priming response (all scenarios other than field studies, natural chemicals, and semi-natural chemicals). The colors in each estimate are purely for illustration purposes.

Discussion

Based on 500 effect sizes from 16 plant species extracted from 34 studies, this meta-analysis confirms that plant defence priming triggered by airborne signals is a common phenomenon, regardless of plant life cycle and genotype, and experimental setups. However, contrary to our hypothesis, the ability to prime defences via airborne signals is similar when comparing annual and perennial plants, as well as cultivated and wild type plants. These groups exhibit priming responses, but there is no discernible difference in their priming capabilities. Experimental conditions in laboratory and greenhouse settings consistently demonstrated a priming effect in plants, unlike field experiments where this effect was undetectable. Plants exhibit priming responses when exposed to airborne signals within specific timeframes, with both medium and short durations inducing defence priming equally. Interestingly, synthetic volatile signals were found to provoke a stronger priming response in plants compared to natural and semi-natural signals.

Plant life cycle

Contrary to our expectations, we found that both annual and perennial plants exhibit the ability to recognize airborne signals that convey information about future herbivory threats, without any distinction between the magnitude of their responses. This outcome agrees with a previous meta-analysis conducted by Karban et al. (2014) whereas perennial plants were as likely as annual plants to exhibit volatile-induced resistance. In the case of annual plants, this outcome may be attributed to their evolution from perennial plants, potentially preserving defensive tactics inherited from perennial plants (Datson et al., 2008) to enhance plant fitness in complex environments (Martinez-Medina et al., 2016). For instance, annual plants may be associated with the constant herbivory pressure they face during their short-growth period (Takahashi and Yamauchi, 2010), particularly in their juvenile stage, which poses significant costs and mortality risks (Friedman and Rubin, 2015). It is understood that annual plants have a higher likelihood of survival when their juvenile stage is protected, whereas perennial plants thrive in environments where juvenile survival is precarious but adult survival is more certain (Stebbins, 1950). Therefore, it was expected that annual plants would have a better perception of herbivory threats, thus efficiently defending themselves during their juvenile stage without compromising their overall fitness (Conrath et al., 2015).

In contrast, perennials can withstand higher rates of herbivory (Doak, 1992) and can delay reproduction under stress conditions (Rubin et al., 2019) since they reproduce multiple times during their lifespan. By temporarily reducing the amount of energy invested in reproduction, perennial plants that delay reproduction may allocate more biomass and resources to vegetative tissues (Bergonzi and Albani, 2011). This is thus essential for the defensive mechanism of plants (Erb, 2018) and such allocation of resources could explain the ability of perennial plants to prime their defence. Such strategic resource allocation through time may explain why perennial species' priming responses do not differ from annual species' priming responses.

Annual and perennial plants must make choices regarding the distribution of resources between vegetative growth and flowering, and the timing of these decisions influences the defensive capabilities of both life cycles (Friedman and Rubin, 2015). This suggests that plants may choose different strategies associated with differing costs, depending on the predictability of the environmental cue (Auge et al., 2023) and are able to prime their defence accordingly. Therefore, this might explain why both life cycles are able to equally prime defence. However, we highlight that while our analysis covered 12 different annual species (data from 28 studies), we obtained data on only three perennial species (data from six studies). While our findings offer valuable information in this regard, further investigation into perennial plants is essential to enhance our comprehension of their ability to detect and react to potential dangers through airborne cues.

Plant genotype

It is well-recognized that herbivores play a significant role in disrupting crop productivity in agricultural ecosystems (Hancock, 2012), despite plants having developed inherent resistance to combat herbivores. While the level of this resistance differs significantly between wild and domesticated varieties (Rosenthal and Dirzo, 1997), it remains highly debated which resistance traits in plants have been altered through the process of domestication (Whitehead et al., 2017). Here we found that wild and domesticated plants similarly prime their defenses in response to airborne cues.

What does explain the comparable levels of defense priming observed in both cultivated and wild plants? Initially, the assumption that domesticated plants would exhibit reduced priming defenses might have been influenced by information on other forms of defense. It's important to note that many studies highlighting decreased defense levels in domesticated plants primarily focus on measuring constitutive and inducible defenses (Moreira et al., 2018), often neglecting the impact of domestication on defense priming. To our knowledge, plant defense priming has not been studied in the context of domestication events. Thus, the assumption that domestication would affect priming defenses is more speculative than based on empirical evidence of reduced priming defense in domesticated plants. A reduction in a plant's constitutive and inducible defenses does not automatically imply a decrease in its priming defenses unless a positive correlation between the two is demonstrated.

Second, it is crucial to consider that artificial selection could have preserved defense characteristics against specific herbivores that have coevolved with cultivated plants during their domestication process (Chen et al., 2015). Gaillard et al., (2018) hypothesized that if farmers and breeders have primarily focused on developing resistance against specialist insect pests, it is likely that traits effective in combating these pests have been preserved, while traits that are ineffective or susceptible to exploitation by these specialists may not have been retained. Indeed, he found that the effect of reduced defenses in cultivated maize is most evident for generalist herbivores and significantly less pronounced for specialist herbivores. This could potentially explain why domesticated plants are still able to prime their defense just as much as their wild ancestors. It’s important to notice that, although our study did not specifically differentiate between specialist and generalist herbivores, most of the studies included in our analysis tended to focus on the priming of defenses through airborne signals influenced by specialist herbivores of the specific plant species being studied rather than generalist ones.

Third, an alternative explanation for the maintenance of defense priming by domesticated plants found here could be rooted in the concept of resource allocation trade-offs (Rosenthal and Dirzo, 1997), suggesting that more expensive defenses are more prone to being diminished during the process of domestication. It is theorized that physical defenses are more costly than chemical defenses (Skogsmyr and Fagerström, 1992). Notably, cultivated plants exhibit reduced trichomes and are recognized to be less tough compared to their wild ancestors (War et al., 2012). In contrast to cultivated plants, wild plants are believed to maintain their defensive capabilities and have a well-established coevolutionary relationship with specialized herbivores (Bruce, 2015). Following this rationale, at the start of the domestication process, one may expect that plant species will first lose high-costly defense such as physical ones instead of cheaper defenses such as the priming responses we assess here.

Importantly, our investigation revealed a limited number of studies, specifically six, examining defense priming via airborne signals in wild species, in contrast to the 28 studies involving cultivated plants. Although we recognize the need to consider this discrepancy in our findings, we emphasize the significance of delving deeper into defense priming in wild plants to enhance our understanding of the range of priming defenses in plants and to differentiate between cultivated and wild varieties.

Experimental conditions

It is evident from our findings that experiments investigating defense priming in plants conducted in controlled laboratory and greenhouse settings are more likely to yield consistent results compared to those carried out in field conditions. Considering only the collection of data, field experiments are known to exhibit greater variability in outcomes (Osier et al., 2000), thus requiring a more controlled environment for an accurate measurement of plant chemical defenses. In contrast, laboratory and greenhouse settings provide a more stable and controlled environment for plant chemical defense studies (Zhou and Jander, 2022), thus facilitating the collection of plant priming defense data. Indeed, a study that measured the priming effect among these three distinct set-ups found that experiments conducted in greenhouse yielded the strongest induction of VOC, followed by the laboratory and then the field (Li and Blande, 2017). The authors attribute this discrepancy to the differences between the controlled conditions of laboratory and greenhouse environments and the more variable conditions faced by plants growing in the field, which are subject to multiple abiotic and biotic stresses. These factors could potentially affect the dynamics of VOC emission by damaged tissues and the perception of these signals by the receiver.

Despite the ease of data collection in various settings, field experiments in this study did not show a priming effect. This could be attributed to several factors. Initially, for plants to activate defense priming, they must detect the airborne cue (Conrath et al., 2006). The perception of these cues is influenced by the distance over which volatile cues can travel (Karban et al., 2006). Recent field investigations revealed that trees suffered less damage when neighboring plants were closer to clipped trees, with damage increasing as the distance between neighboring plants and clipped trees expanded (Hagiwara et al., 2021). In field environments, plants present a wide range of distribution patterns that are affected by environmental factors like climate change, soil differences, and topographical features (Huang et al., 2023). These factors can pose challenges for the spread of volatile compounds, the vapor pressure of compounds, stomatal aperture, enzymatic activity, and availability of precursor molecules thereby having a significant impact on ecological processes (Niinemets et al., 2004).

Nevertheless, one strategy to study priming of defense more accurately would be to utilize agroecological systems. Many studies utilize airborne cues to manage pest populations (Reddy and Guerrero, 2004; Larsson, 2016); however, to date, there is a lack of research assessing plant defense priming in an agroecological context. Agroecological settings may offer a more conducive environment for plant defense priming and potentially provide insights that field experiments have yet to uncover (Chakravarthy, 2020). Therefore, even though the efficacy of plant defense priming under field conditions is still unclear, there is an urgent requirement for forthcoming research conducted in field environments, particularly within agroecological contexts. Furthermore, we propose investigating plant priming defenses using airborne cues as priming agents against various individual and combined abiotic stresses and its practical implementation in crop stress mitigation and ecological relationships (but see Savvides et al., 2016; Aguirre et al., 2023).

Duration of exposure

We observed that providing a plant with short or medium exposure to an airborne signal can trigger plant defense priming without differentiation between the two levels of exposure durations. A possible explanation for this outcome is that short and medium exposures mimic plants' natural environments, where they frequently experience varying stress patterns interspersed with periods of recovery (Höll et al., 2019). Contrary, in other studies it has been shown that plants that are consistently subjected to stress under a long duration exposure showed reduced metabolic stress responses (Huot et al., 2014).

We did not consider longer-term exposure duration in our analysis primarily because there were few effect sizes where plants were exposed to an airborne cue for an extended period (> 7 days), and these few effect sizes came from one single plant species. While numerous studies have assessed how various abiotic cues (e.g. temperature, radiation) influence the priming effect in plants across different durations of exposure (Huot et al., 2014; Liu et al., 2015; Hilker and Schmülling, 2019; Höll et al., 2019; Xu et al., 2019), there is limited understanding of the impact of the difference exposure duration on the plant's capacity to prime their defense against volatile biotic stress. Hence, it is crucial to enhance our comprehension of how an airborne cue triggers a priming reaction in plants across varying durations of time, and whether there is a particular duration that attenuates plant priming defense.

Type of exposure

In line with our prediction, we found that only plants exposed to synthetic signals displayed enhanced defense responses. This discovery is encouraging because semiochemicals are currently widely used in pest control, often proving to be the most practical method for large-scale applications (Reddy and Guerrero, 2004). The creation of compounds through synthesis allows to produce pure enantiomers with higher purities than those occurring naturally (Mori, 2000). Synthesis also enables the confirmation of molecular structures and the utilization of essential bioactive ingredients that may trigger responses in organisms (Regnier, 1971). Semiochemicals serve as environmentally friendly agents for pest management, safeguarding human health, agriculture, and forestry (Larsson, 2016). Further investigation in this field could delve into identifying the specific synthetic compounds that are most effective in priming plant defenses (but see Westman et al., 2019), along with determining the ideal concentrations and durations of exposure. Understanding the potential of synthetic semiochemicals in enhancing plant defense priming could have significant implications for agriculture and ecosystem maintenance.

Conclusion

This meta-analysis has explored the essential factors and extent of plant defense priming through airborne cues. Numerous crucial questions were both answered and raised, providing a promising outlook for utilizing and comprehending airborne cues as priming stimuli in various scenarios. There is still much work to be done to achieve a deeper understanding of how airborne cues that indicate future stress in plants could influence the dynamics of agroecosystems. We emphasize the significance of addressing certain knowledge gaps in this field, such as further investigating airborne cues as priming stimuli in field conditions and conducting more thorough examinations of this phenomenon in wild plant species. A particularly promising avenue for research would be to investigate the priming response in native plants within their natural habitats. This could yield valuable insights into how priming has evolved. Additionally, studies that more precisely determine the timeframe during which airborne signals can effectively enhance plant defense priming would be a worthwhile investment for crop systems and pest management. Despite these challenges, this compilation of variables presents a promising foundation for future directions that upcoming studies should explore further. Nevertheless, this meta-analysis underscores the possibility of utilizing airborne signals as a method for crop protection in agricultural settings and emphasizes the essential aspects that future studies should focus on.

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2. FINAL CONSIDERATION’S

This thesis has provided invaluable insights into the interactions between plants and insects mediated by volatile compounds. It has highlighted the dynamic nature of chemical interactions among organisms and illustrated how different plants can detect and respond to volatile cues as a means of self-protection. The study reveals fascinating findings about how organisms have adapted to coexist and interact. Furthermore, it offers perspectives on more sustainable crop management for two major global crops and presents updated research in this area, emphasizing future directions and potential practical applications. In conclusion, we hope that this thesis provides valuable information to scientists and that applicable research will emerge from the findings presented here.