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Domestication, fertilization, and induced defenses modulate tomato plant resistance to *Tuta absoluta*

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Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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SUMMARY

RESUMO

Domesticação, adubação e defesas induzidas modulam a resistência do tomateiro à *Tuta absoluta*

O tomateiro (*Solanum lycopersicum* L.) é uma cultura de importância global, mas sua produção enfrenta constantes desafios devido à presença de pragas. Entre elas, destaca-se a traça-do-tomateiro, *Tuta absoluta*, um inseto-praga altamente destrutivo, que causa perdas significativas na produção de tomate em todo o mundo. A dependência de inseticidas químicos sintéticos para o controle de pragas levanta preocupações quanto ao impacto ambiental e ao desenvolvimento de resistência a esses inseticidas. Portanto, estão sendo buscadas estratégias alternativas e sustentáveis de manejo, como o aprimoramento da resistência das plantas. Esta tese tem como objetivo abordar várias questões-chave relacionadas aos mecanismos de resistência do tomate e seu potencial no manejo de *T. absoluta*. Primeiramente, foi investigado o impacto da domesticação na perda de características relacionadas à defesa em uma variedade cultivada de tomate, em comparação com seus ancestrais selvagens, e como essas mudanças podem explicar as variações na suscetibilidade à *T. absoluta*. Em segundo lugar, exploramos o potencial de utilização do fungo endofítico *Metarhizium robertsii*, isolado ou em combinação com a rizobactéria *Bacillus amyloliquefaciens*, visando proteger tanto plantas de tomate selvagens quanto cultivadas, reduzindo os ataques de *T. absoluta* e atraindo seus inimigos naturais para o controle biológico. Por último, investigamos a influência da fertilização e da resistência induzida por jasmonato nas características de defesa em tomates cultivados. Além disso, determinamos de que forma esses fatores afetam a resistência das plantas de tomate contra *T. absoluta*. Nossas descobertas evidenciam que os tomates cultivados reduziram suas características defensivas enquanto aprimoraram a qualidade nutricional, tornando-os mais suscetíveis a *T. absoluta*. No entanto, os mecanismos de defesa tanto dos tomates selvagens quanto dos cultivados para diminuir a preferência de *T. absoluta* e atrair seu inimigo natural podem ser fortalecidos por meio de inoculações com *M. robertsii* isoladamente ou em combinação com *B. amyloliquefaciens*. Adicionalmente, observou-se que as aplicações de MeJA aprimoram características defensivas, resultando em uma resistência aumentada contra *T. absoluta*. Em contraste, plantas submetidas a níveis elevados de fertilização exibiram uma maior suscetibilidade à preferência e ao desempenho de *T. absoluta*. Nossos resultados fornecem informações valiosas para aprimorar a resistência do tomate por meio da identificação de características de resistência oriundas de espécies selvagens, a manipulação do estado nutricional das plantas e de suas defesas induzidas utilizando microrganismos benéficos e jasmonato de metila.

Palavras-chave: Resistência de plantas, Plantas selvagens, Disponibilidade de nutrientes, Jasmonato de metila, *Metarhizium robertsii*, *Bacillus amyloliquefaciens*, *Macrolophus basicornis*

ABSTRACT

Domestication, fertilization, and induced defenses modulate tomato plant resistance to *Tuta absoluta*

Tomato (*Solanum lycopersicum* L.) is a globally important vegetable crop, but its production faces challenges due to the presence of pests. Among these, the South American tomato pinworm *Tuta absoluta* stands out as a highly destructive insect pest, causing significant losses in tomato yields worldwide. The reliance on synthetic chemical insecticides for pest control has led to concerns about environmental impact and the development of insecticide resistance. Therefore, alternative sustainable management strategies, such as enhancing plant resistance are being sought. This thesis aims to address several key questions related to tomato resistance mechanisms and their potential in the management of *T. absoluta*. Firstly, we investigated the impact of domestication on the loss of defensive traits in a modern tomato variety compared to its wild ancestors, and how these changes could explain variations in susceptibility to *T. absoluta*. Secondly, we explored the potential of using the endophytic fungus *Metarhizium robertsii*, either alone or in combination with the rhizobacterium *Bacillus amyloliquefaciens*, for the protection of both wild and cultivated tomato plants to reduce *T. absoluta* attacks while also attracting its natural enemy for biological control. Lastly, we investigated the influence of fertilization and jasmonate-induced resistance on defensive traits in cultivated tomatoes, and also determined how these factors impact the resistance of tomato plants against *T. absoluta*. Our findings reveal that cultivated tomatoes have reduced their defensive traits while enhancing nutritional quality, making them more susceptible to *T. absoluta*. However, the defensive mechanisms of both wild and cultivated tomatoes to reduce the preference of *T. absoluta* and attract its natural enemy can be strengthened through inoculations with *M. robertsii* alone or in combination with *B. amyloliquefaciens*. Additionally, applications of MeJA were observed to enhance defensive traits, resulting in increased resistance against *T. absoluta*. Conversely, plants subjected to high levels of fertilization exhibited increased susceptibility to *T. absoluta* preference and performance. Our findings provide valuable insights into improving tomato resistance through the identification of defensive traits from wild counterparts, and the manipulation of plant nutritional status and induced defenses by beneficial microbes and jasmonates.

Keywords: Host-plant resistance, Wild plants, Nutrient availability, Methyl jasmonate, *Metarhizium robertsii*, *Bacillus amyloliquefaciens*, *Macrolophus basicornis*

1. GENERAL INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop in the world, and one of the most widely cultivated commodities, with an annual production of 189 million tons produced across 5.1 million hectares in over 160 countries (FAO 2023). Originating from the South American Andes, tomato cultivation expanded globaly following the Spanish colonization of the Americas (Peralta and Spooner 2007). Currently, tomatoes are grown in both greenhouses and open fields, and their global annual production has steadily increased in the last few decades, primarily driven by China, the European Union, India, Turkey, and the United States of America (FAO 2023). Brazil is ranked as the sixth-larger tomato producer worldwide, making it a key product in its vegetable market. However, the greatest challenge faced in global tomato production is the reduction of pesticide usage for effective pest and disease management (Gatahi 2020).

Tomato plants are susceptible to more than 200 pests and diseases caused by an array of insects, pathogenic fungi, bacteria, viruses, and nematodes (Lange and Bronson 1981; Singh et al. 2017). Among these threats, the South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is considered the most important insect pest worldwide, capable of causing production losses up to 80-100% (Desneux et al. 2010; Biondi et al. 2018). This moth species was initially reported in Peru at the beginning of the 20th century and then spread to other South American countries since 1960 (Vargas 1970; Biondi et al. 2018), reaching Brazil in 1979 (Guedes and Picanço 2012). Over the last few decades, *T. absoluta* has continued to invade more than 100 countries in America, Europe, Africa, and Asia (EPPO 2023), with devastating consequences to tomato growers (Biondi et al. 2018; Han et al. 2019). While *T. absoluta* infest both non-cultivable and economically important solanaceous plants such as potato, tobacco, and eggplant (Bawin et al. 2016; Sylla et al. 2019; Silva et al. 2021), its specialization in tomatoes is notable, as it shares a long-standing evolutionary history with the crop that began centuries ago in South America.

To reduce the losses in tomato production caused by *T. absoluta*, growers heavily rely on synthetic chemical insecticides as their primary tool. However, apart from their increasing cost, these insecticides pose detrimental effects on the environment and non-target organisms, including beneficial insects (Abbes et al. 2015). Furthermore, their injudicious use and over-application has led to the emergence of resistant populations to numerous insecticides (Reyes et al. 2012; Guedes et al. 2019), posing significant challenges in *T. absoluta* control. Despite global efforts towards a sustainable approach for *T. absoluta* management, launched in response to its spread and constant risk of invading new areas (Biondi et al. 2018; Desneux et al. 2022), knowledge gaps remain, particularly regarding the potential of plant resistance. Indeed, enhancing plant defenses can contribute, along with other tactics, to the implementation of sustainable and ecofriendly tomato protection measures to reduce *T. absoluta* damages.

In agroecosystems, plants are continuously exposed to several stress factors, such as attacks by insect pests. To mitigate and reduce damages, plants employ physical and chemical defense mechanisms, which can be constitutive (always present) or induced (produced in response to herbivore damage) (Karban and Myers 1989; War et al. 2012). These defensive traits serve to protect plants directly and indirectly from attackers. Direct defenses, such as trichomes, wax layers, and secondary metabolites, can affect herbivore growth or cause mortality (antibiosis), or make plants less attractive for feeding and attack (antixenosis) (Schoonhoven et al. 2005; Stout 2013). On the other hand, indirect defenses can attract natural enemies of herbivores, such as parasitoids and predators, through herbivore-induced plant volatiles (HIPVs) (Turlings and Ton 2006; Dicke and Baldwin 2010). In tomatoes, glandular trichomes have been extensively studied as a crucial defensive mechanism directly affecting the performance of herbivores (Wang et al. 2020; Marinke et al. 2022). Furthermore, secondary metabolites, such as phenolics and

HIPVs, play significant roles in direct and indirect defense, respectively. High phenolic content in tomatoes have been associated with reduced survival and performance of insect herbivores (Summers and Felton 1994; Wallis and Galarneau 2020). Meanwhile, HIPVs have proven effective in attracting mirid predators, such as *Macrolophus pygmaeus* (Rambur) (Lins et al. 2014; De Backer et al. 2015), *Nesidiocoris tenuis* (Reuter) (Ayelo et al. 2021), *Macrolophus basicornis* Stal, *Engytatus varians* (Distant), and *Campyloneuropsis infumatus* (Carvalho) (Silva et al. 2018), which make them a promising tool to enhance biological control of *T. absoluta.*

However, some of these crucial defensive traits may have been lost during the tomato domestication process, potentially explaining why modern varieties are more susceptible to insect pests compared to their wild ancestors. Nevertheless, wild plants hold great promise as a valuable source of traits that enable tomatoes to whithstand *T. absoluta* attacks. Moreover, the availability of plant nutrients, an important factor that triggers bottom-up effects in plant-insect relationships, can alter the chemical defensive traits in tomatoes (Han et al. 2014; Blazhevski et al. 2018). Additionally, induced defenses caused by beneficial microbes or elicitors can also promote variations in plant chemical defenses, thereby affecting *T. absoluta* and its natural enemies (Strapasson et al. 2014; Silva et al. 2022). In this thesis, we aimed to address the following questions:

- What changes in defensive and nutritional traits occurred during tomato domestication may have increased the susceptibility of modern varieties to *T. absoluta* compared to their wild relatives?
- Can the endophytic fungus *Metarhizium robertsii,* either alone or in combination with the rhizobacterium *Bacillus amyloliquefaciens,* provide protection for both wild and cultivated tomato plants by reducing *T. absoluta* attacks and attracting natural enemies?
- How do the bottom-up effects of fertilization and jasmonate-induced resistance alter plant growth and defensive traits in cultivated tomatoes, and consequently influence tomato resistance against *T. absoluta*?

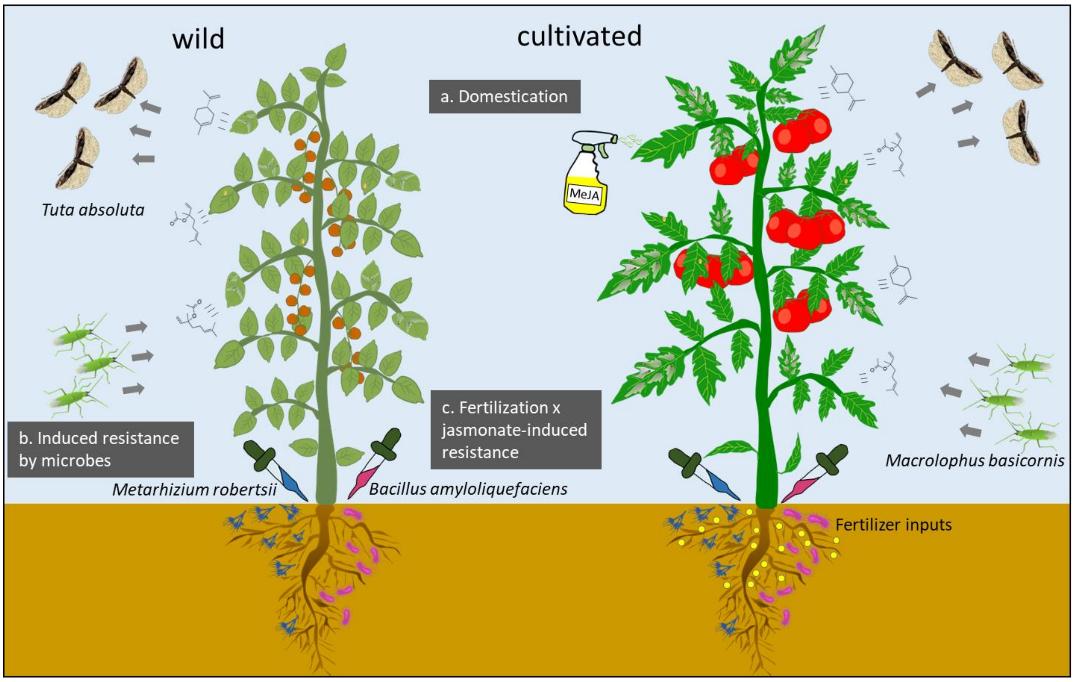

Figure 1. A schematic representation illustrating how tomato's direct resistance through antixenosis and antibiose against *Tuta absoluta*, and indirect resistance through the attraction of the predator *Macrolophus basicornis* can be mediated by a) plant domestication, b) induced resistance by *Metarhizium robertsii* and *Bacillus amyloliquefaciens*, and c) the simultaneous fertilization levels and induced resistance by methyl jasmonate (MeJA).

Each of these questions will be addressed in the following chapters. The second chapter was structured following the formatting guidelines of the journal *Planta*, while the third chapter followed the submission requirements of *Biological control*. Finally, the fourth chapter was prepared in accordance with the guidelines of the *Journal of Plant Interactions*.

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2. DEFENSIVE AND NUTRITIONAL TRAITS SHAPING PLANT RESISTANCE AGAINST *Tuta absoluta* IN WILD AND CULTIVATED TOMATOES

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Abstract

Plant domestication process has selected desirable agronomic attributes that can both intentionally and unintentionally compromise other important traits, such as plant defense and nutritional value. However, how domestication can affect the defensive and nutritional traits of plant organs not exposed to selection and the consequent interactions with specialist herbivores remains unclear. Here, we hypothesized that the modern cultivated tomato has reduced levels of constitutive defense and increased levels of nutritional content compared with its wild relatives, and such differences affect the resistance of the South American tomato pinworm, *Tuta absoluta*, a devastating insect pest that co-evolved with tomato. To test this hypothesis, we compared plant volatile emissions, leaf defensive (glandular and non-glandular trichome density, and total phenolic content), and nutritional traits (leaf nitrogen content) among the cultivated tomato *Solanum lycopersicum* and its wild relatives *S. pennellii* and *S. habrochaites*. We also determined the attraction and ovipositional preference of female moths and larval performance on cultivated and wild tomatoes. Volatile emissions were qualitatively and quantitatively different among the cultivated and wild species. Glandular trichomes density and total phenolics were lower in *S. lycopersicum*. In contrast, this last specie had a greater non-glandular trichome density and leaf nitrogen content. Female moths were more attracted and consistently laid more eggs on the cultivated *S. lycopersicum*. Larvae fed on *S. lycopersicum* leaves had a better performance reaching shorter larval developmental times and increasing the pupal weight compared to those fed on wild tomatoes. Overall, our findings document that agronomic selection for enhanced fruit quality and yields has altered the defensive and nutritional traits in tomato plants, affecting their resistance to *T. absoluta*.

Keywords: Agronomic selection, Leaf nitrogen, Plant resistance, Plant volatiles, Phenolics, Trichomes

2.1. Introduction

Plant domestication is probably one of the most crucial processes in human history and is the nutritional basis for the rise of modern civilization (Gepts 2010). Humans began domesticating plants around 11,000 years ago in diverse geographical regions around the world (Lev-Yadun et al. 2000; Purugganan and Fuller 2009). During this long process that includes the changes imposed by plant breeding, plants were morphologically and physiologically altered, producing crops with desirable agricultural characteristics for human consumption, including palatability and higher yield of the harvested organs (Meyer et al. 2012). However, this anthropogenic selection generates additional unintended consequences on crops, which can result in a lower capacity to resist biotic and abiotic stresses in cultivated plants (Milla et al. 2015) due to a trade-off between growth/productivity and defense, according to the resource allocation theory (Herms and Mattson 1992). In this way, several studies have shown that plant domestication leads to simpler morphology, increased nutritional content of organs under selection, and reduced defense mechanisms (Chen et al. 2015; Moreira et al. 2018).

Before domestication, the interactions among plants, microbes, and herbivorous insects shaped the evolution of plant defenses (Gatehouse 2002; Futuyma and Agrawal 2009). Plants can protect themselves through a wide range of mechanisms, including direct physical (e.g., leaf surface waxes, trichomes, thorns, spines) and chemical traits (e.g., secondary metabolites), that can affect the behavior and performance of attacking herbivores (Alba et al. 2009; War et al. 2018). Given that cultivated plants usually have reduced defenses, it has long been assumed that crops are more susceptible to herbivorous insects than their wild relatives (Whitehead et al. 2017). This reduced resistance against pests is also expected due fundamentally to reduced genetic diversity in crop plants (Reif et al. 2005; Gross and Olsen 2010). A meta-analysis made across 78 plant species showed that domestication does not always affect morphological and chemical defensive traits, but plant resistance to herbivores is consistently reduced (Whitehead et al. 2017). Moreover, the magnitude of this effect depends on the plant organs exposed to the selection and how resistance is measured (Whitehead et al. 2017). In recent years, several studies on maize (Gaillard et al. 2018; Fontes-Puebla et al. 2021; Naranjo-Guevara et al. 2021; Bernal et al. 2023), apple (Whitehead and Poveda 2019), blueberry (Rodriguez-Saona et al. 2019), avocado (Hernández-Cumplido et al. 2021), chili pepper (Chabaane et al. 2022), and potato (Ali et al. 2022) support the domestication-reduced defense hypothesis. However, this pattern is not ubiquitous and can affect generalist and specialist herbivores in distinct ways. Specialists that followed crop plants throughout their domestication and geographical range expansion are known to have mechanisms to tolerate an array of plant defenses, being usually less affected by them (Ali and Agrawal 2012; Haber et al. 2018). In one of the most exhaustive experimental tests on the domestication-reduced hypothesis, the performance of the generalists *Spodoptera exigua* (Hübner) and *Myzus persicae* (Sulzer) was tested on 29 independent domestication events (Turcotte et al. 2014). Despite the negative effect of domestication on the resistance against *S. exigua*, mainly associated with a reduction in plants' morphological and physical defensive traits, domestication did not affect the performance of *M. persicae*. A recent study showed that wild and cultivated squash differ in terms of defensive traits, but the performance of the generalist *Spodoptera latifascia* (Walker) was not affected by these differences (Jaccard et al. 2021). In another example, herbivores performed better on maize than on teosinte and regarding insect specialization, the reduction in performance of specialists was less pronounced than for the generalists (Gaillard et al. 2018). In potatoes, the cultivated *Solanum tuberosum* was severely damaged by the generalists *M. persicae* and *Macrosiphum euphorbiae* (Thomas), while the wild *S. commersonii* was mostly affected by the specialist *Tequus* sp. (Altesor et al. 2014).

In addition to herbivore performance, plant preference/acceptance is a crucial aspect of insect colonization (Le Roux et al. 2014). Host selection is a stepwise process involving behavior sequences that are modulated mainly by plant volatile organic compound (VOC) emissions (Schoonhoven et al. 2005). Indeed, herbivores often use constitutive VOCs as cues to recognize and locate host plants (Thompson and Pellmyr 1991; Anderson and Anton 2014). This decision is particularly important for females, as they should prefer to oviposit on host plants that secure the survival and growth of their offspring (Jaenike 1990, Gripenberg et al. 2010). In this way, changes in morphological and chemical plant traits are expected to determine the location success and ovipositional acceptance for insects (Chen et al. 2015). Although domesticated plants should therefore be more attractive for oviposition due to their often lack of resistance (Chen et al. 2015; Bernal et al. 2023), still little is known about the effects of domestication on VOCs emission and their interactions with herbivores. A recent meta-analysis revealed that cultivated plants emit lower levels of VOCs than their wild relatives, suggesting that domestication can affect volatile emissions (Fernandez et al. 2021). However, the chemotactic responses of herbivores to these VOCs are incredibly variable. For example, while wild tomato VOCs are repellent to the whitefly *Bemisia tabaci* (Genn.) (Bleeker et al. 2009), *Drosophila suzukii* (Matsumura) is more attracted to wild blueberry VOCs compared to its cultivated counterparts (Urbaneja-Bernat et al. 2021).

Domestication can also promote changes in plants' nutritional content (Fernandez et al. 2021). In addition to genotypic variation in domesticated crops, cultivation practices (i.e., use of pesticides and fertilizers) and agricultural intensification can promote strong contrasts between wild and cultivated species (Denison et al. 2003; Fontes-Puebla and Bernal 2020; Fontes-Puebla et al. 2021). Since the production of defenses is energetically costly (Kessler and Baldwin 2002; Hahn and Maron 2016), domestication should favor plants that allocate resources to convert them into growth and reproduction over defenses, suggesting that cultivated plants are more nutritious than their wild relatives (Mondolot et al. 2008). Also, it is noteworthy that environmental conditions, such as resource availability, can drive variations in defense affecting plant tolerance and resistance against antagonists, and shaping the growth–resistance tradeoff among species (Hahn and Maron 2016). Thus, changes in plant nutritional content are also a possible mechanism that explains why domesticated plants suffer greater damage by herbivore insects than wild species (Turcotte et al. 2014; Whitehead et al. 2017). In fact, the nutritional quality of an unsuitable host plant can seriously affect herbivores' survival, growth, and population dynamics (Chen et al. 2010; Wetzel et al. 2016). In contrast, studies have shown that harvested organs (i.e., fruits and seeds) subjected to domestication in cultivated plants had lower nutritional content than their wild relatives (see Fernández et al. 2021). It is, however, still unclear how domestication/breeding alters the nutritional quality in plant tissues other than the harvested organs, and how nutritional content and defensive traits interact in wild and cultivated plants and, in turn, affect interactions with insects.

The cultivated tomato (*Solanum lycopersicum* L.) and its 12 wild relatives are native to western South America along the Andean valleys of Peru and Ecuador (Bergougnoux 2014; Mata-Nicolás et al. 2020). Currently, cultivated tomato is among the most economically important vegetable crops (FAO, 2022). Its several modern cultivars are widespread throughout the world, while most wild tomatoes are often grown in diverse habitats with different weather conditions within the heterogeneous Andes geography (Peralta and Spooner 2007). Although the phylogenetic of the genus *Solanum* section *Lycopersicon* is still unsolved, studies suggest that the wild *Solanum habrochaites* S. Knapp & D. M. Spooner is close-related to *Solanum pennellii* Correll, while *Solanum pimpinellifolium* Mill. ex Dunal and *Solanum galapagense* S. C. Darwin & Peralta are thought to be the closest relatives to the cultivated tomato (Yu et al. 2022; Blanca et al. 2022). These wild relatives of tomato are the main gene source for resistance breeding in modern tomato cultivars, offering genetic and morphological variations, and a vast array of desirable traits such as growth habits, yield, nutritional values, size, the shape of fruits, and defense (Park et al. 2004; Peralta and Spooner 2007; Seong et al. 2020). On the other hand, cultivated tomato also experienced a severe genetic bottleneck causing a reduction in genetic diversity (Bauchet and Causse 2012). In consequence, modern cultivars may have lost important resistance traits to herbivores compared to their wild relatives. For example, certain wild species contain a broad range of glandular trichomes providing resistance against several pest insects (Alba et al. 2009; Rakha et al. 2017). Still, many other mechanisms of tomato resistance associated with domestication and breeding are poorly understood.

The South American tomato pinworm, *Tuta absoluta* (Meyrick), is a specialist leafminer pest in its native area (South America) and is currently considered the major threat to tomato production worldwide (Biondi et al. 2018; Han et al. 2019; Colmenárez et al. 2022). Recent hypotheses suggest that *T. absoluta* originated in the Peruvian central Andes (Biondi et al. 2018), then spread to other Latin American countries around 1960, and in the last decades to Europe, Africa, and Asia, causing serious economic losses in both greenhouse and open-field tomato production (Desneux et al. 2010; Mansour et al. 2018; Han et al. 2019). Due to the same geographical origin, *T. absoluta* likely followed the tomato crop throughout its domestication in the central Andes. Although its preferred host is *S. lycopersicum*, *T. absoluta* has been reported to feed on other solanaceous plants, both cultivated and wild species (Desneux et al. 2010; Bawin et al. 2016; Silva et al. 2021).

Here, we hypothesized that cultivated tomato has reduced levels of constitutive defense and increased levels of nutritional value compared with its wild relatives, and such differences affect *T. absoluta* preference and performance. To test this hypothesis, we used the cultivated *S. lycopersicum* cv. Santa Clara, a modern cultivar developed by plant breeding programs and widely cultivated in Brazil since the late 1980s (Melo et al. 2008), and its wild relatives *S. habrochaites* and *S. pennellii*. In this study, our specific objectives were to:

1) Determine whether differences in constitutive VOCs profiles in both cultivated and wild tomato plants can modulate the attraction and oviposition preference (antixenosis) of *T. absoluta* moths;

2) Compare whether physical (glandular and non-glandular trichomes) and chemical (total phenolics) traits in wild and cultivated tomato leaves may explain possible resistance to *T. absoluta* larvae (antibiosis); and

3) Evaluate the nutritional quality (nitrogen content) of wild and cultivated tomato leaves.

2.2. Material and Methods

2.2.1. Plants and insects

Seeds of the wild tomato species *S. pennellii* accession LA0716 and *S. habrochaites* accession PI134417 were obtained from the active germplasm bank of the Agronomic Institute (Campinas, SP, Brazil), while the seeds of the cultivated tomato *S. lycopersicum* cv. Santa Clara were purchased from Isla Sementes Ltda. (Porto Alegre, RS, Brazil). All seeds were sown in plug trays filled with Basaplant® soil (Base Agro, Artur Nogueira, SP, Brazil), then transferred to 1.9-L plastic pots (3 weeks after sowing) containing Basaplant® soil and coconut fiber (3:1) fertilized with 3 g of Basacote® Mini 6M 16-8-12 (+2) (Compo Expert, Sumaré, SP, Brazil). Plants were maintained in the greenhouse under natural light and temperature (25 ± 5.5 °C) and watered as needed. Plants used in the experiments were 5-week-old with similar height (~40 cm).

Tuta absoluta was obtained from a laboratory colony started in 2018 from larvae and pupae collected from commercial tomato farms (Paulínia, SP, Brazil). The colony was initially maintained on tomato plants, cv. Santa Cruz, for over 30 generations in the Insect Biology Laboratory at ESALQ/USP (Piracicaba, SP, Brazil). Larvae of this original population were then reared on an alternative host plant (*Solanum melongena* cv. Napoli) for at least four generations to avoid previous experience and habituation to the tomato species used in the experiments. Larvae and adults were kept in separate cages (60 × 30 × 30 cm) covered with fine nylon mesh under controlled conditions (25 ± 0.5 °C, 70 ± 1% RH, 12:12 L:D). An aqueous honey solution (10%) was provided as a food source for adults on a small ball of cotton wool inside the cage.

2.2.2. Collection and analysis of plant volatiles

Headspace volatiles were collected from tomato plants (*n* = 5) over a 12-hour period, from 8:00 pm to 8:00 am, under laboratory conditions (25 ± 1 °C, 70 ± 10% RH). The plastic pots and soil were covered with aluminum foil to reduce the collection of volatiles from these sources. Then, plants were individually enclosed in a glass chamber (50 cm width × 36 cm height) and connected to a volatile collection system (Analytical Research Systems Inc., Gainesville, FL, USA). Charcoal-filtered and humidified air was pumped in at 0.8 L min^{-1} and drawn out at 0.6 L min^{-1} through a Hayesep-Q trap (50 mg, 80-100 mesh; Alltech Associates, Bannockburn, IL, USA) connected to the system via PTFE tubing. The adsorbent tubes were eluted with 0.5 mL of redistilled hexane and the headspace samples were concentrated to 50 μL under a gentle N_2 flow. Samples were stored in glass vials at −30 °C until analysis.

Tomato volatiles were analyzed on a Shimadzu GC-2010 equipped with a non-polar Rtx-1 column (25 mm × 30 m × 25 µm, RESTEK, Bellefonte, PA, USA) and a flame ionization detector (GC-FID) operated at 270 °C. The oven temperature was maintained at 50 °C for 2 min, programmed to 5 °C min^{-1} to 180 °C, held for 0.1 min, then 10 °C min^{-1} to 250 °C, and held for 20 min. The injector was at 250 °C. Nonyl acetate (Sigma Aldrich, St. Louis, MO, USA) was added (10 µL of a 10 ng μL^{-1} solution) to each sample as an internal standard. A 2-µL aliquot of each sample was injected on a splitless injector, with helium as the carrier gas (24 cm s^{-1}). The relative amount of the compounds in each sample was determined based on a comparison of their peak area with that of the internal standard, and standardized per unit of dry shoot biomass (g) for each of the replicates. Data were collected with GC Solution.

For volatile identification, the most representative sample of each tomato species was further analyzed on a Shimadzu GCQP-2010 Ultra quadrupole mass spectrometer coupled to a gas chromatograph (Shimadzu GC2010) equipped with a non-polar Rxi-1MS column (25 mm × 30 m × 25 µm, RESTEK, Bellefonte, PA, USA), a splitless injector and helium as the carrier gas. Ionization was by electron impact (70 eV, source temperature at 250 °C). The injector was at 250 °C using the same temperature program as in GC-FID analysis. Data were collected with GCMS Solution. Identifications were made by comparison of the mass spectra with mass spectral library database (NIST11) and using the Kováts retention index. Authentic standards were used to confirm the identities of α-pinene, β-myrcene, β-pinene, α-phellandrene, limonene, o-cymene, γ-terpinene, α-humulene, β-caryophyllene, and 2-undecanone (Sigma-Aldrich, Merck KGA, St. Louis, Missouri, USA); and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT; synthesized and kindly donated by Dr. Michael A. Birkett from Rothamsted Research, Harpenden, UK).

2.2.3. Closed arena bioassay

A closed arena was used to determine the olfactory responses of female *T. absoluta* to cultivated and wild tomato plant volatiles. The experimental arena consisted of a metal cage (100 × 70 × 50 cm) covered with fine nylon mesh and two 15-L glass vessels that were used to enclose the plants on opposite sides of the arena (Fig. 1). The glass vessels were wrapped in aluminum foil to avoid any visual cues. A funnel-like hole (3 cm diameter) on the top of each vessel allowed moths to enter but reduced their exit from it. A sticky trap was also placed inside each vessel to prevent females to escape. Twenty mated females (2-3 day-old) were released in the arena and their positions were recorded after 12 h (20:00 to 8:00) under controlled laboratory conditions (25 ± 0.5 °C, 70 ± 1% RH, 12:12 L: D). Attraction responses were assessed to odor sources emitted from (*i*) blank (empty vessel) vs *S. lycopersicum*; (*ii*) blank vs *S. pennellii*; (*iii*) blank vs *S. habrochaites;* (*iv*) *S. pennellii* vs *S. lycopersicum*; and (*v*) *S. habrochaites* vs *S. lycopersicum*. The experiment was replicated 10 times and each replicate employed fresh plants and moth sets.

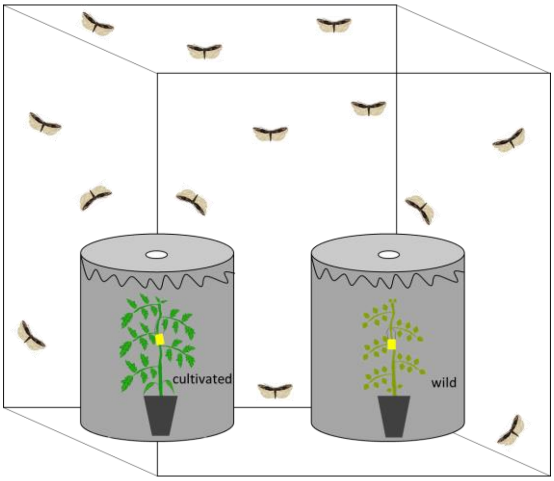

Fig. 1 Closed arena used for dual-choice attraction test of *Tuta absoluta* females to volatile organic compounds from cultivated tomato *Solanum lycopersicum* and its wild relatives *Solanum pennellii* and *Solanum habrochaites*

2.2.4. Oviposition preference

To examine oviposition preferences between cultivated and wild tomato species, experiments were conducted in metal cages (100 × 70 × 50 cm) covered with fine nylon mesh in a greenhouse under natural conditions of light and temperature (25 ± 5.5 °C). Three 2-3-day-old mated couples, with no previous oviposition experience, were released in the middle of the cage and allowed access to the plants for 48 h. Care was taken to select plants with similar heights (~40 cm). Thereafter, the number of eggs laid on plants was counted. The experiment was replicated 10 times. For the no-choice test, a single plant of either *S. lycopersicum, S. pennellii,* or *S. habrochaites* was placed in the center of the cage. For the dual-choice test, one cultivated and one wild tomato plant were placed approximately 60 cm apart (*S. lycopersicum* vs. *S. pennellii* and *S. lycopersicum* vs. *S. habrochaites*).

2.2.5. Trichomes

To determine the trichome density of each tomato species, all glandular and non-glandular trichomes in an area of 15 mm^2 on the abaxial and adaxial sides of a leaf collected from the middle to upper level of the plant were counted. Two pictures were taken close to the middle portion of the leaf at both sides of the main vein using a camera (Leica DF 295) attached to a stereomicroscope (Leica M205 C). Then, an average of the two measurements was expressed for each species. Eight plants of each species were assessed.

2.2.6. Leaf nitrogen content

Leaf samples (*n* = 5) from the middle to the plant's upper level were taken from each tomato species and dried in an oven at 60 °C for 72 h. Dried samples were ground and 150 g was submitted to sulfuric digestion to determine nitrogen concentrations by the Kjeldahl method (Nelson and Sommers 1980).

2.2.7. Leaf phenolic content

The total phenolic content in tomato leaves was determined based on a colorimetric assay following a slightly modified Folin-Ciocalteu method (Ainsworth and Gillespie 2007). Briefly, leaf samples (*n* = 5) were flash-frozen in liquid nitrogen, homogenized, and 0.3 g were placed into a 15-mL glass tube with 4 mL of methanol (50% vol vol^{-1}). The extract was then incubated in a water bath for 1.5 h at 80 °C and centrifuged at 20,000 *g* for 15 min at room temperature. The supernatant was collected and placed in a new tube. A 150 µL of this extract was mixed with 150 µL of F-C reagent (Sigma Aldrich, Merck, Germany), previously diluted in water (1:1), and allowed to stand for 5 min. Then, 3 mL of Na_2CO_3 (2%) was added. After 5 min, the mixture was measured at 750 nm on a spectrophotometer (Hitachi U-1900, Tokyo, Japan). A standard curve was prepared on chlorogenic acid concentrations of 0, 10, 20, 30, 40, 50, 100, 150, and 250 µg mL^{-1}. The total phenolic content was calculated as chlorogenic acid equivalents using a regression equation.

2.2.8. Larval performance

To determine *T. absoluta* larval performance on tomato plant species, three eggs (60-72-h-old) were placed on a plant in different fully-developed leaves using a fine brush. For each evaluated parameter, the mean values of these three individuals was considered as a replicate. Fifteen plants were used for each tomato species. Plants were placed in a metal cage (50 × 50 × 50 cm) covered with fine mesh and maintained in a greenhouse under natural light and temperature variations (25 ± 5.5 °C). Eggs were inspected daily until they were hatched. Larval survival was recorded, and then we measured the larval and pupal developmental time (days), and pupal weight (mg) in alive individuals.

2.2.9. Statistical analyses

Normality was tested using the Shapiro-Wilk test and the homogeneity of variances was verified by Levene's test. A Generalized Linear Model (GLM) with Gamma distribution was used to compare VOC emissions and total phenolics from cultivated and wild tomato species, and differences in *T. absoluta* pupal weight. Larval survival, dual-choice oviposition preference, and olfactory responses were analyzed using a GLM with quasi-Binomial distribution, while the no-choice oviposition preference, leaf trichome density, leaf nitrogen content, and larval and pupal developmental time were analyzed using a GLM with quasi-Poisson distribution. Means were compared using Tukey's *post hoc* tests ($P < 0.05$). A principal component analysis (PCA) was applied to the multivariate data to evaluate the influence of all VOCs in separating the tomato species. PCA was performed using a correlation matrix and comparison between groups. All analyses were performed using R software version 3.02.3 (R Core Team 2022).

2.3. Results

2.3.1. Plant volatile profile

Chemical analyses of VOCs revealed 23 major compounds emitted during the nighttime from three tomato species with qualitative and quantitative differences among them (Table 1). Only α-pinene and β-caryophyllene were common to all species, while the monoterpenes β-pinene, β-myrcene, 2-carene, β-phellandrene, and β-ocimene were exclusively emitted by *S. lycopersicum*. Moreover, the monoterpene α-terpinene and the sesquiterpene β-humulene were emitted only by *S. pennellii*; and the ester methyl salicylate, the sesquiterpene α-copaene, and the methyl ketones 2-undecanone, 2-dodecanone, tetradecanone, 2-pentadecanone, and 2-nonadecanone were produced only by *S. habrochaites*. Total VOCs emission rates were also significantly different among species (GLM $\chi^2 = 89.16$; df = 2; $P < 0.001$) (Table 1). The emission of total VOCs in the wild tomato *S. habrochaites* was higher in comparison to *S. pennellii* and *S. lycopersicum*.

The first two PCA components explained 70% of the total variance. A clear separation of species according to the VOCs profiles was shown in the PCA (Fig. 2). The separation of the wild *S. pennellii* was mainly influenced by α-terpinene and β-humulene, while *S. habrochaites* was influenced by the high concentration of methyl ketones, such as 2-tridecanone, 2-undecanone, tetradecanone, 2-nonadecanone, 2-pentadecanone, and 2-dodecanone. On the other hand, the separation of the cultivated *S. lycopersicum* was influenced by the presence of several terpenes.

Table 1 Concentrations of volatile organic compounds (mean ng g^{-1} DW ± SE, *n* = 5) emitted from 8:00 pm to 8:00 am by cultivated tomato *Solanum lycopersicum* and its wild relatives *Solanum pennellii* and *Solanum habrochaites*. Means followed by different letters in total emission indicate a significant difference among species (GLM, $P < 0.05$)

No.	**Compounds**	**RI**	**Cultivated**	**Wild species**	
			Solanum lycopersicum	***Solanum pennellii***	***Solanum habrochaites***
Aromatic heterocyclic					
1	Methyl salicylate	1163	n.d	n.d	0.49 ± 0.29
Terpenes					
2	α-Pinene	929	2.28 ± 0.53	0.25 ± 0.05	0.88 ± 0.44
3	β-Pinene	967	1.68 ± 0.96	n.d	n.d
4	β-Myrcene	981	2.15 ± 1.67	n.d	n.d
5	2-Carene	991	8.87 ± 2.28	n.d	n.d
6	α-Phellandrene	993	1.30 ± 0.33	0.94 ± 0.17	n.d
7	α-Terpinene	1006	n.d	0.03 ± 0.005	n.d
8	o-Cymene	1008	0.88 ± 0.41	0.01 ± 0.002	n.d
9	β-Phellandrene	1016	24.04 ± 5.86	n.d	n.d
10	Limonene	1018	5.77 ± 1.35	0.51 ± 0.06	n.d
11	β-Ocimene	1038	1.14 ± 0.03	n.d	n.d
12	γ-Terpinene	1046	0.12 ± 0.02	0.71 ± 0.14	n.d
13	α-Copaene	1369	n.d	n.d	0.07 ± 0.04
14	β-Caryophyllene	1409	1.22 ± 0.33	0.15 ± 0.05	27.43 ± 12.57
15	α-Humulene	1442	0.22 ± 0.065	n.d	3.76 ± 1.81
16	β-Humulene	1490	n.d	0.17 ± 0.08	n.d
17	TMTT	1565	n.d	0.02 ± 0.01	3.30 ± 1.00
	Total terpenes		49.7 ± 13.8	2.8 ± 0.6	35.4 ± 15.8
Methyl ketones					
18	2-Undecanone	1272	n.d	n.d	32.25 ± 12.39
19	2-Dodecanone	1373	n.d	n.d	0.68 ± 0.37
20	2-Tridecanone	1476	n.d	0.35 ± 0.20	321.57 ± 121.43
21	Tetradecanone	1648	n.d	n.d	32.60 ± 14.05
22	2-Pentadecanone	1678	n.d	n.d	7.92 ± 2.99
23	2-Nonadecanone	1736	n.d	n.d	1.98 ± 0.90
	Total methyl ketone		n.d	0.35 ± 0.2	397.0 ± 152.1
	Total emission		48.5 ± 13.8 b	3.2 ± 0.8 c	432.9 ± 168.2 a

RI Retention index; n.d Not detected

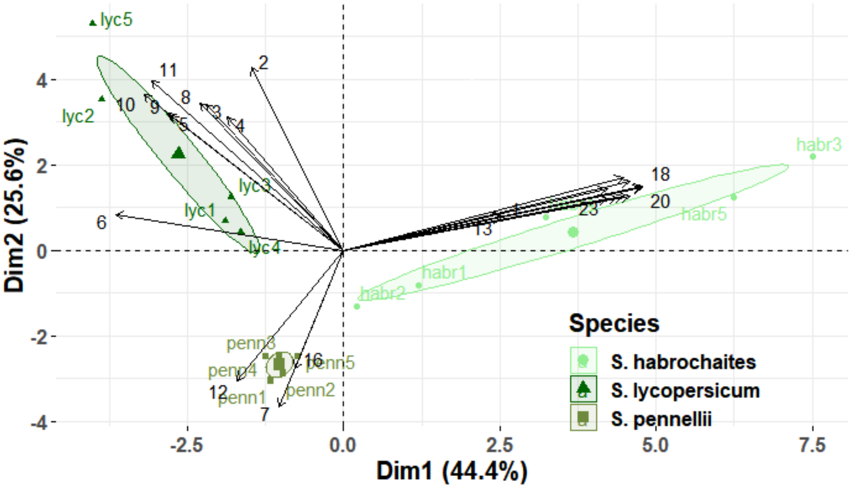

Fig. 2 Principal component analysis (PCA) of volatile organic compounds from cultivated tomato *Solanum lycopersicum* and its wild relatives *Solanum pennellii* and *Solanum habrochaites*. Vector numbers correspond to the compound listed in Table 1. The ellipses enclose the data points for each tomato species

2.3.2. *Tuta absoluta* response to tomato plant VOCs

In dual-choice test, females were more attracted to VOCs emitted by *S. lycopersicum* (GLM χ^2 = 94.66; df = 1; *P* < 0.001), *S. pennellii* (GLM χ^2 = 23.40; df = 1; *P* < 0.001), and *S. habrochaites* (GLM χ^2 = 5.65; df = 1; *P* = 0.017) than control (empty vessel) (Fig. 3a). However, when the cultivated tomato was tested against both wild species, females were significantly more attracted to *S. lycopersicum* (vs. *S. pennellii* GLM χ^2 = 24.60; df = 1; *P* < 0.001 and vs. *S. habrochaites* GLM χ^2 = 10.39; df = 1; *P* = 0.001) (Fig. 3a). Comparing female responses from dual-choice tests of each tomato species against blank control, a gradient of preference was noticed showing that they were significantly more attracted to *S. lycopersicum* than *S. pennellii* or *S. habrochaites* (GLM χ^2 = 46.59; df = 2; *P* < 0.001) (Fig. 3b).

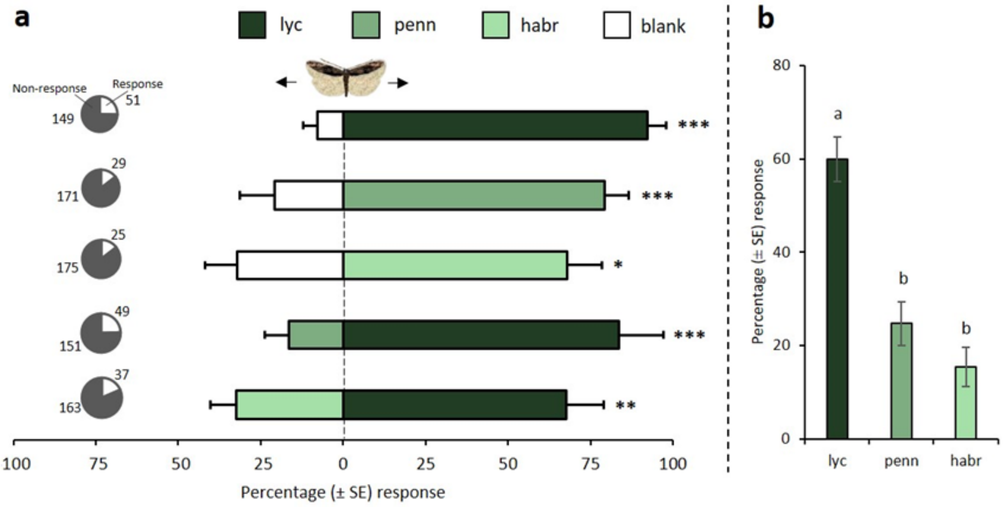

Fig. 3 Percentage (mean ± SE, *n* = 10) of attraction of *Tuta absoluta* females in **(a)** a dual-choice test to volatile organic compounds from cultivated tomato *Solanum lycopersicum* (lyc) and its wild relatives *S. pennellii* (penn) and *S. habrochaites* (habr), and blank: empty vessel and **(b)** comparison of response among tomato species. Pie charts on the left side of the figure show proportions of responsive (choice) and non-responsive (no choice) females in the assay. GLM: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and different letters indicate significant differences among treatments ($P < 0.05$)

2.3.3. Oviposition preference

In the no-choice test, female *T. absoluta* laid eggs on all three species (Fig. 4a). However, tomato species significantly affected *T. absoluta* oviposition preference (GLM $\chi^2 = 12.31$; df = 2; $P = 0.002$), as females laid more eggs on *S. lycopersicum* and *S. habrochaites* than on *S. pennellii*. When given a choice, *T. absoluta* females showed a clear preference to oviposit on the cultivated *S. lycopersicum* than on their wild relatives. Thus, females laid 376% and 154% more eggs on *S. lycopersicum* than on *S. pennellii* (GLM $\chi^2 = 78.36$; df = 1; $P < 0.001$) and *S. habrochaites* (GLM $\chi^2 = 10.81$; df = 1; $P = 0.001$), respectively (Fig. 4b).

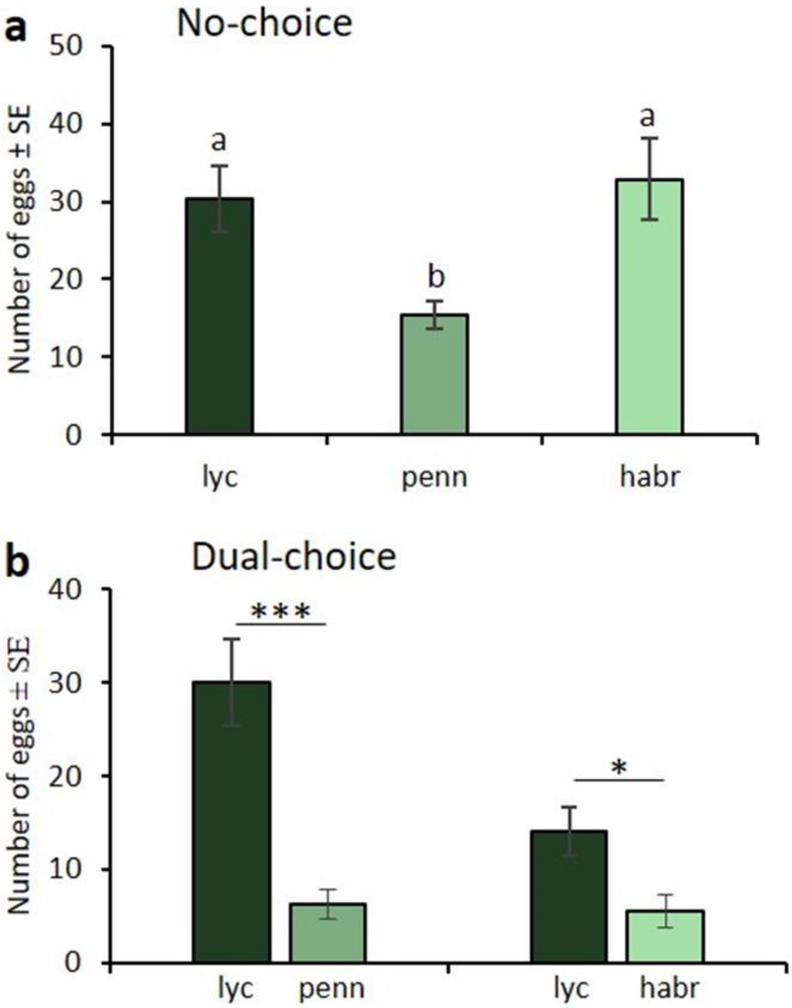

Fig. 4 Number of eggs laid by *Tuta absoluta* (mean ± SE, *n* = 10) on cultivated tomato *Solanum lycopersicum* (lyc), and its wild relatives *Solanum pennellii* (penn), and *Solanum habrochaites* (habr) in a no-choice **(a)**, and dual-choice test **(b)**. Different letters indicate significant differences among treatments. * $P < 0.05$, *** $P < 0.001$

2.3.4. Trichome density

Trichome density varied among tomato species. Regarding glandular trichomes density, both adaxial (GLM $\chi^2 = 324.94$; df = 2; $P < 0.001$) and abaxial (GLM $\chi^2 = 78.67$; df = 2; $P < 0.001$) leaflet surfaces differed among species (Fig. 5a). The wild tomato species *S. pennellii* and *S. habrochaites* produced, respectively, 11 and 5 times more glandular trichomes than the cultivated *S. lycopersicum*. Non-glandular trichome density also significantly varied among the species in both adaxial (GLM $\chi^2 = 247.29$; df = 2; $P < 0.001$) and abaxial (GLM $\chi^2 = 233.84$; df = 2; $P < 0.001$) leaflet surfaces; however, *S. lycopersicum* produced 10 and 14 times more non-glandular trichomes than *S. habrochaites* and *S. pennellii*, respectively (Fig. 5b).

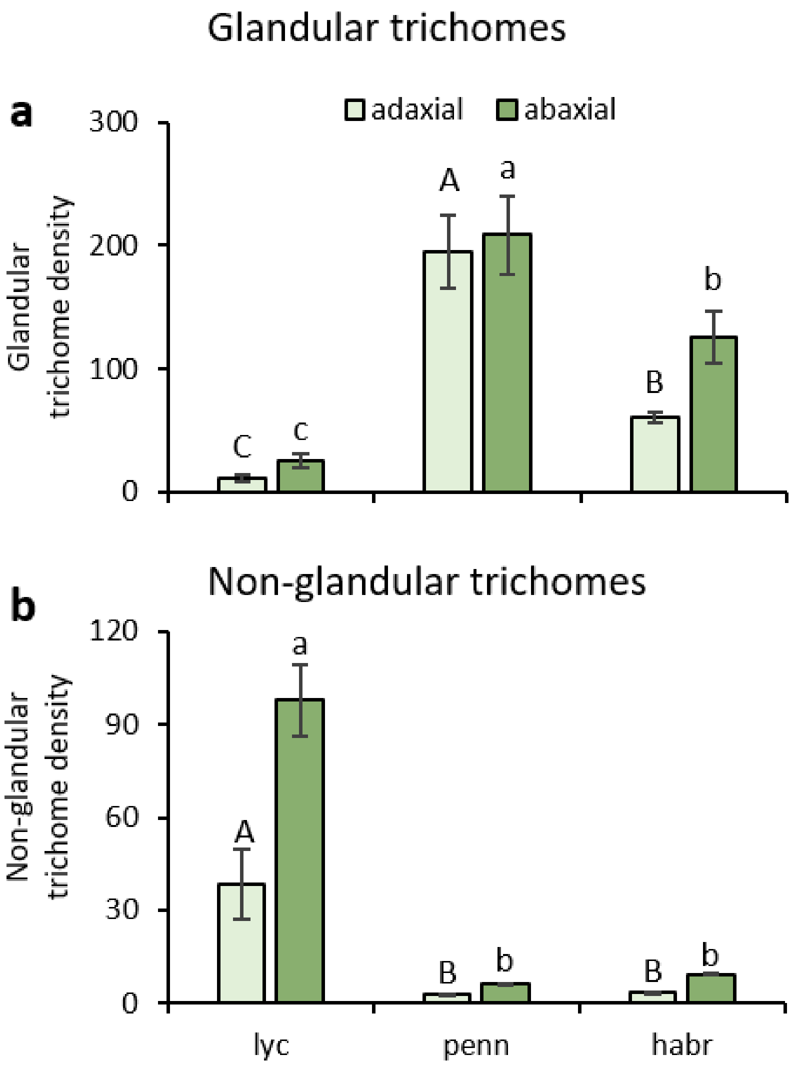

Fig. 5 Density (mean number/15 mm^2 leaf ± SE, $n = 8$) of glandular **(a)**, and non-glandular trichomes **(b)** on adaxial and abaxial surfaces of cultivated tomato *Solanum lycopersicum* (lyc), and its wild relatives *Solanum pennellii* (penn), and *Solanum habrochaites* (habr) leaflets. Means with different letters are statistically different ($P < 0.05$). Capital letters correspond to comparisons on the adaxial surface, while lowercase letters correspond to comparisons on the abaxial surface

2.3.5. Leaf phenolic and nitrogen content

Total phenolics content in leaves was significantly different among tomato species (GLM $\chi^2 = 13.32$; df = 2; $P = 0.0012$). The wild *S. habrochaites* produced the highest level of total phenolics and *S. pennellii* and *S. lycopersicum* had the lowest (Fig. 6a). Foliar nitrogen levels were also significantly different among tomato species (GLM $\chi^2 = 13.06$; df = 2; $P = 0.0054$) and *S. lycopersicum* had 8% and 14% more nitrogen content than *S. pennellii* and *S. habrochaites*, respectively (Fig. 6b).

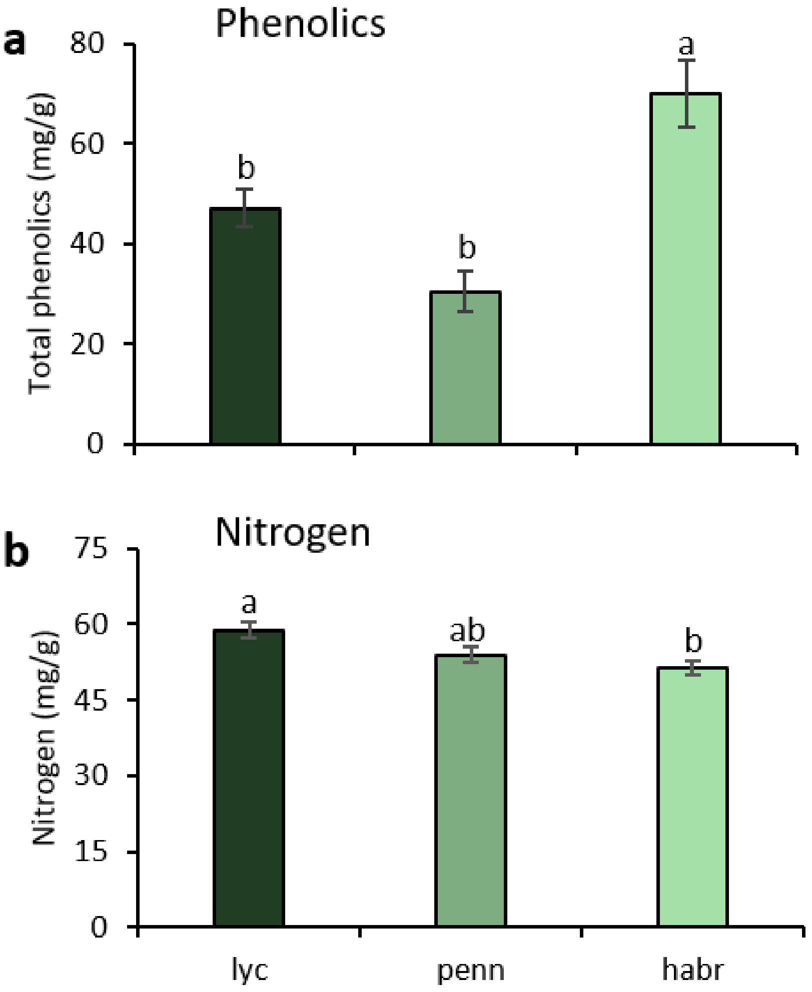

Fig. 6 Total phenolic content (mean mg/g FW ± SE, *n* = 5) **(a)**, and nitrogen concentration (mean mg g^{-1} FW ± SE) **(b)** in the leaves of cultivated tomato *Solanum lycopersicum* (lyc), and its wild relatives *Solanum pennellii* (penn), and *Solanum habrochaites* (habr). Different letters indicate significant differences among treatments (GLM: $P < 0.05$)

2.3.6. Larval performance

There was no difference in larval survival among tomato species (GLM $\chi^2 = 3.41$; df = 2; $P = 0.181$) (Fig. 7a). However, larval developmental time was significantly affected by the tomato plant species on which they fed (GLM $\chi^2 = 34.44$; df = 2; $P < 0.001$). The developmental times were 11% and 6% longer in *S. habrochaites* and *S. pennellii*, respectively, than in *S. lycopersicum* (Fig. 7b). In contrast, tomato plant species did not affect pupal developmental time (GLM $\chi^2 = 3.71$; df = 2; $P = 0.155$) (Fig. 7c), but the pupal weight was significantly affected by tomato plant species (GLM $\chi^2 = 7.37$; df = 2; $P = 0.025$) as the larvae that fed on *S. lycopersicum* resulted in pupae 4% and 15% heavier than those fed on *S. pennellii* and *S. habrochaites*, respectively (Fig. 7d).

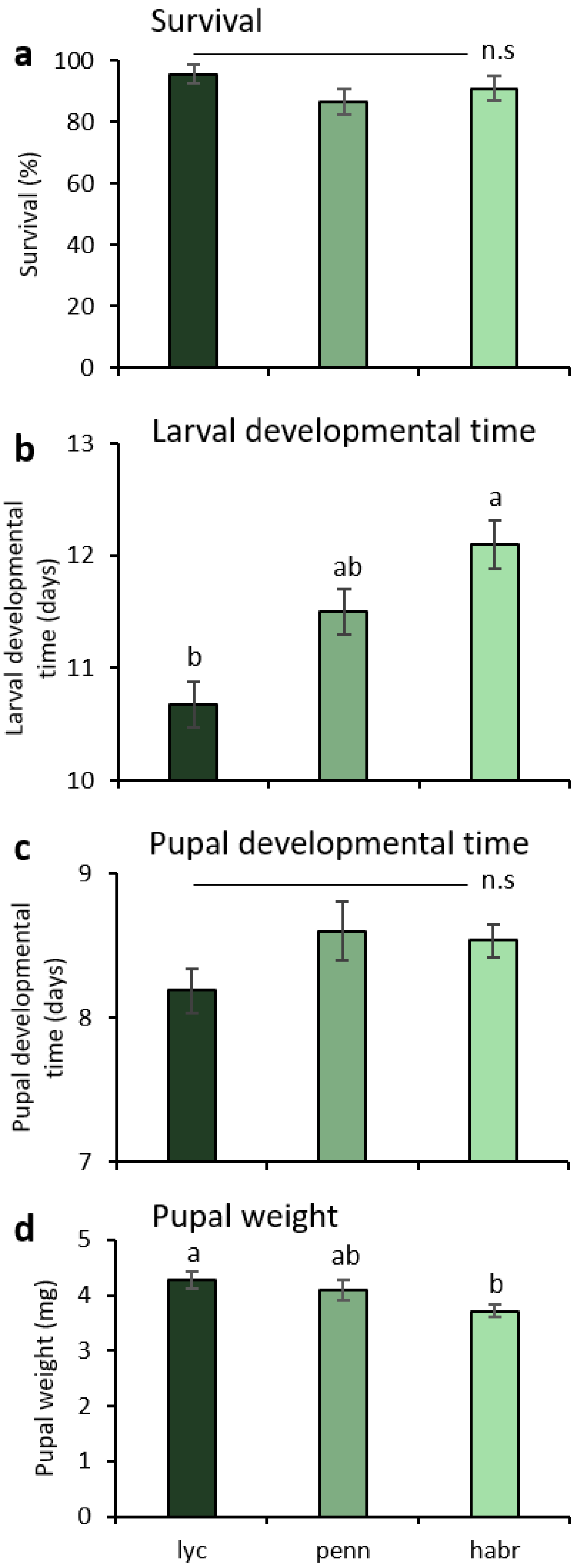

Fig. 7 Larval survival (percent ± SE, *n* = 15) **(a)**, larval developmental time (mean days ± SE) **(b)**, pupal developmental time (mean days ± SE) **(c)**, and pupal weight (mean mg ± SE) **(d)** of *Tuta absoluta* feeding on cultivated tomato *Solanum lycopersicum* (lyc), and its wild relatives *Solanum pennellii* (penn), and *Solanum habrochaites* (habr). Different letters indicate significant differences among treatments (GLM: $P < 0.05$)

2.4. Discussion

Agronomic selection for increased yields has altered plant defense mechanisms, influencing resistance against herbivores in complex and not always predictable ways (Turcotte et al. 2014; Chen et al. 2015). We explored the differences between a modern cultivated tomato and its wild relatives regarding constitutive defensive and nutritional traits, and their effect on the preference (antixenosis) and performance (antibiosis) of the specialist *T. absoluta*. The results corroborate our hypothesis, and four key findings can be drawn from this study: 1) there are remarkable differences in the VOC profiles of cultivated and wild tomato species; 2) enhanced ovipositional preference of *T. absoluta* on the cultivated tomato; 3) low glandular trichome density and total phenolic concentrations and high nitrogen content and non-glandular trichome density in cultivated tomato compared to its wild relatives, and; 4) negative effects of wild tomato on *T. absoluta* larval developmental time and pupal weight.

Chemical analysis of constitutive VOCs revealed a different pattern among wild and cultivated tomato species. The VOCs identified in the species studied here are largely in accordance with those obtained from previous studies (Bleeker et al. 2009; Antonious and Snyder 2015; Anastasaki et al. 2018; Silva et al. 2018; Paudel et al. 2019). Overall, the wild *S. habrochaites* had higher levels of constitutive VOCs compared to the cultivated *S. lycopersicum*; another piece of evidence supporting that cultivated crops have reduced levels of constitutive defensive chemicals compared to their wild relatives (Turcotte et al. 2014; Moreira et al. 2018; Paudel et al. 2019). The same pattern is also true for *S. pimpinellifolium* (Paudel et al. 2019) and *S. lycopersicum* var. *cerasiforme* (Miano et al. 2022). Interestingly, *S. pennellii* had the lowest VOC emission compared to the other species. This species is endemic to arid habitats of the Andean region and this might be an adaptation mechanism to cope with such conditions, as this kind of environment can lead to lower constitutive emission of VOCs (Plaza et al. 2005; Peralta and Spooner 2007; Lavoir et al. 2009). The volatile chemical profile of *S. lycopersicum* was dominated by terpenoids, mainly α-pinene, β-myrcene, 2-carene, limonene, and β-phellandrene. In similar studies with other cultivated genotypes, β-phellandrene, and 2-carene were also the major components of the blend (Bleeker et al. 2009; Paudel et al. 2019; Miano et al. 2022). In contrast, *S. habrochaites* emitted greater levels of methyl ketones. Type IV glandular trichome synthesizes and accumulates high levels of the methyl ketones 2-undecanone, 2-tridecanone, and 2-pentadecanone (Fridman et al. 2005). This kind of glandular trichome is restricted to wild tomato species, such as *S. habrochaites* and *S. pennellii* (Bergau et al. 2015). Indeed, *S. pennellii* also emitted 2-tridecanone, but the cultivated *S. lycopersicum* did not produce detectable amounts of methyl ketones. A previous study has shown that the methyl ketones are a remarkable pheromone antagonist of *T. absoluta* (Dominguez et al. 2016), and also have a repellent effect on the spider mite *Tetranychus urticae* Koch (Antonious and Snyder 2015), but no had effect on the whitefly *B. tabaci* (Bleeker et al. 2009). Regarding *S. pennellii* VOCs profile, the major components were α-phellandrene and γ-terpinene, which were associated with reduced attractiveness for whiteflies to tomato plants (Bleeker et al. 2009).

Domestication/breeding can modify the quality and quantity of VOC emissions (Gols et al. 2011) and affect the attraction and ovipositional acceptance of both generalist and specialist insect herbivores (Kelly and Bowers 2016). Previous studies have shown that the generalist moths *Helicoverpa armigera* (Hübner) and *Manduca sexta* (L.) preferentially oviposit on *S. lycopersicum* compared to its wild relatives (Li et al. 2018; Paudel et al. 2019). The specialist *T. absoluta* is also more attracted and prefers to oviposit on *S. lycopersicum* over the wild tomato *S. habrochaites* (Proffit et al. 2011; Rakha et al. 2017). In line with these patterns, our study demonstrated that female moths oriented preferentially to VOCs emitted by *S. lycopersicum* over *S. pennellii* and *S. habrochaites,* corroborating that *T. absoluta* can recognize and differentiate the volatile emissions of cultivated and wild tomatoes. Females also showed oviposition preference for the cultivated tomato, indicating that in addition to VOCs, visual and/or tactile short-range cues are involved in host selection.

Plant morphological defensive traits can act as physical barriers restricting herbivores' movement and delaying their access to the epidermis (such as non-glandular trichomes), or as appendages to produce toxic metabolites that affect herbivores performance (i.e., glandular trichomes) (Tian et al. 2012; Kariyat et al. 2019). Although plant domestication is assumed to reduce trichome density, this trend has shown inconsistent patterns across crops (Bellota et al. 2013; Turcotte et al. 2014). For example, Paudel et al (2019) found greater levels of constitutive glandular and non-glandular trichome densities in cultivated tomatoes than in the wild *S. lycopersicum* var. *cerasiforme* and *S. pimpinellifolium*. In contrast, our study revealed greater glandular trichome density in both *S. pennellii* and *S. habrochaites* than in the cultivated tomato, but higher non-glandular trichome density in *S. lycopersicum*. Our findings agree with most previous studies reporting higher glandular and lower non-glandular trichome densities in wild tomatoes over cultivated species (Savi et al. 2019; Almeida et al. 2023). Different trichome types are associated with the production of terpenes and methyl ketones which have a great potential to be used in plant breeding for resistance against insect pests (Bleeker et al. 2009; Bergau et al. 2015; Wang et al. 2020).

One of the most important consequences of plant domestication on defenses is the reduction of secondary metabolites that are associated with a decrease in the survival and development of herbivores (Meyer et al. 2012; Chen et al. 2015b). Among these compounds, phenolics play a crucial role in tomato plants' chemical defense, acting as feeding deterrents against herbivores (Isman and Duffey 1982). Several studies on defense losses during domestication/breeding have shown highly variable patterns regarding phenolic content (Parker et al. 2010; Rodriguez-Saona et al. 2019; Jaiswal et al. 2020). For example, Paudel et al. (2019) found a negative effect of domestication on total phenolics content in tomato leaves, while Jaiswal et al. (2020) found no differences in 25 genotypes among wild and cultivated tomatoes. We showed that agronomic selection for increased yields might have affected *S. lycopersicum* total phenolics content, as its wild relative *S. habrochaites* produced higher levels of these compounds. However, *S. pennellii* produced similar amounts of phenolics as *S. lycopersicum*, indicating that there are also variations in defensive traits among wild tomatoes. Although our study focused on total phenolics, we acknowledge that specific phenolic compounds might have different patterns between cultivated and wild species, and further studies are still needed to clarify the implications of domestication on defense losses.

Nitrogen is essential in several plant processes such as photosynthesis. Moreover, it has been positively correlated with amino acid and protein content (Sinclair and Horie 1989), but it often shows an inverse correlation with secondary metabolite concentration (i.e., terpenoids, phenolics, and glycoalkaloids) (Bot et al. 2009; Royer et al. 2013). The cultivated *S. lycopersicum* presented greater leaf nitrogen content than the wild *S. habrochaites*, suggesting that the agronomic selection of tomatoes might have altered the plant's capacity to allocate nutritional resources. Because our study was conducted in a greenhouse under the same soil, fertilization, and watering conditions, intrinsic physiological differences (i.e., uptake, assimilation, and translocation of nutrients) probably led to leaf nitrogen variations between cultivated and wild tomatoes. We expected a possible trade-off between growth and defense in tomato leaves, i.e., nitrogen and phenolic allocation. Corroborating this hypothesis, our results demonstrated that *S. lycopersicum* leaves had a higher constitutive nitrogen level and a lower total phenolics content. This expected chemical divergence may explain the resource allocation strategy favoring the increased biomass and yield in cultivated tomatoes. Similarly, Mondolot et al. (2008) found a lower foliar concentration of tannins (chemical defense) and a lower C/N ratio (an indicator of growth and quality) in leaves of domesticated cassava. Such patterns, however, are not consistent across domestication processes, but changes in defense and nutritional traits could increase crop susceptibility to herbivores attacks (Fernández et al. 2021).

Previous studies have shown that survival and growth of *T. absoluta* in tomato plants can be negatively correlated to high glandular trichomes densities (Sohrabi et al. 2017; Resende et al. 2022), high levels of phenolic compounds (Larbat et al. 2016) and low leaf nitrogen content (Han et al. 2014; Salazar-Mendoza et al. 2023). Here, we found that lower levels of glandular trichome density and total phenolics, and greater nitrogen content in cultivated tomato leaves might have caused the shorting larval developmental time and increased pupal weight of *T. absoluta*. Larvae fed on *S. habrochaites* leaves were more strongly affected than those fed on *S. pennellii*, indicating variability between these two wild tomato species regarding constitutive resistance against *T. absoluta*. Even though native specialist herbivores are usually well adapted and may perform better on their co-evolved wild hosts (Chacón-Fuentes et al. 2015), larvae fed on wild tomatoes had longer developmental time and decreased pupal weight compared to those fed on cultivated tomato. Although other important biological parameters, such as survival and pupal developmental time of *T. absoluta* were not different between cultivated and wild species, female oviposition preference, and larval performance corroborate the preference-performance hypothesis, indicating that chemical and morphological variations in tomato might benefit population outbreaks of this herbivore in agricultural environments.

To summarize, our findings demonstrated that artificial selection for enhanced fruit quality and yield in tomatoes has increased nitrogen content in leaves, and also reduced plant constitutive defensive traits as predicted by the plant domestication-reduced defense hypothesis, which in turn, enhanced *T. absoluta* preference and performance on the cultivated *S. lycopersicum*. As we used a single cultivar/accession to represent each species, we suggest that future studies should include a broader array of cultivars and accessions to bolster the conclusions drawn from our study. Moreover, further studies are still necessary to investigate the role of induced plant defenses mediated by tomato domestication on *T. absoluta* and other herbivore insects and the potential trade-offs between constitutive and induced resistance. Tomato domestication can also affect higher trophic interactions, such as natural enemies of pests through changes in herbivore-induced plant volatiles, which still needs investigation. Despite the several remaining knowledge gaps, our study attests to the importance of assessing the effects of agronomic selection on plant constitutive defense and nutritional traits to better understand resistance mechanisms against herbivores. Addressing these challenges will contribute to achieving the United Nations' sustainable development global goals.

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3. ENHANCING PLANT RESISTANCE TO *Tuta absoluta* IN WILD AND CULTIVATED TOMATOES BY AN ENDOPHYTIC FUNGUS INDIVIDUALLY AND IN COMBINATION WITH A GROWTH-PROMOTING RHIZOBACTERIA

This chapter has been formated for submission according to the guidelines of the journal *Biological Control.*

Abstract

Several microbe species are capable of inducing plant defensive mechanisms that enhance their resistance against herbivores. Therefore, they are considered a promising strategy for pest management in agroecosystems. However, it remains unclear whether different beneficial microbes can work together to enhance defenses in wild plants, similar to how they induce resistance against insect pests in cultivated plants. In this study, we investigated the effect of the endophytic fungus *Metarhizium robertsii,* both when applied individually and in combination with the growth-promoting rhizobacteria *Bacillus amyloliquefaciens,* on plant growth and volatile emissions in the cultivated *Solanum lycopersicum* and its two wild parents, *S. pimpinellifolium* and *S. habrochaites*. We also assessed the ovipositional preference of the devastating pest *Tuta absoluta*, and the olfactory responses of its natural enemy, the predator *Macrolophus basicornis* towards these treatments. Wild and cultivated plants inoculated with *M. robertsii* enhanced growth and emitted higher levels of volatile compounds compared to non-inoculated plants. Furthermore, *Tuta absoluta* females laid fewer eggs on *S. lycopersicum* and *S. habrochaites* inoculated with *M. robertsii.* In addition, the inoculation of this beneficial fungus resulted in increased attraction of *M. basicornis* to *S. lycopersicum* and *S. pimpinellifolium*. Interestingly, the combined inoculation of *B. amyloliquefaciens* and *M. robertsii* did not yield an additive effect on plant growth, volatile emissions, and resistance against *T. absoluta*, when compared to wild and cultivated plants inoculated by *M. robertsii* alone. These results suggest that the application of *M. robertsii* has great potential in protecting tomato plants against *T. absoluta* moths and enhancing attraction of their natural enemy, *M. basicornis.*

Keywords: Bacillus amyloliquefaciens*, Indirect plant defense,* Macrolophus basicornis*,* Metarhizium robertsii*, Microbe-plant-insect interactions, Microbe-induced plant volatiles*

3.1. Introduction

Plants have a close association with a diverse array of microbes, both aboveground and belowground, which significantly influence their growth and reproduction (Finkel et al., 2017; Shikano et al., 2017). Within this microbial community, certain groups of beneficial fungi and bacteria provide essential services within agroecosystems (Pava-Ripoll et al., 2011; Wagg et al., 2019; Ahmad et al., 2020). For instance, several entomopathogenic fungi, which are frequently used in biological control programs, can establish endophytic colonization within plant tissues. This colonization provides additional ecological benefits, including increased resilience against adverse environmental conditions, promotion of plant growth, and enhanced plant protection against biotic stressors such as insect herbivores and pathogens (Lewis 2004; Shikano et al., 2017; Dara 2019). The presence of endophytes can induce or increase the production of essential plant growth-related phytohormones and secondary metabolites (Schulz et al., 2002; Kusari et al., 2013). Consequently, the activation of such defensive mechanisms in plants can affect insects' survival, reducing their growth and reproductive rates, and influencing many other performance parameters (Jaber and Ownley, 2018; Jensen et al., 2020).

In addition to fungal endophytes, plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria commonly found in the rhizosphere of various plant species, and they can also establish endophytic colonization within host plants (Afzal et al., 2019; Dahmani et al., 2020). PGPR have the capacity to synthesize many plant phytohormones and induce systemic resistance, thereby promoting plant growth and stimulating plant defense against herbivores (Pangesti et al., 2015; Asaf et al., 2017; Cappellari et al., 2017; Friman et al., 2021). Combining endophytic fungi and PGPR can offer novel perspectives in integrated pest management (IPM) to reduce the use of synthetic chemical pesticides for control of insect pests (Verma et al., 2019; Vishwakarma et al., 2020). However, interactions among different microbial species, such as synergism or competition, can either enhance or diminish their beneficial effects on host plants (Barea et al., 2005; Wang et al., 2015; Pangesti et al., 2017), and consequently modify plant-insect interactions.

Plants release specific volatile organic compounds (VOCs) constitutively, which function as chemical signals for herbivores to recognize and locate suitable host plants (Freeny et al., 1999). In response to herbivore attacks, plants can change their emission profiles of chemical compounds, known as herbivore-induced plant volatiles (HIPVs). These volatiles serve as detectable cues for a wide range of natural enemies to locate their host or prey (Turlings et al., 1990; Paré and Tumlinson, 1999; Dicke and Baldwin, 2010). Exploring HIPVs has been widely suggested as a promising strategy for enhancing biological control within an IPM framework (Peñaflor and Bento, 2013; Ingrao et al., 2019; Ayelo et al., 2021). Moreover, certain microbes, such as fungal endophytic and PGPR, can also induce changes in plant VOCs (Sharifi et al., 2018; Friman et al., 2021), thereby influencing interactions with their ecological surroundings. Beneficial microbe-induced plant volatiles can enhance plant defensive responses against herbivores, modifying their oviposition behavior (Zhu et al. 2021), and attracting their natural enemies (Pangesti et al., 2015).

Plant domestication has led to significant alterations in morphological and physiological traits in order to maximize crop yields (Meyer et al., 2012; Chen et al., 2015). However, this domestication process has also resulted in a notable reduction in chemical defense mechanisms, often leading to lower resistance to herbivores in modern cultivated varieties (Futuyma and Agrawal, 2009; Turcotte et al., 2014; Whitehead et al., 2017). Specifically, constitutive VOCs emitted by cultivated species have undergone substancial modifications and are strongly associated with increased attraction and acceptance of insect herbivores for oviposition, when compared to their wild counterparts (Paudel et al., 2019; Cloonan et al., 2019; Salazar-Mendoza et al., 2023a). Since inducible VOCs emitted by wild species are typically less complex than cultivated plants (Rowen and Kaplan, 2016), it remains unclear whether beneficial microbes can alter VOCs in wild plants to enhance their resistance against herbivores by modifying their behavior and attracting their natural enemies, as the effects of microbes on host-plant genotype can be highly specific (Long et al., 2008).

The South American tomato pinworm, *Tuta absoluta,* is a highly destructive pest in tomato production worldwide, often resulting in significant yield losses ranging from 80-100% (Biondi et al., 2018; Mansour et al., 2018; Han et al., 2019). In addition, this moth can also lay eggs and develop in other economically important solanaceous crops, as well as their wild relatives (Proffit et al., 2011; Bawin et al., 2016; Sylla et al., 2019). Conventional control methods based on insecticide application are challenging due to the leaf-mining activity of *T. absoluta* larvae whithin the leaf mesophyll (Tropea Garzia et al., 2012; Biondi et al., 2018). Therefore, there is a need for environmentally friendly alternatives for *T. absoluta* control. A promising natural enemy for biological control of *T. absoluta* is the neotropical mirid *Macrolophus basicornis* Stal, known for its high preying and reproductive capacity (Silva et al., 2016; Van Lenteren et al., 2018; Bueno et al., 2023). However, since *M. basicornis* exhibits zoophytophagous habits, relying on the host plant for both feeding and oviposition, any changes in plant chemistry, such as VOCs, caused by beneficial microbe colonization, may influence its behavior and selection.

In this study, we aimed to investigate the effects of the fungal endophyte *Metarhizium robertsii* Bischoff, Rehner & Humber (Hypocreales: Clavicipitaceae), both alone and in combination with the PGPR *Bacillus amyloliquefaciens* (Fukumoto) (Bacillales: Bacillaceae), on the growth of wild and cultivated tomato plants. Additionally, we examined the influence of these microbial species on the emission of VOC emissions by plants. We hypothesized that these microbial inoculations would impact plant growth and VOC emission, subsequently affecting the ovipositional preference of *T. absoluta*, and the attraction of its predator, *M. basicornis*. Previous studies have shown the beneficial effects of *M. robertsii* on plant growth promotion (Siqueira et al., 2020) and induced resistance against *T. absoluta* by *B. amyloliquefaciens* (Magalhães et al. 2023) in tomato. Specifically, we asked the following questions: (1) Can *M. robertsii* enhance plant growth and induce VOC emissions to provide protection, both directly and indirectly, against *T. absoluta* in wild and cultivated tomatoes? (2) Does the combination of *M. roberstii* with *B. amyloliquefaciens* further improve plant growth and VOC emissions, enhancing protection against *T. absoluta* in wild and cultivated tomatoes? By investigating how beneficial microbes can modulate the plant defensive responses to alter plant-insect interactions in tomatoes and their wild relatives, our study aims to provide practical insights for biological control strategies targeting *T. absoluta*.

3.2. Materials and Methods

3.2.1. Bacterial and fungal suspensions

The microorganisms were selected from the collection "Prof. Sérgio Batista Alves" in the "Laboratory of Pathology and Microbial Control of Insects" at USP/ESALQ (Piracicaba, SP, Brazil). The PGPR *B. amyloliquefaciens* strain GB03 was streaked onto Tryptic Soy Agar (TSA) plates and incubated at at 25±1 °C in darkness for 48 h. After, *B. amyloliquefaciens* colonies were transferred from TSA plates into Falcon tubes with 5 mL of Tryptic Soy Broth (TSB) medium and cultured under 25±1 °C and 150 rpm for 48 h. The final bacterial suspension concentration was measured by optical density (OD_{600} = 0.7) and adjusted to yield 10^9 colony forming units mL^{-1} through a series of dilutions.

Metarhizium robertsii strain ESALQ 1635 was cultivated in petri dishes (60 x 10 mm) containing potato dextrose agar (Difco®) supplemented with Pentabiotic® (5mg/L) and Derosal® (10 µl/L). After 14 days, conidial suspensions were collected and kept in a sterile aqueous solution containing 0.05% Tween® 80. The fungal concentration was adjusted to a suspension of 10^8 conidia mL^{-1} by quantifying the number of conidia using a Neubauer chamber. An aliquot of each suspension was sampled to determine conidial viability, as previously described by Oliveira et al. 2015.

3.2.2. Plants and treatments

We grew the cultivated tomato *Solanum lycopersicum* L. cv. Santa Clara, which is a modern variety widely cultivated in Brazil, along with its two wild relatives: the sister ancestor *Solanum pimpinellifolium* (Jusl.) Mill (accession PI126931), and the relative *Solanum habrochaites* S. Knapp & D. M. Spooner (accession PI134417). Seeds of *S. lycopersicum* were obtained from Isla Sementes Ltda. (Porto Alegre, RS, Brazil), while the seeds of the wild species were kindly provided by Dr. André Luiz Lourenção from the active germplasm bank of the Agronomic Institute (Campinas, SP, Brazil). All seeds were washed once with a solution of sodium hypochlorite (50% aqueous solution) and distilled water (1:1) for 5 min. Then, they were rinsed three times with distilled water under laboratory conditions. These tomato species were subjected to different inoculation treatments: inoculated once with *M. robertsii* only (MR) (Siqueira et al., 2020), co-inoculated with B. *amyloliquefaciens* twice (Magalhães et al., 2023) and once with *M. robertsii* (BA+MR), or mock-inoculated (Control) (Fig. 1). For BA+MR treatments, sterilized seeds were immersed in a suspension of *B. amyloliquefaciens* for 60 min, while seeds of MR treatments and control were immersed in a suspension solution consisting of water and 0.05% of Tween 80 (v/v). Subsequently, the seeds were sown in plug trays filled with Basaplant® soil (Base Agro, Artur Nogueira, SP, Brazil). Bacterial inoculation was carried out immediately after sowing by applying 1mL of the respective solution. When the seedlings reached 5–7 cm high (15 days after sowing), they were transferred to 1.9-L plastic pots containing Basaplant® soil and coconut fiber (5:1). One day after transplanting, a second bacterial inoculation was performed at the base of each seedling for BA+MR treatment (Fig. 1). Two days after transplanting, a 1 mL-fungal inoculation was applied to both MR and BA+MR treatments (Fig. 1). The plants were maintained in a greenhouse under natural light and temperature conditions (25 ± 5.5 °C). They were irrigated as needed and used in experiments 32-36 days after sowing, with similar height among all treatment groups.

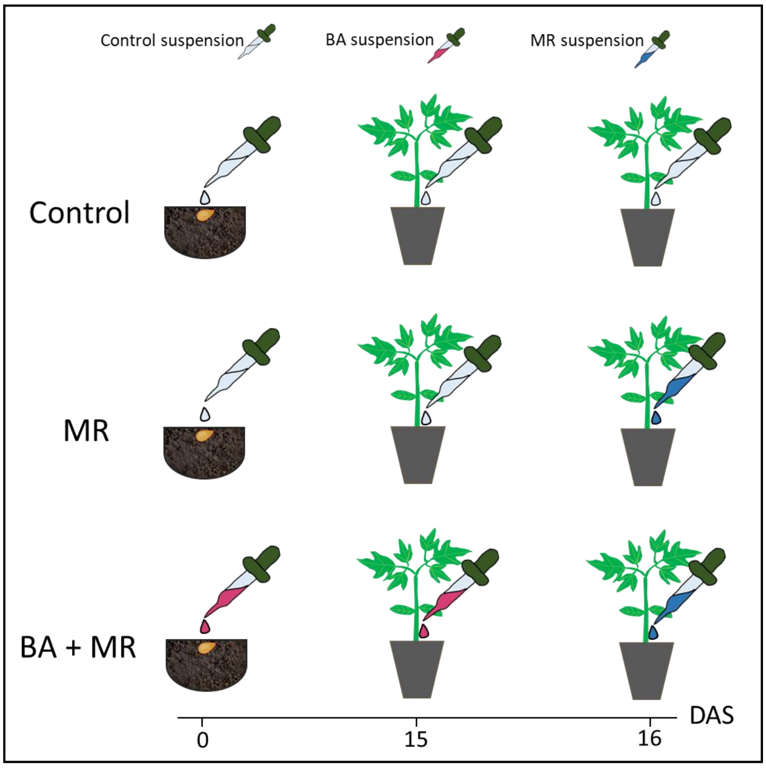

Figure 1. Protocol schedule of the tomato plant inoculation by beneficial microbes. The top section shows the suspension solutions used for plant inoculation. The control solution consisted of water and Tween 80 at 0.05%. MR: represents a suspension of the endophytic fungus *Metarhizium robertsii*. BA + MR: consists of two suspensions; the first one involves the application of the growth-promoting rhizobacteria *Bacillus amyloliquefaciens* twice, and second one involves the application of *M. robertsii* once. DAS: days after seeding.

3.2.3. Insects

Colonies of *T. absoluta* and *M. basicornis* were maintained in the Department of Entomology and Acarology at USP/ESALQ (Piracicaba, SP, Brazil), under controlled conditions (25 ± 5 °C, 70 ± 5% RH, 12:12 L:D). *Tuta absoluta* colony originated from larvae and pupae collected from commercial tomato farms (Paulínia, SP, Brazil). Larvae were reared on tomato plants, cv. Santa Cruz, in cages (60 × 30 × 30 cm) covered with fine nylon mesh separate from the adults. An aqueous honey solution (10%) was provided as a food source for adults on a small ball of cotton wool inside the cage. *Macrolophus basicornis* colony initiated in 2011 from nymphs and adults collected from commercial tobacco farms (Ribeirão Vermelho, MG, Brazil). Nymphs and adults were kept in separate cages (50 x 30 x 30) on tobacco plants, cv. TNN, and were fed with frozen eggs of *Ephestia kuehniella* (Zeller) sprinkled on tobacco leaves.

3.2.4. Determination of *M. robertsii* colonization on tomato plants

All plant treatments were collected, and to remove soil particles, they were washed with distilled water. Subsequently, they were cut into small fragments (2-3 cm) to detect the presence of *M. robertsii*. These plant samples (roots, stems and leaves), underwent surface sterilization by immersion in 70% ethanol for 1 min, followed by 1% sodium hypochlorite for 2 min and 70% ethanol for 1 min. Afterward, the samples were rinsed three times with distilled water and dried on sterile filter paper. The sterilized plant samples were then individually placed in Petri dishes (9 x 1.5 cm) containing 20 ml of PDA supplemented with 0.5 g/L of cycloheximide, 0.2 g/L of chloramphenicol, 0.5 g/L of Dodine (65%), and 0.01 g/L of Crystal Violet following the method described in Behie et al. (2015). The Petri dishes were incubated in darkness at 25 °C for 15 days. After the incubation period, the presence of *M. robertsii* fungal colinazation was determined visually observing the emergence of white hyphal growth from the plant sections.

3.2.5. Plant growth

Aboveground plants (n = 12) from each of the three treatment in all tomato species were harvested from the pots and placed in paper bags. They were dried in a drying oven (Quimis Q317M32, São Paulo, Brazil) at 60 °C for 72 h. After drying, samples were weighed to determine differences in plant growth among treatments within each specie.

3.2.6. Oviposition preference

We conducted free-choice and non-choice tests to compare the oviposition preference of *T. absoluta* among non-inoculated plants, plants inoculated with MR, and plants inoculated with BA+MR from each tomato species. In the free-choice test, plants from each treatment were placed 0.3 m apart in metal cages (110 × 70 × 50 cm) under controlled conditions (25 ± 5 °C, 70 ± 5% RH, 12:12 L:D). For the non-choice test, a single plant from each treatment was placed in the center of the cage. Six and two coupled females (2-3-day-old), respectively, were released into the cage and allowed access to the plants for 24 h. The number of eggs laid on the plants was then counted. The experiment was replicated 12 times.

3.2.7. Olfactometer assays

To determine the preference of *M. basicornis* for the VOCs emitted by both wild and cultivated plants, non-inoculated and inoculated with the microorganims, we conducted a choice experiment using a glass Y-tube olfactometer (4 cm in diameter, main arm 9 cm long, side arms 10 cm long, 70° angle between side arms). The olfactometer was positioned vertically, and each of the two arms was connected to a cylindrical glass vessel (50 cm width × 36 cm height) via Teflon tubing (5 mm diameter x 40 cm length). An ARS volatile collection system (Analytical Research System, Gainesville, FL, USA) was used to provide a constant airflow (0.8 L/min/arm) over the plant in the glass chamber, which then entered the arms of the olfactometer. To prevent visual detection of plants by *M. basicornis*, the glass vessels were kept behind a black panel. Nine supplemental 120-cm lights (GreenPower LED DR/W 18 W, Philips, Amsterdam, Netherlands) were positioned 50 cm above the olfactometer to provide uniform light (total light intensity of 40 µmoles m^{-2} s^{-1}) throughout the test period. The assay was conducted in a dark room (25 ± 2 °C and 70 ± 10% RH) between 10:00 and 14:00 h.

This choice assay was conducted using the following combinations to each tomato specie: (*i*) blank (empty glass chamber) vs non-inoculated plant, (*ii*) blank vs MR-inoculated plant, (*iii*) blank vs BA+MR-inoculated plant, (*iv*) non-inoculated plant vs MR-inoculated plant, (*v*) non-inoculated plant vs BA+MR-inoculated plant, and (*vi*) MR-inoculated vs BA+MR-inoculated plant. For each trial, a single female *M. basicornis*, aged 4-10 days was starved for 2 h. The female was then introduced into the main arm of the olfactometer, and observed for up to 10 min. A female was considered to have made a choice when it crossed a line drawn 8 cm from the branching point of the Y-tube olfactometer. Females that did not choose a side arm within 10 min were considered non-responsive and excluded from the statistical analysis. Each female was tested only once, and after five replicates, the plant combinations were replaced. To avoid positional bias, the side of odors in the olfactometer was rotated between trials, and the olfactometer was cleaned with water and ethanol after each trial. Females were assayed until a total of 30 individuals responded (positive chemotaxis).

3.2.8. Collection and analysis of plant volatile emissions

Plant volatiles were collected from *S. lycopersicum*, *S. pimpinellifolium* and *S. habrochaites*, both non-inoculated and inoculated with MR and BA+MR (n = 6), for 8 h during the daytime under laboratory conditions (25 ± 1 °C, 70 ± 10% RH). The plastic pots containing the plants and soil were wrapped with aluminum foil to reduce trapping volatiles from these sources. Then, plants were individually enclosed in a glass chamber (50 cm width × 36 cm height) and connected to a volatile collection system (Analytical Research Systems Inc., Gainesville, FL, USA). Charcoal-filtered and humidified air was pumped into the chamber at 0.8 L/min and drawn out at 0.6 L/min through an adsorbent polymer trap (50 mg, Hayesep Q, Alltech Associates, Bannockburn, IL, USA) connected to the system via PTFE tubing. After collection, the adsorbent tubes were eluted with 0.5 ml of distilled hexane, and the headspace samples were concentrated to 50 µL under a gentle N_2 flow. Samples were stored in glass vials at −30 °C until analysis.

Tomato volatiles were analyzed on a Shimadzu GC-2010 equipped with a non-polar Rtx-1 column (25 mm × 30 m × 25 µm, RESTEK, Bellefonte, PA, USA) and a flame ionization detector (GC-FID) operating at 270 °C. The oven temperature was programmed to start at 50 °C for 2 min, then increased at 5 °C/min to 180 °C, held for 0.1 min, then 10 °C/min to 250 °C, and held for 20 min. The injector was at 250 °C. Nonyl acetate (Sigma Aldrich, St. Louis, MO, USA) was added as an internal standard (10 µL of a 10 ng/µL solution) to each sample. A 2-µL aliquot of each sample was injected on a splitless injector, with helium as the carrier gas (24 cm/s). The relative amount of the compounds in each sample was determined by comparing their peak area with that of the internal standard, and results were standardized per unit of dry shoot biomass (g) for each replicate. Data were collected with GC Solution.

For volatile identification, the two most representative samples of each treatment in all tomato species were further analyzed on a Shimadzu GCQP-2010 Ultra quadrupole mass spectrometer coupled to a gas chromatograph (Shimadzu GC2010) equipped with a non-polar Rxi-1MS column (25 mm × 30 m × 25 µm, RESTEK, Bellefonte, PA, USA), a splitless injector and helium as the carrier gas. Ionization was by electron impact (70 eV, source temperature at 250 °C). The injector was at 250 °C using the same temperature program as in GC-FID analysis. Data were collected with GCMS Solution. Identifications were made by comparing the mass spectra with the mass spectral library database (NIST11), and Kováts retention index. Authentic standards were used to confirm the identities of α-pinene, β-myrcene, β-pinene, α-phellandrene, limonene, o-cymene, γ-terpinene, α-humulene, β-caryophyllene, and 2-undecanone (Sigma-Aldrich, Merck KGA, St. Louis, Missouri, USA); and (*E, E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT; synthesized and kindly donated by Dr. Michael A. Birkett from Rothamsted Research, Harpenden, UK).

3.2.9. Data analyses

For the olfactometer experiments, the preference of *M. basicornis* for plant volatiles was analyzed using a generalized linear model (GLM) with a quasi-Binomial distribution and log link function. For the oviposition test, the number of eggs laid by *T. absoluta* moths in both the free-choice and non-choice tests were analyzed using a GLM with a quasi-Poisson distribution and log link function. To compare the effects of treatments on plant growth and VOCs emission, a GLM with a Gamma distribution and log link function was used. The assessment of goodness-of-fit was conducted by employing half-normal plots with a simulated envelope, using the "hnp" package. Prior the analyzes, all data were checked for normality of variances using the Shapiro-Wilk test and homogeneity of variances using the Levene test. All data analyses were performed using R software version 3.02.3 (R Core Team 2022).

3.3. Results

3.3.1. Endophytic colonization by *Metarhizium robertsii* on tomato plants

Microbiological techniques confirmed endophytic colonization by *M. robertsii* in leaf tissue, stems, and roots of tomato plant species that had received either single or combined inoculation with *B. amyloliquefaciens*. In contrast, no colonization of *M. robertsii* was observed in any of the samples collected from the control plants.

3.3.2. Plant growth

The inoculation of plants with MR and BA+MR had a significant effect on the dry weight of *S. lycopersicum* (GLM χ^2 = 7.09; df = 2, 35; P = 0.028), *S. pimpinellifolium* (GLM χ^2 = 6.17; df = 2, 35; P = 0.045), and *S. habrochaites* (GLM χ^2 = 7.87; df = 2, 35; P = 0.019). In *S. lycopersicum,* the dry weight of plants inoculated with MR and BA+MR was 24% and 35% higher, respectively, compared to non-inoculated plants (Fig. 2A). In *S. pimpinellifolium*, plants inoculated with MR and BA+MR exhibited a dry weight that was 14% and 30% higher, respectively, than non-inoculated plants (Fig. 2B). Similarly, *S. habrochaites,* the dry weight of plants inoculated with MR and BA+MR was 25% and 36% higher than non-inoculated plants (Fig. 2C).

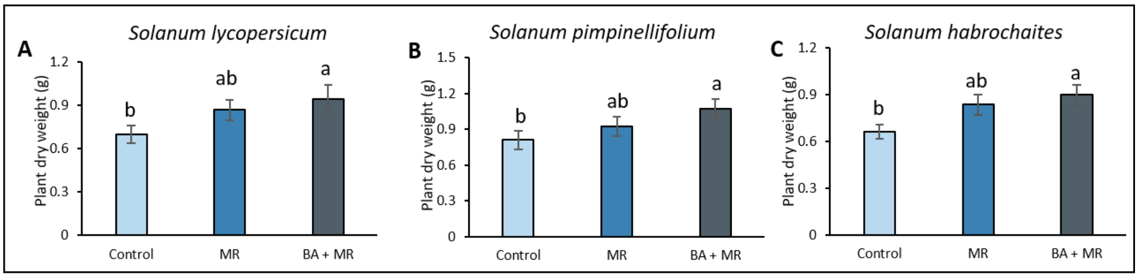

Figure 2. Plant dry weight (mean g ± SE, n = 12) of non-inoculated plants (control), plants inoculated with *Metarhizium robertsii* (MR), and co-inoculated with *Bacillus amyloliquefaciens* and *M. robertsii* (BA+MR) in the cultivated tomato *Solanum lycopersicum* (A), and its wild relatives *S. pimpinellifolium* (B), and *S. habrochaites* (C). Different letters indicate significant differences among treatments (GLM: $P < 0.05$).

3.3.3. Oviposition preference

In the non-choice test, the presence or absence of inoculations with MR or BA+MR did not significantly affect *T. absoluta* oviposition preference in *S. lycopersicum* (GLM χ^2 = 2.05; df = 2, 35; P = 0.358) (Fig 3.A), *S. pimpinellifolium* (GLM χ^2 = 0.59; df = 2, 35; P = 0.744) (Fig. 3B), or *S. habrochaites* (GLM χ^2 = 1.30; df = 2, 35; P = 0.521) (Fig. 3C). However, in the choice-test, *T. absoluta* females clearly showed a preference for ovipositing on non-inoculated plants compared to those inoculated with MR and BA+MR in *S. lycopersicum* (GLM χ^2 = 7.09; df = 2, 35; P = 0.028) (Fig. 3D), and *S. habrochaites* (GLM χ^2 = 7.87; df = 2, 35; P = 0.019) (Fig. 3F), although no significant effect was observed in *S. pimpinellifolium* (GLM χ^2 = 4.02; df = 2, 35; P = 0.133) (Fig. 3E). Females laid 58% and 80% more eggs on non-inoculated *S. lycopersicum* plants than on those inoculated with MR and BA+MR (Fig. 3D). Similarly, females laid 41% and 131% more eggs on non-inoculated *S. habrochaites* plants than on treated plants with MR and BA+MR (Fig 3F).

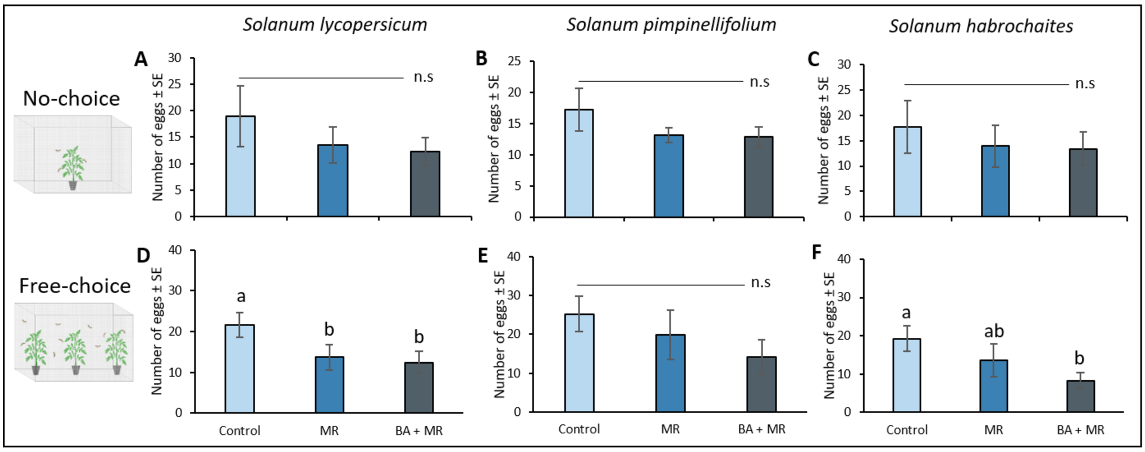

Figure 3. Number of eggs laid by *Tuta absoluta* (mean ± SE, n = 10) on control (non-inoculated plants), plants inoculated with *Metarhizium robertsii* (MR), and plants co-inoculated with *Bacillus amyloliquefaciens* and *M. robertsii* (BA+MR) in the cultivated tomato *Solanum lycopersicum* (A, D), and its wild relatives *S. pimpinellifolium* (B, E), and *S. habrochaites* (C, F) in no-choice and free-choice tests. Different letters indicate significant differences among treatments (GLM: $P < 0.05$). n = 10.

3.3.4. Response of *Macrolophus basicornis* to plant VOC emissions

Macrolophus basicornis females discriminated among the odors from blank and non-inoculated (GLM χ^2 = 9.04; df = 1, 29; P = 0.0026), MR-inoculated (GLM χ^2 = 6.40; df = 1, 29; P = 0.011), and BA+MR-inoculated *S. lycopersicum* plants (GLM χ^2 = 9.04; df = 1, 29; P = 0.0026) (Fig. 4A). They showed a preference for odors from BA+MR-inoculated compared to non-inoculated *S. lycopersicum* (GLM χ^2 = 7.45; P = 0.0060), but did not exhibit a preference between odors from non-inoculated and MR-inoculated plants (GLM χ^2 = 1.06; df = 1, 29; P = 0.303), or between MR-inoculated and BA+MR-inoculated *S. lycopersicum* (GLM χ^2 = 0.26; df = 1, 29; P = 0.605) (Fig. 4A).

In *S. pimpinellifolium*, *M. basicornis* females showed a greater preference for odors from BA+MR-inoculated compared to blank (GLM χ^2 = 6.79; df = 1, 29; P = 0.0091), but did not show a preference between odors from non-inoculated plants and blank (GLM χ^2 = 2.36; df = 1, 29; P = 0.123), or between odours from MR-inoculated and blank (GLM χ^2 = 1.06; df = 1, 29; P = 0.303) (Fig. 4B). They preferred odors from MR-inoculated (GLM χ^2 = 4.16; df = 1, 29; P = 0.041) and BA+MR-inoculated plants (GLM χ^2 = 6.40; df = 1, 29; P = 0.0011) over non-inoculated plants (Fig. 4B). There was no significant difference in female attraction between MR-inoculated and BA+MR-inoculated plants (GLM χ^2 = 0.266; df = 1, 29; P = 0.605) (Fig. 4B).

Non-inoculated *S. habrochaites* plants (GLM χ^2 = 9.04; df = 1, 29; P = 0.0026) and MR-inoculated plants (GLM χ^2 = 6.40; df = 1, 29; P = 0.011) were more attractive to *M. basicornis* than the blank, but there was no preference observed for BA+MR-inoculated and the blank (GLM χ^2 = 0.26; df = 1, 29; P = 0.600) (Fig. 4C). The predator was more attracted to odors emitted by MR-inoculated plants compared to BA+MR-inoculated plants (GLM χ^2 = 8.52; df = 1, 29; P = 0.0035) (Fig. 4C). However, there was no significant difference between MR-inoculated plants (GLM χ^2 = 2.36; df = 1, 29; P = 0.12) and BA+MR-inoculated plants (GLM χ^2 = 2.36; df = 1, 29; P = 0.12) when compared to non-inoculated plants (Fig. 4C).

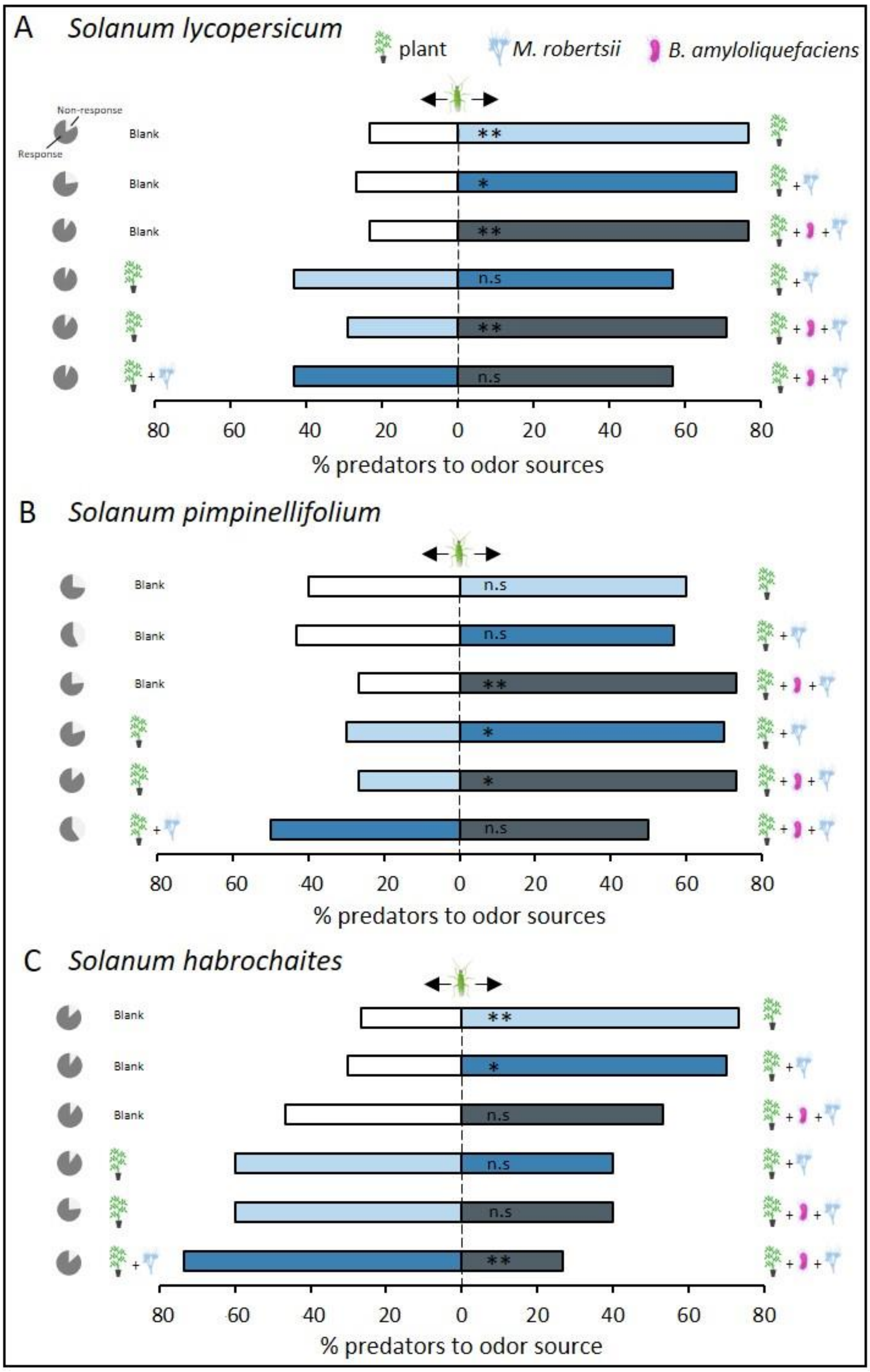

Figure 4. Response of *Macrolophus basicornis* females to volatiles from different treatments: blank (without plant), non-inoculated plants (control), plants inoculated with *Metarhizium robertsii*, and plants co-inoculated with *Bacillus amyloliquefaciens* and *M. robertsii* in the cultivated tomato *Solanum lycopersicum* (A), and its wild relatives *Solanum pimpinellifolium* (B), and *Solanum habrochaites* (C). Different letters indicate significant differences among treatments (GLM: $P < 0.05$). *$P < 0.05$, ** $P < 0.01$, n.s $P > 0.05$.

3.3.5. Plant VOC emissions

Chemical analyses of plant VOCs revealed no qualitative differences among compounds emitted during daytime from non-inoculated, MR-inoculated, and BA+MR-inoculated in all tomato species. The microorganisms inoculations increased the total VOCs emission in all three species. In *S. lycopersicum,* we detected the same 13 major terpene compounds in all treatments (Table 1). However, 2-carene, α-phellandrene, o-cymene, and β-phellandrene were quantitatively emitted at lower levels in non-treated plants compared to the other treatments (Table 1).

Table 1. Concentration of volatile organic compounds (mean ng/g DW ± SE) emitted during daytime by non-inoculated (Control), *Metarhizium robertsii*-inoculated (MR), and *Bacillus amyloliquefaciens* and *M. robertsii*-inoculated (BA+MR) *Solanum lycopersicum*. Means with different letters indicate a significant difference among treatments (GLM, $P < 0.05$).

Compounds	Control	MR	BA+MR	GLM	*P*
α-Pinene	4.18 ± 1.29	7.63 ± 3.01	5.96 ± 1.85	1.544	0.462
β-Pinene	4.09 ± 2.20	5.48 ± 3.40	3.28 ± 2.18	0.358	0.836
β-Myrcene	1.80 ± 0.52	1.24 ± 0.07	1.68 ± 0.47	1.362	0.506
2-Carene	9.10 ± 2.43 b	18.43 ± 5.70 a	23.63 ± 5.13 a	6.276	**0.043**
α-Phellandrene	1.76 ± 0.26 b	3.54 ± 0.96 a	4.02 ± 1.03 a	6.754	**0.034**
α-Terpinene	0.25 ± 0.14	1.14 ± 0.69	0.78 ± 0.31	3.881	0.143
o-Cymene	1.33 ± 0.77 b	5.53 ± 3.51 a	0.74 ± 0.22 b	7.902	**0.019**
β-Phellandrene	21.36 ± 5.18 b	47.45 ± 14.96 ab	61.40 ± 14.25 a	7.726	**0.021**
Limonene	15.69 ± 6.39	17.85 ± 5.04	17.34 ± 3.93	0.091	0.955
β-Ocimene	0.35 ± 0.14	0.46 ± 0.12	0.66 ± 0.14	2.296	0.317
γ-Terpinene	0.73 ± 0.35	1.26 ± 0.70	0.61 ± 0.11	1.555	0.459
β-Caryophyllene	0.68 ± 0.13	0.60 ± 0.25	1.35 ± 0.28	4.749	0.093
α-Humulene	0.06 ± 0.03	0.13 ± 0.06	0.14 ± 0.06	2.272	0.321
Total	61.37 ± 10.59	110.73 ± 28.08	121.58 ± 22.37	5.959	0.051

Similarly, ten VOCs were detected in *S. pimpinellifolium*, but only the emission of α-terpinene was lower in non-inoculated plants (Table 2).

Table 2. Concentration of volatile organic compounds (mean ng/g DW ± SE) emitted during daytime by non-inoculated (Control), *Metarhizium robertsii*-inoculated (MR), and *Bacillus amyloliquefaciens* and *M. robertsii*-inoculated (BA+MR) *Solanum pimpinellifolium*. Means with different letters indicate a significant difference among treatments (GLM, $P < 0.05$).

Compounds	Control	MR	BA+MR	GLM	*P*
α-Pinene	3.05 ± 0.88	6.03 ± 2.72	5.97 ± 2.47	1.833	0.399
4-Carene	18.78 ± 6.69	51.33 ± 31.61	46.76 ± 28.06	1.879	0.391
α-Phellandrene	3.26 ± 1.21	10.56 ± 6.57	10.22 ± 6.30	2.524	0.283
α-Terpinene	0.07 ± 0.03 b	0.31 ± 0.06 a	0.24 ± 0.06 a	11.58	**0.003**
o-Cymene	0.94 ± 0.12	2.53 ± 1.12	2.60 ± 1.07	4.586	0.101
β-Phellandrene	45.63 ± 15.68	138.01 ± 88.25	129.82 ± 81.41	2.172	0.337
Limonene	10.82 ± 3.86	24.38 ± 11.81	17.98 ± 7.62	1.781	0.411
β-Caryophyllene	1.96 ± 1.26	3.36 ± 1.85	3.25 ± 1.56	0.545	0.762
α-Humulene	0.36 ± 0.25	0.53 ± 0.25	0.50 ± 0.24	0.263	0.877
TMTT	0.88 ± 0.54	2.77 ± 1.19	1.34 ± 0.49	2.971	0.226
Total	85.75 ± 28.82	239.83 ± 143.10	218.68 ± 127.79	2.098	0.351

In *Solanum habrochaites*, 12 detectable VOCs were identified. Among them, the terpenes β-caryophyllene and α-humulene, as well as the methyl ketones 2-undecanone and 2-pentadecanone, were emitted at higher levels in plants inoculated with MR and BA+MR compared to non-inoculated plants (Table 3).

Table 3. Concentration of volatile organic compounds (mean ng/g DW ± SE) emitted during daytime by non-inoculated (Control), *Metarhizium robertsii*-inoculated (MR), and *Bacillus amyloliquefaciens* and *M. robertsii*-inoculated (BA+MR) *Solanum habrochaites*. Means with different letters indicate a significant difference among treatments (GLM, $P < 0.05$).

Compounds	Control	MR	BA+MR	GLM	*P*
α-Pinene	0.60 ± 0.46	0.76 ± 0.42	0.82 ± 0.43	0.137	0.934
Methyl salicylate	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	1.821	0.402
α-Copaene	0.67 ± 0.53	0.89 ± 0.43	0.57 ± 0.29	0.281	0.869
2-Undecanone	12.82 ± 3.12 b	29.27 ± 6.71 ab	43.96 ± 21.59 a	6.087	**0.047**
2-Dodecanone	0.15 ± 0.05	0.51 ± 0.22	0.55 ± 0.33	3.883	0.143
β-Caryophyllene	9.64 ± 2.85 b	30.31 ± 6.21 a	23.02 ± 7.28 a	8.326	**0.015**
α-Humulene	1.39 ± 0.40 b	4.05 ± 0.76 a	3.07 ± 0.91 a	8.216	**0.016**
2-Tridecanone	139.35 ± 34.84	294.07 ± 63.12	327.72 ± 122.29	4.736	0.094
TMTT	0.63 ± 0.18	1.34 ± 0.20	1.10 ± 0.24	5.469	0.0649
Tetradecanone	0.91 ± 0.78	0.20 ± 0.14	0.34 ± 0.30	1.789	0.409
2-Pentadecanone	3.38 ± 0.92 b	7.12 ± 1.33 a	7.86 ± 2.17 a	6.212	**0.045**
2-Nonadecanone	0.94 ± 0.44	1.46 ± 0.43	1.45 ± 0.53	0.808	0.668
Total	170.46 ± 40.59	370.00 ± 74.86	410.44 ± 152.83	5.26	0.072

3.4. Discussion

Although plant species often interact with multiple microbes belowground and aboveground, which can influence their growth and interactions with herbivores, most studies on plant defense have primarily focused on investigating these interactions using single beneficial microbe species (Pangesti et al., 2015). However, the rhizosphere's synergistic and competitive interactions among different microbe species can have diverse and unpredictable effects on host plants, with significant implications for agroecosystem management (Gadhave et al., 2016; Jaber and Ownley, 2018). Here, we explored the effects of the endophytic fungus *M. robertsii*, when inoculated alone and in combination with the PGRP *B. amyloliquefaciens* on promoting plant growth and enhancing resistance against herbivores in wild and cultivated tomatoes. Four key findings on the effects of microbe inoculation on tomato plants and their interactions with insects can be drawn from our study: (1) Inoculation with *M. robertsii* promoted plant growth and increased the emission of various VOCs in both cultivated and wild tomatoes; (2) *M. robertsi*-inoculated plants exhibited a reduced ovipositional preference by *T. absoluta* in *S. lycopersicum* and in the wild *S. habrochaites*; (3) *M. robertsi*-inoculated plants increased the attraction of the predator *M. basicornis* in *S. lycopersicum* and in the wild *S. pimpinellifolium*; and (4) Overall, combined inoculation with both microbe species did not enhance plant growth or the defensive response against *T. absoluta* compared to single *M. robertsii* inoculation in both wild and cultivated tomatoes.

A previous study has shown that plants inoculated with the same strain of *M. robertsii* exhibited increased plant height, root length, and dry weight in the dwarf tomato Micro-Tom (*Solanum lycopersicum* cv. Micro-Tom)

(Siqueira et al., 2020). This finding was expected, as the authors also found that *M. robertsii* induced the expression of hormone-regulated gene and increased the production of enzymes and hormones associated with plant growth. In our study, wild and cultivated tomato plants inoculated with *M. robertsii* also exhibited higher plant dry weight compared to non-inoculated plants. Similar results have been reported for Arabidopsis (Liao et al., 2017), maize (Ahmad et al., 2022), rice (Jiang et al., 2022), bean species (Sasan and Bidochka, 2012; Canassa et al., 2019, Barelli et al., 2020), and cover crop species (Ahmad et al., 2020). In our study, the endophytic colonization by *M. robertsii* may have enhanced nutrient acquisition from the soil and activated plant growth-related signaling pathways in both cultivated and wild tomato plants. However, further studies are still needed to investigate whether *M. robertsii* alters physiological processes differently in wild and cultivated species.

Beneficial microbes have been shown to enhance the emission of VOC by plants, potentially resulting in increased plant resistance by modifying the oviposition behavior of insect herbivores and enhancing the attraction of their natural enemies (Pangesti et al., 2015). While recent studies have explored VOC alterations in plants induced by *Beauveria bassiana* (Gonzáles-Mas et al., 2020; Zhu et al., 2022), our study demonstrated that although *M. robertsii* inoculation did not qualitatively alter the blend of VOCs, it did increase the emission of specific plant VOCs in both wild and cultivated tomatoes. Notably, terpenes such as 2-carene, α-phellandrene, β-phellandrene, and o-cymene were detected at higher concentrations in *S. lycopersicum* inoculated with *M. robertsii* than in non-inoculated plants. These compounds have also been found to be emitted at high concentrations in response to larval infestation of *T. absoluta* (Silva et al. 2017) or exogenous application of the defensive compound methyl jasmonate (Salazar-Mendoza et al. 2023b). In addition to the increased VOC emission, factors such as visual cues, trichomes, and plant growth may have contributed to a reduced egg-laying behavior of *T. absoluta* on inoculated plants. Previous studies have suggested that changes in tomato plant VOC emissions caused by *T. absoluta* larval damage or exogenous application of defensive methyl jasmonate can confer antixenotic resistance in tomato plants against *T. absoluta* females (Anastasaki et al., 2018; Salazar-Mendoza et al., 2023b). Indeed, the avoidance of *T. absoluta* oviposition on microbe-inoculated plants suggests that its offspring may not perform well, in line with the preference-performance hypothesis (Jaenike 1990; Silva et al. 2021; Salazar-Mendoza et al., 2023a). Further studies are needed to determine whether the inoculation of *S. lycopersicum* with microbes can induce additional defensive traits, including morphological characteristics and non-volatile compounds, which can affect larval performance. In contrast to the avoidance of *T. absoluta*, the presence of specific VOCs at high concentrations in *S. lycopersicum* plants inoculated with both microbes was found to be strongly correlated with a preference for these plants by *M. basicornis*. A recent study showed that *Nesidiocoris tenuis* (Reuter), a zootphytophagous hemipteran predator of *T. absoluta* in Europe, is attracted to α-pinene, α-phellandrene, 3-carene β-phellandrene, and β-ocimene (Ayelo et al., 2021). In our study, most of these compounds were emitted at high levels in plants induced by *M. robertsii,* which could explain the greater preference for *M. basicornis*. Surprisingly, higher o-cymene emissions were only detected in plants inoculated with *M. robertsii* alone, but this was not correlated with a higher preference for *M. robertsii* compared to non-inoculated plants. One possible explanation for this finding is that the increase in o-cymene could have altered the proportions of major compounds in the VOC blend, consequently reducing the attractiveness of *M. basicornis*.

Altough our understanding of potential variations in VOCs induced by microbes or herbivores in wild tomatoes is limited, our study demonstrated that wild tomato plants inoculated with *M. robertsii*- also undergo changes in their VOC emissions, characterized by increased levels of specific compounds. In *S. pimpinellifolium* plants inoculated with *M. robertsii,* a notable increase in the emission of a single compound, α-terpinene, was observed at high levels. Conversely, *M. robertsii* inoculation on *S. habrochaites* led to higher concentrations of β-caryophyllene, α-humulene, 2-undecanone, and 2-pentadecanone. Previous studies have shown greater emissions of α-terpinene and β-caryophyllene in *S. lycopersicum* in response to herbivore infestation (Silva et al., 2017), while the increase of methyl ketones in *Solanum* spp. has not been extensively reported. Both wild tomato species inoculated with *M. robertsii* caused a variable response to insects. While *M. basicornis* showed a stronger attraction to *M. robertsii*-inoculated *S. pimpinellifolium* compared to non-inoculated plants, there were no effects on the oviposition preference of *T. absoluta*. Conversely, in *S. habrochaites*, microbe inoculation resulted in fewer *T. absoluta* eggs compared to control plants, but it did not affect the olfactory responses of *M. basicornis*. Previous studies have shown that methyl ketones exhibit repellent activities against pest insects (You et al., 2014; Antonious and Snyder, 2015), including *T. absoluta* (Dominguez et al., 2016), but their effects on natural enemies have not been studied. On the other hand, *M. basicornis* females preferred plants with higher emissions of α-terpinene, indicating that this compound could also play a key role in attracting them.

Other studies have attempted to investigate the effect of co-inoculated microbes compared to single microbe inoculation, revealing variable responses (Wang et al., 2015; Wazny et al., 2018; Batool et al., 2020). For example, in wheat, the mycorrhizal fungus *Rhizophagus irregularis* and the PGPR *Pseudomonas putida* exhibited synergistic effects on plant growth and systemic defense against pathogens (Pérez-de-Luque et al., 2017). In contrast, co-inoculation of *P. simiae* and *P. fluorescens* resulted in lower biomass and did not ehnance the resistance of Arabidopsis to *Mamestra brassicae* compared to single-specie inoculation (Pangesti et al., 2017). A recent study has shown that co-inoculation of *Bacillus thuringiensis* and *Azospirillum brasilense* on maize increased indole production but did not affect plant growth compared to single microbe inoculation (Almeida et al., 2021). In contrast to these findings, our study demonstrated that combined inoculation of *B. amyloliquefaciens* and *M. robertsii* on cultivated and wild tomato plants did not enhance plant growth and VOC emissions, or resistance to *T. absoluta*, when compared to single *M. robertsii* inoculation. Further studies are required to elucidate the potential competitive and antagonist interactions between these beneficial microbes on tomato plants.

3.5. Conclusion

In this study, we investigated the effects of individual inoculation with *M. robertsii* and its combined inoculation with *B. amyloliquefaciens* on wild and cultivated tomato plants. We found that these inoculations led to alterations in plant biomass and VOC emission, ultimately resulting in increased resistance against *T. absoluta*. However, the combined inoculation did not enhance these plant traits or interactions with insects compared to the effects caused by *M. robertsii*-alone. The significant increase of specific VOCs induced by *M. robertsii* in *S. lycopersicum* was correlated with a reduced ovipositional preference of *T. absoluta* and an increased attraction of *M. basicornis*. These findings suggest that fungal inoculation with *M. robertsii* could be a promising and ecologically safe strategy for biological control of *T. absoluta* in tomato crops. In addition, the high levels of methyl ketones induced by *M. robertsii* in *S. habrochaites* hold potential as a repellent against *T. absoluta*. It is important to investigate the persistence of induced resistance over time and under varying field conditions. Furthermore, confirming any potential antagonistic interactions between *M. robertsii* and *B. amyloliquefaciens* and understanding their implications for plant protection against herbivores should be a focus of future studies.

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4. NUTRITIENT AVAILABILITY AND JASMONATE-INDUCED RESISTANCE INDEPENDENTLY AFFECT THE INTERACTIONS BETWEEN TOMATO PLANTS AND *Tuta absoluta*

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Abstract

Whether nutrient availability interacts with induced resistance to alter plant chemistry and, consequently, the preference and performance of herbivores on plants remains unclear. We hypothesized that changing fertilizer inputs modulates responses induced by exogenous application of the defensive phytohormone methyl jasmonate (MeJA) against *Tuta absoluta*, a devastating pest of tomatoes. We found that tomato plants grown at or 2-fold above optimal fertilizer rates had higher nutrient content and were more preferred by *T. absoluta* females for oviposition and were better hosts for their offspring. MeJA increased phenolic content and volatile emissions in plants, which correlated with lower *T. absoluta* oviposition preference and offspring performance. However, we found no significant interactions when fertilizer and MeJA were applied simultaneously, such that induced responses against *T. absoluta* in tomatoes were similar regardless of fertilizer rate. These results provide novel insights into the bottom-up effects of fertilization and induced resistance on plant-insect herbivore interactions.

Keywords: plant-insect interactions, methyl jasmonate, fertilization, preference-performance, bottom-up effects, plant volatiles.

4.1. Introduction

Most plants must frequently deal with a variety of biotic stresses, such as the attack by insect herbivores, and in response, they can protect themselves through physical and chemical resistance traits to avoid or reduce herbivore damage (Karban and Myers 1989; War et al. 2012). These resistance traits can be constitutive, i.e., always present, or inducible, i.e., only produced or mobilized in response to damage by herbivores (Karban and Myers 1989). Induced plant resistance occurs upon recognition of an herbivore attack and may often increase the levels of physical and chemical defensive traits that, in turn, reduce the preference (i.e., antixenosis) and performance (i.e., antibiosis) of herbivores on plants (Bezemer et al. 2005; Eyles et al. 2010; Alba et al. 2011; Poelman 2015). The expression of many of these plant resistance-related traits is regulated through hormones (Stella de Freitas et al. 2019). Specifically, the activation of inducible resistance in plants against chewing insects and necrotrophic pathogens is mainly regulated by the jasmonic acid (JA) defensive pathway (Thaler et al. 2001; Howe and Jander 2008; Erb et al. 2012; Acevedo et al. 2015; Tortorici et al. 2022). For instance, exogenous application of jasmonates, i.e., JA or its volatile derivative methyl jasmonate (MeJA), often induces the production of secondary metabolites in plants that deter herbivore feeding, reduce their survival, or inhibit their digestion, i.e., direct resistance traits, in a manner like those induced by chewing herbivores (Chen et al. 2005; Wei et al. 2021). In addition, exogenous jasmonate application induces volatile emissions in plants that can modify the herbivore's behavior (Rodriguez-Saona et al. 2001; Cao et al. 2014) and attract their natural enemies, i.e., indirect resistance traits (Kessler and Baldwin 2001; Yu et al. 2018). Thus, these jasmonates are often used to mimic herbivory and induce plant resistance (Thaler et al. 2001; Tortorici et al. 2022).

In addition to biotic stresses, abiotic stresses can also affect plant chemistry, leading to changes in trophic cascades through bottom-up forces (Power 1992; Hunter and Price 1992; Amtmann et al. 2008; Chen et al. 2010; Han et al. 2019a). One important factor that could trigger strong bottom-up effects in plant-insect interactions is plant nutrient availability (Poelman 2015; Han et al. 2022). However, despite its importance, plant nutrient availability has received little attention within the practical context of integrated pest management (IPM) (Han et al. 2022). In agroecosystems, fertilizer application often alters plant quality via changes in the plant's nutritional status, which in turn may affect the survival, growth, and behavior of insect herbivores (Joern et al. 2012; Han et al. 2014; Islam et al. 2017; Li et al. 2021). For instance, nitrogen is an essential nutrient in regulating leaf photosynthesis (Hou et al. 2019) that has been widely studied in terms of its impact on insect herbivores (Sétamou et al. 1993; Han et al. 2022). Low nitrogen levels in plant tissues are known to reduce herbivore survival, performance, and/or fertility (Awmack and Leather 2002; Schoonhoven et al. 2005; Han et al. 2014). In contrast, high nitrogen levels in plants often result in increased performance of herbivores, which may subsequently alter their population dynamics, leading to rapid herbivore outbreaks (Throop and Lerdau 2004; Schoonhoven et al. 2005).

Despite this knowledge, it is still unclear how plants cope with simultaneous biotic and abiotic stressors, such as protection against herbivores under limited nutrient availability. Because the activation of defense pathways can demand considerable resources, induced resistance in plants is assumed to be energy-intensive (Gershenzon 1994; Kessler and Baldwin 2002; Hahn and Maron 2016). In this way, plants continuously deal with the dilemma of how to allocate their resources to growth or defense, particularly when these resources are limited (Herms and Mattson 1992). According to the resource availability hypothesis, fast-growing plants that are adapted to resource-rich environments should have high foliar nutrient concentrations, high tissue turnover rates, and low investment in antiherbivore defenses and, consequently, should experience high levels of herbivore damage (Coley et al. 1987). In contrast, plants in resource-poor environments should show slow growth rates and enhanced resistance to herbivores. Thus, according to this hypothesis, we expect trade-offs between nutrient availability and plant resistance to herbivores.

In tomato (*Solanum lycopersicum* L.) plants, exogenous MeJA application increased defensive proteins, including trypsin proteinase inhibitors and polyphenol oxidases, that reduced the performance of insect herbivores such as the corn earworm, *Helicoverpa zea* (Boddie) (Paudel et al. 2014) and the cotton bollworm, *Helicoverpa armigera* Hübner (Tan et al. 2012). Additionally, low nitrogen inputs applied to tomato plants increase concentrations of soluble phenolics, glycoalkaloids, polyphenol oxidases, and volatile compounds (Stout et al. 1998; Larbat et al. 2016; Islam et al. 2017; Becker et al. 2021) and impair the performance (Han et al. 2014; Larbat et al. 2016), longevity (Blazhevski et al. 2018), and fertility (Blazhevski et al. 2018) of the South American tomato pinworm *Tuta absoluta* (Meyrick), as well as the attraction of the whitefly *Bemisia tabaci* (Gennadius) (Islam et al. 2017). However, the interactive effects of jasmonate-induced resistance and nutrient availability on the preference-performance relationship of *T. absoluta* in tomato plants have not been studied.

In this study, we hypothesized that nutrient availability interacts with jasmonate-induced resistance to affect plant chemical defenses and the preference and performance of *T. absoluta* on tomato plants. Specifically, we asked the two following questions: (1) What are the bottom-up effects of fertilization and MeJA on tomato plant growth and resistance traits (direct and indirect) and on the preference and performance of *T. absoluta*? (2) Do fertilization and MeJA interact to affect tomato plant growth and resistance traits and the preference and performance of *T. absoluta*? Our study aims to better understand how nutrient availability may modulate plant resistance traits against a specialist herbivore of tomato and other solanaceous plants, which can have practical implications within an IPM framework.

4.2. Materials and Methods

4.2.1. Plants and insects

We tested our hypothesis in a system that consisted of tomato plants and the herbivore *T. absoluta* (Lepidoptera: Gelechiidae), a serious insect pest of tomatoes in its native range (South America) that has recently invaded new areas around the world (Desneux et al. 2010; Mansour et al. 2018; Han et al. 2019b).

Tomato plants, *S. lycopersicum* cv. Santa Clara, were grown from seeds in a greenhouse under natural light and temperature conditions (24 ± 5.5°C) in Basaplant soil (Base Agro, São Paulo, Brazil) in plastic cells; the cultivar Santa Clara is highly susceptible to *T. absoluta* (Proffit et al. 2011). Seedlings at 6–7 cm high (15–20 days after sowing) were transferred to 1.9-L round plastic pots (17 × 15 × 12 cm) containing Basaplant and coconut fiber (1:1). Plants were maintained in the greenhouse, irrigated as needed, and used in experiments (see 'MeJA treatment' below) when they had six fully expanded leaves (40–45 days after sowing).

Tuta absoluta was obtained from a laboratory colony initiated in 2018 from larvae and pupae collected from commercial tomato farms located in Paulínia (São Paulo, Brazil). The colony was maintained on tomato plants, cv. Santa Cruz, in the Department of Entomology and Acarology at ESALQ/USP (Piracicaba, São Paulo, Brazil). Larvae and adults were kept in separate cages (60 × 60 × 30 cm) covered with a fine mesh under controlled conditions (25 ± 2°C, 70 ± 10% relative humidity [RH], 12:12 light:dark [L:D]). An aqueous honey solution (10%) was provided on a cotton wool as a food source for adults inside the cage. Adults used in oviposition studies were 2–3 days old, and eggs used in performance studies were 60–72 h old.

4.2.2. Fertilization regimes

After being transplanted to the round plastic pots, 15–20 days after sowing as described above, plants were grown in soil treated with one of the following four different rates of Basacote® Plus 6M nitrogen, phosphorus, and potassium (NPK) 16:8:12(+2) (Compo, Münster, Germany), a slow-release fertilizer: 1) low = 0.05 g/L (0.025×), 2) medium = 1 g/L (0.5×), 3) optimal = 2 g/L (1×), and 4) high = 4 g/L (2×). These rates were based on recommendations of the manufacturer (https://www.compo-expert.com/sites/default/files/2020-01/Folder_Basacote_EN_2018.pdf). Because tomato plants do not grow well without fertilizer, the lowest fertilizer rate used was 0.025× the optimal rate; in addition, we tested a rate below and a rate above the optimal rate following methods in de Lange et al. (2019). Fertilizer applications were made once. Different sets of plants were used to study the effects of fertilization on the preference and performance of *T. absoluta* and on plant chemistry (see details below).

4.2.3. MeJA treatment

To determine a MeJA concentration that affects *T. absoluta* performance on tomatoes, we conducted a preliminary study to test the effects of different MeJA concentrations on *T. absoluta*. Plants (40–45 days old) were individually sprayed 25 days after the fertilizer treatment with suspensions of 15 mL MeJA (Sigma-Aldrich, St. Louis, MO, USA) at 0 (control), 1, 2, or 3 mM dissolved in 0.1% Tween-20. Control treatments were sprayed with 0.1% Tween-20 only. Treatments were applied to plants using an atomizer until run-off. To avoid contamination, MeJA and control sprays were performed in different greenhouses maintained under the same environmental conditions mentioned above, and plants remained in separate greenhouses until the start of the experiments. Three days after the MeJA and control treatments, four *T. absoluta* eggs were individually placed on different leaves within plants; 16 plants (replicates) were used for each treatment. After larval development was completed, pupal weights were recorded.

Based on the results from these preliminary studies (see Supplemental Figure 1), we conducted all the experiments on the effects of MeJA application on the preference and performance of *T. absoluta* and on plant chemistry (see details below) using 15 mL of a 2 mM MeJA solution per plant. This (2 mM) was the lowest MeJA concentration that negatively affected *T. absoluta* pupal weight. All these experiments were conducted three days after the MeJA treatment, as in Tan et al. (2012), and different sets of plants were used for each experiment.

4.2.4. Oviposition preference

Three choice experiments were carried out to determine *T. absoluta* oviposition preference in response to fertilizer rates and induction by MeJA on tomato plants. All experiments were conducted in an empty greenhouse under natural light and temperature conditions (24 ± 5.5°C). In all experiments, *T. absoluta* adults were removed from the plants after 24 h and the number of eggs laid on each plant was counted. Each choice experiment was replicated 8 times.

- **Two-choice tests**. We conducted two-choice tests to compare the oviposition preference of *T. absoluta* between MeJA-induced plants and non-induced (control) plants, under the same fertilization rate. Plants from each treatment were placed 0.8 m apart on opposite sides of a cage (100 × 70 × 50 cm), covered with fine netting, in a greenhouse. Cages were at least 2 m from each other and 6 *T. absoluta* adults (3 males and 3 females) were released inside each cage.

- **Four-choice tests.** We conducted four-choice tests to compare the oviposition preference of *T. absoluta* among plants exposed to the four different fertilization rates (low, medium, optimal, and high) under the same induction status (i.e., plants induced by MeJA or non-induced plants). The four plants were arranged randomly in a square pattern at 1 m from each other inside a greenhouse (4 × 4 × 4 m). Twelve *T. absoluta* adults (6 males and 6 females) were then released inside the greenhouse. Each replicate was run on a separate day.

- **Multiple-choice test.** We conducted multiple-choice tests to compare the oviposition preference of *T. absoluta* among all eight treatment combinations (plants under four different fertilization regimes that were induced and not induced by MeJA). Plants were arranged in a (3.5 m diameter) circle inside a greenhouse (4 × 4 × 4 m), such that a plant from each treatment combination was placed randomly at about 1 m from each other. Following, 24 *T.*

absoluta adults (12 males and 12 females) were released inside the greenhouse. Each replicate was run on a separate day.

4.2.5. Offspring performance

To assess *T. absoluta* offspring performance on plants, four *T. absoluta* eggs were placed with a fine bush on plants inside of cages (45 × 30 × 30 cm) covered in fine netting and kept in a greenhouse under natural light and temperature conditions (24 ± 5.5°C). The eggs were individually placed in different leaves within the plants and were inspected daily until they hatched. If an egg failed to hatch, a newly emerged larva was placed on the same leaf. Larval survival, developmental time (days) from egg to pupa, and pupal weights were recorded. Thirteen plants (replicates) were used for each of the 8 treatment combinations, i.e., plants under different fertilization regimes treated or not treated with MeJA.

4.2.6. Plant growth

Aboveground plant dry weight and plant height were used as proxy for plant growth. Plants (n = 8) for each of the 8 treatment combinations were cut just above the soil surface from the pots, their lengths were measured, and were then dried in paper bags in a drying oven (Quimis Q317M32, São Paulo, Brazil) at 60°C for 72 h. After drying, samples were weighed to determine differences on dry plant biomass among treatments.

4.2.7. Soil nitrogen levels

To quantify the amounts of nitrogen in the soil, soil samples (~100 g, n = 5) were taken from pots from each of the 8 treatment combinations. Soil samples were dried at 45°C for 72 h and sieved (~2-mm mesh), and total nitrogen was quantified by the Kjeldahl method (Nelson and Sommers 1980).

4.2.8. Nutrient levels in leaves

Leaf samples (140 g, n = 5) were taken for each of the eight treatment combinations and dried in a stove at 60°C for 72 h. Dried samples were ground and then subjected to sulfuric digestion to determine nitrogen concentrations by the Kjeldahl method (Nelson and Sommers 1980) and subjected to nitric-perchloric digestion to determine the concentration of phosphorus, potassium, calcium, magnesium, and sulfur (Zarcinas et al. 1987).

4.2.9. Total phenolics

We measured levels of total phenolics as proxy of a direct resistance trait. Total phenolic content was determined by a modification of the Folin-Ciocalteu method (Ainsworth and Gillespie 2007). In short, leaf samples (0.3 g) were frozen in liquid nitrogen and placed into 15-mL glass tubes with 4 mL methanol (50%). The extract was incubated in a water bath (Marconi MA156, São Paulo, Brazil) for 1.5 h at 80°C and centrifuged (Sorvall instruments RC5C centrifuge, Dupont, DE, USA) at 20,000 g for 15 min at room temperature, and then the supernatant was collected in a new tube (Kofalvi and Nassuth 1995). A total of 150 μL of this extract was mixed with 150 μL Folin reagent (Sigma Aldrich, Merck, Germany), previously diluted with water (1:1), and allowed to stand for 5 min. Then, 3 mL of Na_2CO_3 (2%) was added. After 5 min, the mixture was measured at 750 nm on a spectrophotometer (Hitachi U-1900, Tokyo, Japan). A standard curve was prepared with chlorogenic acid at concentrations of 0, 10, 20, 30, 40, 50, 100, 150, and 250 μg/mL (Bray and Thorpe 1954). Five samples (replicates) of each of the 8 treatment combinations were analyzed.

4.2.10. Collection and analysis of volatile emissions

We measured volatile emissions as a proxy of an indirect resistance trait. Headspace volatile samples were taken from tomato plants exposed to one of the four fertilizer regimes and one of the two induction treatments for 4 h during daytime under laboratory conditions (25 ± 1°C, 70 ± 10% RH). Pots containing the plants were wrapped with aluminum foil to avoid trapping volatiles from plastic and soil. After that, plant shoots were individually enclosed in a 15-L glass chamber and connected to a volatile collection system (Analytical Research Systems Inc., Gainesville, FL, USA). Clean humidified air was pushed at 0.8 L/min into the glass chamber connected to a column containing an adsorbent polymer (30 mg; Hayesep-Q, Alltech Associates, Deerfield, IL, USA) that trapped the volatiles, which was connected to a vacuum pump pulling air at the same flow rate. Following, the polymer column was eluted with 150 μL of distilled hexane, and the samples were stored in glass vials at −30°C until analysis. Dry weight of the plant shoots was determined. Five replicates for each of the 8 treatment combinations were sampled.

Volatile quantification was performed by gas chromatography coupled to a flame ionization detector (GC-FID; GC2010 Shimadzu, Kyoto, Japan) operated at 280°C. Nonyl acetate (Sigma Aldrich, St. Louis, MO, USA) was added (10 μL of a 10-ng/μL solution) in each sample as an internal standard. A 2-μL aliquot of each sample was injected in the column with helium as the carrier gas (24 cm/s). The relative amount of the compounds in each sample was determined based on a comparison of their peak area with that of the internal standard and standardized per unit of fresh shoot biomass (g) for each of the replicates. For volatile identification, the most representative sample of each treatment was further analyzed by gas chromatography coupled to a mass spectrometer (GC-MS; Agilent 6890 Series GC system G1530A), following the same set-up of the GC-FID analysis. Volatile compounds were tentatively identified by comparing the mass spectra obtained in the GC-MS run with the NIST11 library database and by comparison with published Kovats retention indices. Authentic standards were used to confirm the identities of α-pinene, β-myrcene, α-phellandrene, D-limonene, γ-terpinene, and β-caryophyllene (Sigma-Aldrich, Merck KGA, St. Louis, Missouri, USA).

4.2.11. Data analyses

The experimental design was 4×2 factorial, with four fertilizer regimes and two induction treatments for a total of eight treatment combinations. Prior to the analyses, all data were checked for normality using the Shapiro-Wilk test and homogeneity of variances using the Levene's test. All data analyses were performed in Minitab version

17 (Minitab 2013) and R software version 2022.02.3 (R Core Team 2022) using the packages “hnp”, “emeans”, and “multcomp”.

Two-choice tests to determine *T. absoluta* oviposition preference were analyzed using Student’s *t* tests. Four-choice and multiple-choice tests were analyzed using two-way analysis of variance (ANOVA), testing for the effects of fertilizer rates, MeJA-induced resistance, and the interaction between them (fixed factors); replicate was included in the model as a random factor.

Larval survival, egg-larva developmental times, pupal weights, plant growth, nitrogen in the soil, and total phenolics in leaves were analyzed using two-way ANOVA with fertilizer rates, MeJA-induced resistance, and the interaction between them as independent factors. Prior to the analysis, data on offspring performance were averaged for each plant (replicate), and data on percent larval survival were arcsine-square-root transformed. Replicate was included in the model as a random factor.

Since the components of the nutrient and volatile profiles are not independent, we used two-way multivariate analysis of variance (MANOVA) to analyze the effects of fertilizer rates, MeJA-induced resistance, and their interaction on the levels of leaf nutrients and volatile emissions. A significant MANOVA effect was then followed by generalized linear model (GLM) with a gamma distribution (log link) to assess which individual component within the nutrient and volatile profiles was affected by treatment.

If needed, data were transformed prior to ANOVA and MANOVA analysis by using ln (x+1) to meet assumptions of normality. Significant ANOVA effects were subjected to pairwise comparison by Tukey post-hoc tests ($P < 0.05$).

4.3. Results

4.3.1. Oviposition preference

- **Two-choice tests.** Induced resistance by MeJA had a significant effect on *T. absoluta* oviposition preference under the low ($t = 2.50$; df = 1, 17; $P = 0.041$) and optimal ($t = 2.63$; df = 1, 17; $P = 0.034$) fertilizer rates; females laid 112% and 85% more eggs, respectively, on non-induced (control) plants than on MeJA-induced plants (Figure 1a). Although the trend in oviposition preference between MeJA-induced and control plants was similar at the medium and high fertilizer rates, the difference was not significant (Medium: $t = 1.10$; df = 1, 17; $P = 0.308$; High: $t = 2.15$; df = 1, 17; $P = 0.068$) (Figure 1a).
- **Four-choice tests.** Fertilizer regime significantly affected *T. absoluta* oviposition preference on non-induced (control) plants ($F = 9.33$; df = 3, 31; $P < 0.001$) and on plants induced by MeJA ($F = 4.81$; df = 3, 31; $P = 0.011$). On non-induced plants, *T. absoluta* laid 138%, 269%, and 215% more eggs under medium, optimal, and high fertilizer rates, respectively, than at the low rate (Figure 1b). Similarly, on MeJA-induced plants, the numbers of eggs laid were 64%, 72%, and 64% higher under medium, optimal, and high fertilizer rates, respectively, than at the low rate (Figure 1b).
- **Multiple-choice tests.** The amount of fertilizer significantly affected *T. absoluta* oviposition preference ($F = 10.76$; df = 3, 63; $P < 0.001$). Females laid 66%, 100%, and 114% more eggs on plants under the medium, optimal, and high fertilizer rates, respectively, than at the lower rate (Figure 1c). In contrast, MeJA application had no effect on adult oviposition preference ($F = 2.88$; df = 1, 63; $P = 0.096$), and there was no interaction effect between fertilizer rates and induced resistance by MeJA ($F = 0.54$; df = 3, 63 $P = 0.657$).

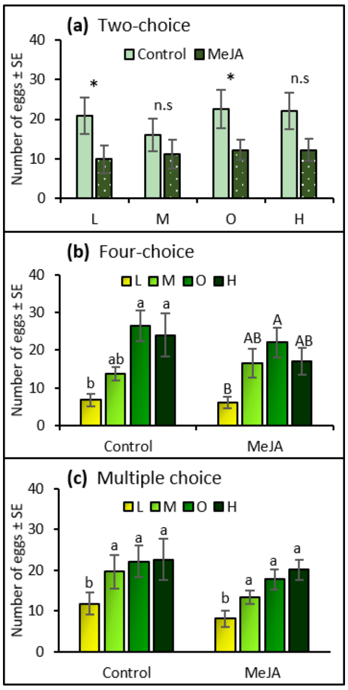

Figure 1. Number of eggs laid by *Tuta absoluta* females (mean ± SE) on tomato plants exposed to four fertilizer regimes (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA) in two-choice (a), four-choice (b), and multiple-choice tests (c). An asterisk (*) indicates significant differences between treatments. Different letters indicate significant differences among treatments (two-way analysis of variance, $P < 0.05$). n = 8; n.s, not significant.

4.3.2. Offspring performance

- **Survival.** Fertilizer rates affected larval survival of *T. absoluta* significantly ($F = 2.85$; df = 3, 103; $P = 0.041$). Larval survival was 15%, 26%, and 19% greater on plants under the medium, optimal, and high fertilizer rates, respectively, than at the lower rate (Figure 2a). However, MeJA application had no effect on larval survival ($F = 1.92$; df = 1, 103; $P = 0.169$), and there was no interaction effect between fertilizer rates and induced resistance by MeJA ($F = 0.40$; df = 3, 103; $P = 0.755$).

- **Larval developmental time.** The amount of fertilizer ($F = 18.83$; df = 3, 103; $P < 0.001$) and induced resistance by MeJA ($F = 6.47$; df = 1, 103; $P < 0.01$) had a significant effect on *T. absoluta* developmental time. Larval developmental time was 5–7% longer when plants received low fertilizer rates than under the medium, optimal, and high rates (Figure 2b). Induced resistance by MeJA also significantly extended larval developmental times by 1.7% compared to control plants. There was no interaction effect between fertilizer rates and induction ($F = 0.11$; df = 3, 103; $P = 0.952$).

- **Pupal weight.** Both fertilizer rates ($F = 20.73$; df = 3, 103; $P < 0.001$) and induced resistance by MeJA ($F = 6.62$; df = 1, 103; $P = 0.012$), but not their interaction ($F = 0.44$; df = 3, 103; $P = 0.723$), had a significant effect on *T. absoluta* pupal weight. Pupal weights were 24%, 31%, and 30% higher in plants under the medium, optimal, and high fertilizer rates, respectively, than at the lower rate (Figure 2c). In contrast, pupal weights were 7% lower in plants induced by MeJA than in non-induced plants (Figure 2c).

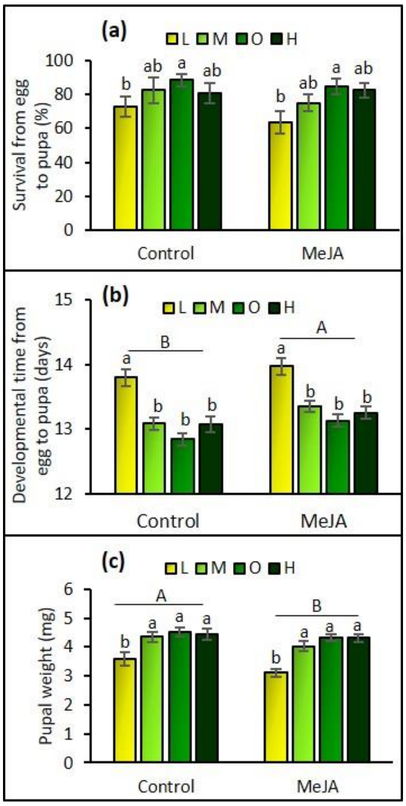

Figure 2. Larval survival (percent ± SE) (a), developmental time from egg to pupa (mean days ± SE) (b), and pupal weights (mean mg ± SE) (c) of *Tuta absoluta* feeding on tomato plants exposed to different fertilizer regimes (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA). Uppercase letters indicate significant differences between induced and non-induced plants, while lowercase letters indicate significant difference among fertilizer regimes (two-way analysis of variance, $P < 0.05$). n = 13.

4.3.3. Plant growth

Plant dry weight was significantly affected by fertilizer rates ($F = 16.34$; df = 3, 63; $P < 0.001$). Plant dry weight under the optimal fertilizer input was 113%, 25%, and 29% higher than at the low, medium, and high rates (Figure 3a). Plants under the low fertilizer rate had the lowest weight of all the treatments. There were no effects of induced resistance by MeJA ($F = 0.01$; df = 1, 63; $P = 0.919$) or an interaction effect between fertilizer and induction on the dry weight of tomato plants ($F = 0.18$; df = 3, 63; $P = 0.906$).

Plant height was also significantly affected by fertilizer rates ($F = 35.0$; df = 3,63; $P < 0.001$). Plant height under the optimal and medium fertilizer inputs was higher than at the low and high rates (Figure 3b). There were no effects of MeJA induction ($F = 0.02$; df = 3,63; $P = 0.896$) or an interaction effect between fertilizer and induction on the height of tomato plants ($F = 0.15$; df = 3,63; $P = 0.926$)

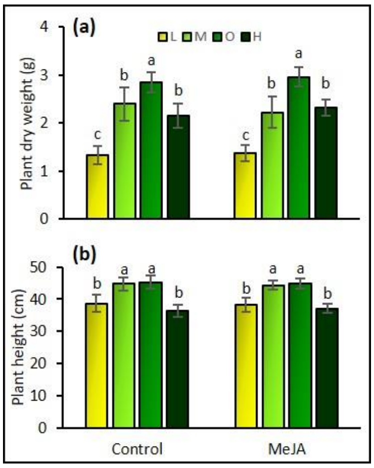

Figure 3. Dry weight (mean g ± SE) (a) and height (mean cm ± SE) (b) of tomato plants exposed to four fertilizer regimens (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA). Different letters indicate significant differences among treatments (two-way analysis of variance, $P < 0.05$). n = 8.

4.3.4. Soil nitrogen levels

As expected, higher fertilizer rates led to significantly higher nitrogen levels in the soil ($F = 42.64$; df = 1, 40; $P < 0.001$) (Figure 4). Also, as expected, there were no effects of MeJA induction ($F = 0.22$; df = 1, 40; $P = 0.805$) or an interaction effect between fertilizer regime and induction on the level of nitrogen in the soil ($F = 0.65$; df = 3, 40; $P = 0.937$).

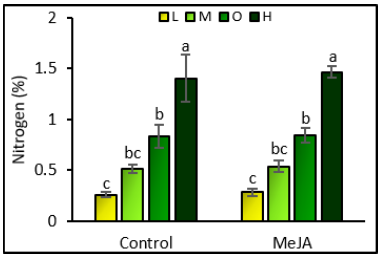

Figure 4. Percentage of nitrogen in the soil (mean percent ± SE) exposed to four fertilizer regimens (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA). Different letters indicate significant differences between treatments (two-way analysis of variance, $P < 0.05$). n = 5.

4.3.5. Nutrient levels in leaves

The amount of fertilizer affected the levels of nutrients in tomato leaves (Wilks' Lambda = 0.058; $F = 7.416$; df = 18, 76; $P < 0.001$). However, neither MeJA induction (Wilks' Lambda = 0.726; $F = 1.698$; df = 6, 27; $P = 0.160$) nor the interaction between fertilizer and induction (Wilks' Lambda = 0.844; $F = 0.263$; df = 18, 76; $P = 0.999$) affected nutrient levels. Levels of nitrogen, phosphorus, potassium, calcium, and magnesium in leaf tissue were significantly altered by fertilizer rates (Table 1). Plants under high fertilizer rates had 52%, 18%, and 13% higher levels of nitrogen than under low, medium, and optimal rates, respectively (Figure 5a). In the same way, phosphorus levels in high fertilizer plants were 88%, 52%, and 32% higher than at the low, medium, and optimal rates, respectively (Figure 5b). Plants under the optimal fertilizer rate had 24%, 10%, and 11% higher concentration of potassium than the low, medium, and high rates, respectively (Figure 5c). Plants under the low fertilizer rate had the lowest levels of nitrogen, phosphorus, and potassium of all fertilizer treatments (Figure 5c). Conversely, plants under the low fertilizer rate had the highest concentration of calcium and magnesium in leaf tissue of all fertilizer treatments (Figure 5d, 5e). In both cases, plants under the high fertilizer rate had the lowest concentration of calcium and magnesium. The amount of fertilizer did not affect sulfur concentration in leaves (Table 1, Figure 5f).

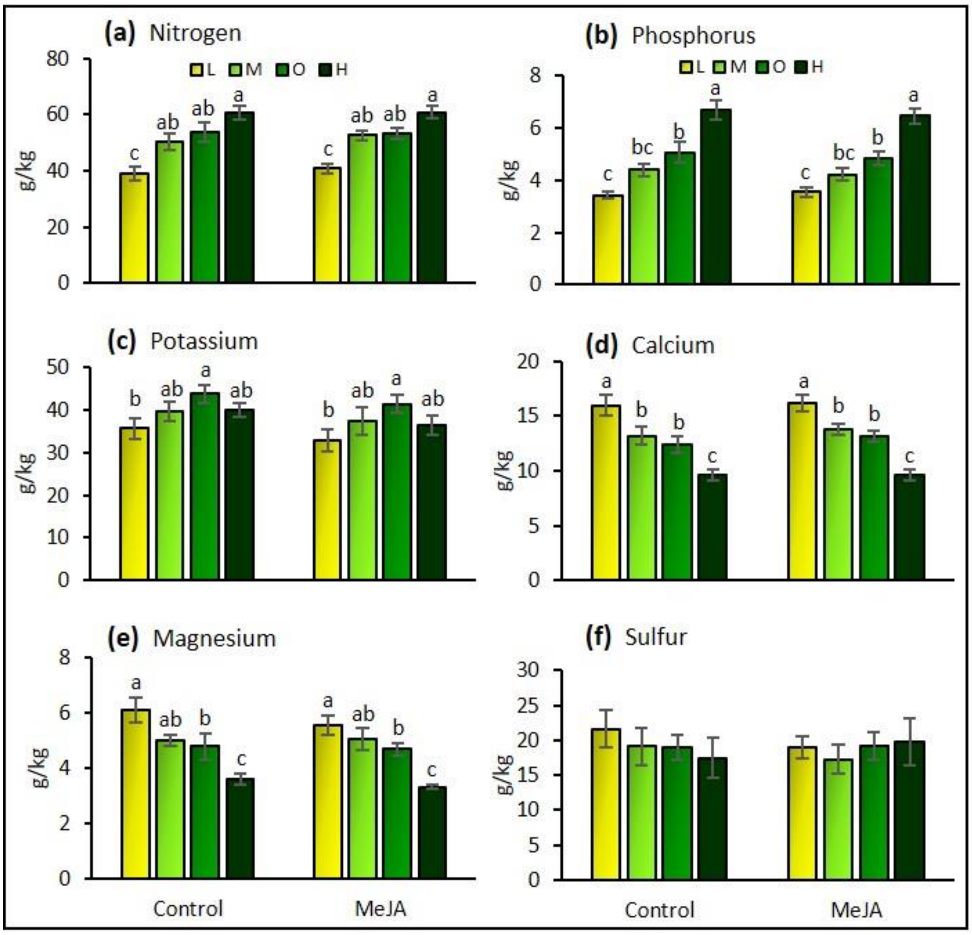

Figure 5. Concentrations of nitrogen (a), phosphorus (b), potassium (c), calcium (d), magnesium (e), and sulfur (f) in the leaves of tomato plants (mean g/kg ± SE) exposed to four fertilizer regimens (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA). Different letters indicate significant differences among treatments (two-way multivariate analysis of variance, $P < 0.05$). n = 5.

Table 1. Results of two-way analysis of variance (ANOVA) for the effects of fertilizer rates ('Fertilizer') and induction by methyl jasmonate ('MeJA'), and the interaction between these two factors on the levels of nutrients in tomato leaves.

Source of variation	**df**[a]	**Nitrogen**		**Phosporus**		**Potassium**		**Calcium**		**Magnesium**		**Sulfur**	
		F	***P***[b]	***F***	***P***[b]	***F***	***P***[b]	***F***	***P***[b]	***F***	***P***[b]	***F***	***P***[b]
Fertilizer	3	26.48	**< 0.001**	43.92	**< 0.001**	4.03	**0.015**	29.81	**< 0.001**	15.42	**< 0.001**	0.41	0.744
MeJA	1	0.26	0.62	0.29	0.59	2.87	0.1	0.67	0.42	1.06	0.31	0.05	0.82
Fertilizer x MeJA	3	0.16	0.92	1.93	0.9	0.07	0.98	0.14	0.93	0.27	0.85	0.32	0.81

[a] Error df = 32

[b] Numbers in bold indicate significant effects at α = 0.05

4.3.6. Total phenolics

Total phenolic levels in leaf tissue were not affected by fertilizer rates (*F* = 1.79; df = 3,39; *P* = 0.169). However, MeJA increased total phenolic levels by 14% compared to non-induced plants (*F* = 5.67; df = 1,39; *P* = 0.023) (Figure 6). There was no interaction effect between fertilizer rates and MeJA induction on the levels of total phenolics in tomato plants (*F* = 0.05; df = 3,39; *P* = 0.985).

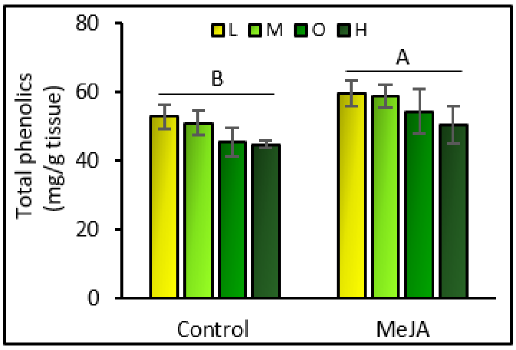

Figure 6. Total phenolic content (mean mg de chlorogenic acid/g fresh tissue ± SE) in tomato leaves exposed to four fertilizer regimes (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA). Different letters indicate significant differences between treatments (two-way analysis of variance, $P < 0.05$). n = 5.

4.3.7. Volatile emissions

Volatile analyses identified 14 compounds among all treatments. Volatile emissions were affected by MeJA induction (Wilks' Lambda = 0.371; *F* = 2.305; df = 14, 19; *P* = 0.046) but not by fertilizer regime (Wilks' Lambda = 0.15; *F* = 1.217; df = 42, 57; *P* = 0.243) or the interaction between fertilizer regime and MeJA induction (Wilks' Lambda = 0.146; *F* = 1.244; df = 42, 57; *P* = 0.220). MeJA induction increased total volatile emissions and the emissions of 12 terpene compounds (α-pinene, β-pinene, β-myrcene, 2-carene, α-phellandrene, α-terpinene, o-cymene, β-phellandrene, D-limonene, β-ocimene, γ-terpinene, and caryophyllene) in tomato plants compared to non-induced plants (Table 2; Figure 7.

Table 2. Results of two-way analysis of variance (ANOVA) for the effects of fertilizer rates ('Fertilizer') and induction by methyl jasmonate ('MeJA'), and the interaction between these two factors on the amounts of volatiles emitted from tomato plants.

Compound	Factor	χ^2	*P*[a]	Compound	Factor	χ^2	*P*[a]	Compound	Factor	χ^2	*P*[a]
(*Z*)-3-Hexenol	Fertilizer	0.054	0.997	α-Phellandrene	Fertilizer	0.763	0.858	β-Ocimene	Fertilizer	2.916	0.405
	MeJA	0.067	0.797		MeJA	7.271	**0.007**		MeJA	13.678	**0.001**
	Fertilizer x MeJA	0.222	0.974		Fertilizer x MeJA	2.072	0.558		Fertilizer x MeJA	2.127	0.546
α-Pinene	Fertilizer	1.744	0.627	α-Terpinene	Fertilizer	1.324	0.723	γ-Terpinene	Fertilizer	1.786	0.618
	MeJA	7.743	**0.005**		MeJA	5.907	**0.015**		MeJA	4.501	**0.034**
	Fertilizer x MeJA	1.575	0.665		Fertilizer x MeJA	2.004	0.572		Fertilizer x MeJA	1.137	0.768
β-Pinene	Fertilizer	1.505	0.681	O-cymene	Fertilizer	0.941	0.815	β-Caryophyllene	Fertilizer	1.786	0.901
	MeJA	4.688	**0.030**		MeJA	10.628	**0.001**		MeJA	4.045	**0.044**
	Fertilizer x MeJA	2.811	0.422		Fertilizer x MeJA	0.644	0.886		Fertilizer x MeJA	0.491	0.921
β-Myrcene	Fertilizer	1.633	0.652	β-Phellandrene	Fertilizer	1.233	0.745	Humulene	Fertilizer	0.545	0.909
	MeJA	10.18	**0.001**		MeJA	6.766	**0.009**		MeJA	3.579	0.058
	Fertilizer x MeJA	2.88	0.41		Fertilizer x MeJA	1.748	0.626		Fertilizer x MeJA	0.555	0.907
2-Carene	Fertilizer	1.139	0.768	D-Limonene	Fertilizer	0.902	0.825	Total	Fertilizer	1.129	0.770
	MeJA	6.998	**0.008**		MeJA	5.433	**0.020**		MeJA	6.77	**0.009**
	Fertilizer x MeJA	1.814	0.612		Fertilizer x MeJA	1.668	0.644		Fertilizer x MeJA	1.626	0.653

[a] Numbers in bold indicate significant effects at α = 0.05.

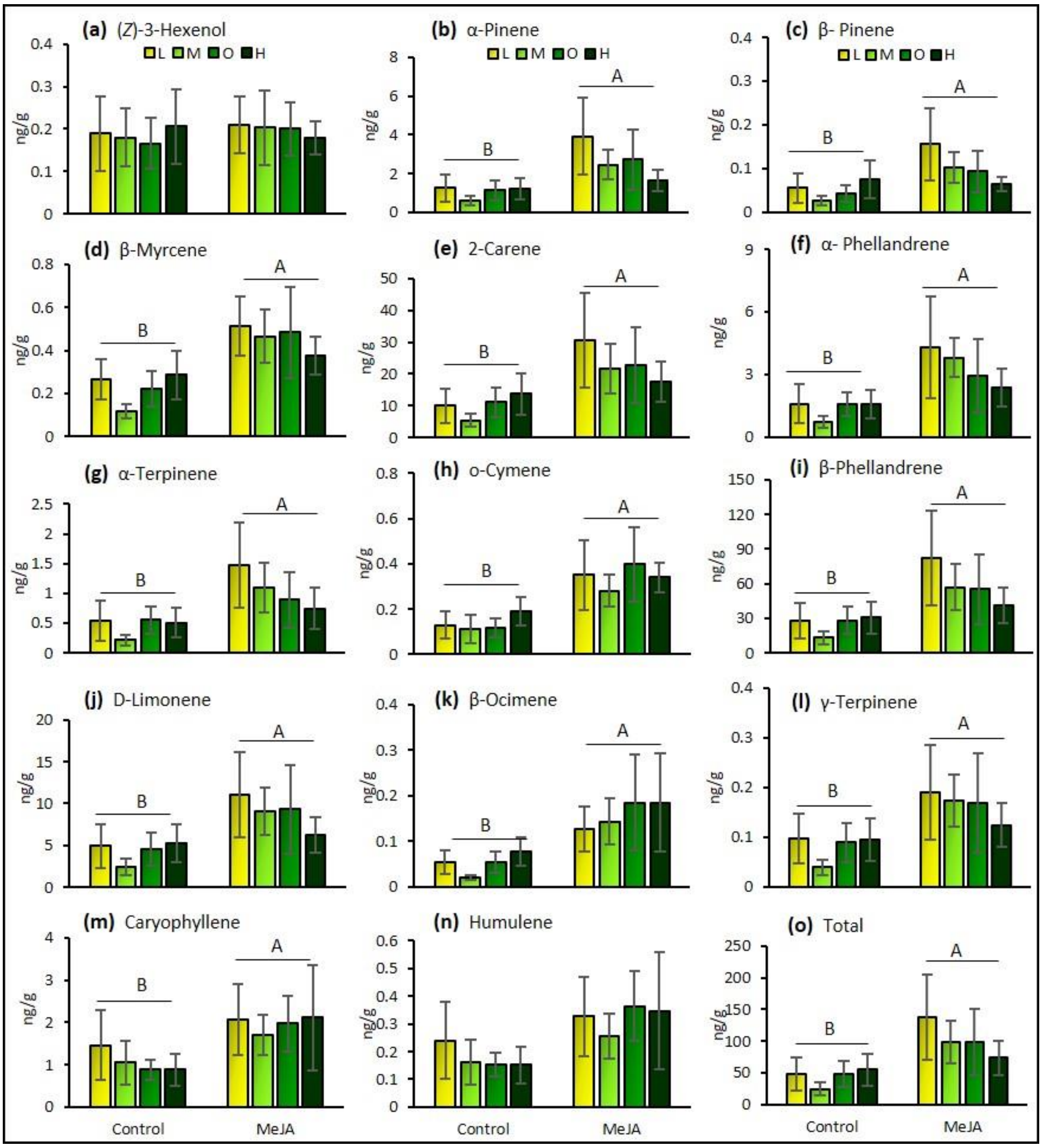

Figure 7. Amounts (mean ng/g fresh tissue ± SE) of (*Z*)-3-hexenol (a), α-pinene (b), β-pinene (c), β-myrcene (d), 2-carene (e), α-phellandrene (f), α-terpinene (g), o-cymene (h), β-phellandrene (i), D-limonene (j), β-ocimene (k), γ-terpinene (l), caryophyllene (m), humulene (n), and total volatiles (o) emitted from tomato plants under four fertilizer regimes (L = low, M = medium, O = optimal, and H = high) with and without induction by methyl jasmonate (MeJA). Means followed by different letters indicate significant differences among treatments (GLM, $P < 0.05$). n = 5.

4.4. Discussion

Although plants often deal with multiple stresses that can affect their growth and defensive traits and subsequently affect their interactions with herbivores (Chapin et al. 1987), most research has focused on understanding these interactions under a single stress condition. Here, we explored how nutrient availability, a potential abiotic stress, modulates induced resistance triggered by the elicitor MeJA against *T. absoluta* in tomato plants. Our study demonstrated that (1) fertilization increased leaf nutrient content of nitrogen, phosphorus, and potassium (NPK), which resulted in greater preference and performance of *T. absoluta*, but had no effect on defensive traits (total phenolic and volatile emissions); (2) MeJA increased levels of defensive traits, which correlated with decreased preference and performance of *T. absoluta*; and (3) fertilization did not interact with MeJA to affect plant growth and nutrient content, defensive traits, or the preference and performance of *T. absoluta*.

Fertilization triggered bottom-up effects that influenced tomato-*T. absoluta* interactions. In our study, tomato plants under an optimal fertilizer rate had higher growth rates than plants under insufficient (i.e., below optimal) and excessive (i.e., above optimal) fertilizer rates. Nutrient deficiencies can negatively affect plant growth, while an excess of nutrients can be associated with high soluble salt concentrations that can interfere with plant growth (Marschner 1983). These bottom-up effects positively affected *T. absoluta*. In accordance with the preference-performance (or 'mother knows best') hypothesis (Jaenike 1978), we observed a strong correlation between *T. absoluta* oviposition preference and larval performance. Females preferred to oviposit and larvae performed better, i.e., had increased survival rates, reduced developmental times, and higher pupal weights, on plants under optimal and high nutrient content. By contrast, plants with low fertilization rates showed low oviposition preference by females and reduced larval performance. These findings were expected since the development success of insect herbivores is often positively correlated with the nitrogen content in their host plants (Sétamou et al. 1993; Awmack and Leather 2002; Shah 2017). In fact, recent studies have also shown that *T. absoluta* females prefer to oviposit on host plants that enhance their offspring performance (Sylla et al. 2019; Silva et al. 2021). *Tuta absoluta* females likely respond behaviorally to visual and chemical changes in host plants due to fertilization, which is an example of antixenosis triggered by bottom-up effects. Interestingly, fertilization rates above optimal reduced plant growth even when nitrogen levels in the soil and leaves were the highest; however, despite this increase in nitrogen levels, *T. absoluta* preference and performance were comparable between optimal and high fertilizer rates, indicating that too much fertilizer did not benefit either the plant or the pest.

The positive effects of fertilization on *T. absoluta* preference and performance can be attributed to changes in plant nutrients and secondary metabolites (Herms and Mattson 1992; Herms 2002). As expected, our study showed that plants under higher fertilizer rates had increased levels of NPK in their leaves. These three elements are essential for the proper growth and development of tomato plants (Adams 1986) and of insects (Dale 1988; Schoonhoven et al. 2005; Amtmann et al. 2008; Chan et al. 2021). For instance, nitrogen is positively correlated with amino acid and protein content and its deficiency may limit insect growth (Schoonhoven et al. 2005). Potassium deficiency may disrupt protein synthesis, which can result in an increase in amino acids and a decrease in sugar content (Dale 1988), and phosphorus can also play an important role in plant resistance against herbivores (Chan et al. 2021). Higher fertilizer rates also decreased levels of calcium and magnesium, and these imbalances in macro-elements could trigger bottom-up effects that affect the performance of *T. absoluta* on tomato plants (Queiroz et al. 2022); however, compared to NPK, these two elements appear to be less critical to tomato-*T. absoluta* interactions because their levels were not correlated with *T. absoluta* preference and performance. As predicted by the resource availability hypothesis, we expected tomato plants under high fertilizer regimes to have low investment in anti-herbivore defenses (Coley et al. 1987). However, our study did not show changes in levels of total phenolics (direct resistance) or volatile emissions (indirect resistance) in response to fertilization. It is possible that other resistance traits not measured here, such as specific phenolic compounds, glycoalkaloids, and defensive enzymes, could have increased under low nutrition since nitrogen content may affect the levels of these compounds in tomato plants (Larbat et al. 2016; Han et al. 2016; Becker et al. 2021).

Jasmonates (i.e., JA and MeJA) are elicitors of plant resistance against chewing herbivores such as caterpillars (Chen et al. 2005; Tortorici et al. 2022). Here, we demonstrated that *T. absoluta* females lay fewer eggs on tomato plants treated with MeJA. Previous studies showed that *T. absoluta* females prefer to oviposit on undamaged plants than on plants damaged by conspecific larvae (Anastasaki et al. 2018), which could be mediated by plant volatiles. Indeed, plant volatiles are cues used by females to locate suitable oviposition sites (Webster and Cardé 2017) and to avoid competition by conspecific and heterospecific herbivores (De Moraes et al. 2001; Knolhoff and Heckel 2014). In fact, tomato plants infested by herbivores altered the emission of volatiles, which modified *T. absoluta* oviposition (Anastasaki et al. 2018, Silva et al. 2022). In our study, MeJA-induced plants increased the emission of 12 terpene volatiles and many of them are also increased after *T. absoluta* larval feeding (Anastasaki et al. 2018; Silva et al. 2022). Likewise, tomato seeds treated with JA increased the emission of methyl salicylate and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (Smart et al. 2013). By contrast, a decrease in volatile emission was found when tomato seeds were applied with a low concentration of MeJA (0.8 mM) (Strapasson et al. 2014). Our MeJA treatment increased volatile emissions during the daytime, which could attract the natural enemies of *T. absoluta* and thus increase indirect resistance in tomatoes. For instance, tomato plants damaged by *T. absoluta* emitted higher amounts of α-pinene, α -phellandrene, 3-carene, β-ocimene, and β-phellandrene that increased attraction of the predator *Nesidiocoris tenuis* (Reuter) (Ayelo et al. 2021). Additional studies are needed to determine if MeJA also induces volatile emissions in tomatoes at nighttime.

Previous studies showed that *T. absoluta* larval performance is negatively affected by MeJA application to tomato seeds (Strapasson et al. 2014). In accordance with the preference-performance hypothesis, our study showed that lower oviposition on MeJA-treated tomato plants is correlated with reduced pupal weights and increased larval developmental times. Our MeJA treatment increased total phenolic content in leaves, which could partially explain the reduced *T. absoluta* performance on these plants. Phenolic compounds play an important role in plant resistance against insect herbivores (Summers and Felton 1994; Wallis and Galarneau 2020). Other studies also report increases of defensive enzymes after MeJA treatment in tomato plants (Tan et al. 2012; Paudel et al. 2014). In our study, we used a MeJA concentration that induces a relatively low, but significant, defensive response in tomato plants against *T. absoluta*; however, whether this concentration induces a defensive response similar to that caused by herbivore damage remains unknown. It is also unclear whether stronger bottom-up effects of tomato plants on *T. absoluta* could be induced by higher concentrations of MeJA (> 2 mM), and if these effects could persist over a long period.

We expected potential trade-offs between resistance responses of plants when simultaneously exposed to abiotic and biotic stresses. However, our study showed no interactions between nutrient availability and jasmonate-mediated induced resistance such that each plant stressor acted independently to affect antixenotic and antibiotic resistance against *T. absoluta* in tomato plants, i.e., there were no fertilizer regime × MeJA induction interaction effects. This finding suggests that in our bottom-up tomato-*T. absoluta* system, resources allocated for jasmonate-mediated induced resistance did not compete with resources allocated for plant growth. Other studies have also attempted to identify trade-offs between nutrient availability and induced plant resistance and found variable evidence (i.e., negative, neutral, and positive) for these trade-offs (Stout et al. 1998; Cipollini and Bergelson 2001; Schmelz et al. 2003; Lou and Baldwin 2004; Mason et al. 2022; Wang et al. 2022). For example, limited nutrient availability reduced induced resistance in rapeseed plants, but not under high nutrient availability (Cipollini and Bergelson 2001). By contrast, low nutrient availability resulted in increased induction of defense-related plant terpenes and phytohormones in maize (Schmelz et al. 2003) and increased herbivore-induced defenses in cotton (Chen et al. 2008). Recent studies in maize showed that high nitrogen supply improves induced resistance (Wang et al. 2022), while low fertilization reduces induced defenses but only in a resistant genotype (Mason et al. 2022). These studies, together with ours, highlight that predicting the outcomes of bottom-up effects on induced resistance against herbivores can be difficult because these effects depend on many factors, such as the type and strength of the fertilizer treatment (i.e., amount, composition, and rate) and the inducer of plant resistance (i.e., mechanical, herbivory, or elicitor), as well as on the plant's genotype. Future studies using standardized methodologies across multiple crops may help in better predicting the outcomes of these bottom-up effects.

In summary, we demonstrated that, under our testing conditions, the combined bottom-up effects of fertilization and MeJA independently affect the interactions between tomato plants and *T. absoluta*. Managing fertilizer inputs and the level of induced resistance in plants can be strategies to control insect pests in agroecosystems (Han et al. 2022). For instance, we found a lower preference and performance of *T. absoluta* on tomato plants under low fertilizer inputs and on plants treated with MeJA, which was correlated with lower nutrient content and higher levels of induced phenolics and terpene emissions, respectively. These two treatments could, however, negatively affect plant growth and yield. Although low fertilizer regimes are expected to reduce plant growth and yield, the effects of jasmonates on tomato growth and yield are not clear (Thaler 1999; Redman et al. 2001). It is even less clear how simultaneous stressors, such as limited nutrient availability and the activation of induced resistance, affect crop yield. In our study, *T. absoluta* performed well even at half the optimal fertilizer rate, which suggests that lower rates would be needed for its control but to the detriment of plant growth. In contrast, MeJA-induced resistance reduced *T. absoluta* preference and performance without any negative (short term) effects on plant growth, which suggests that manipulating induced resistance in tomatoes could serve as a potential control strategy against this pest. Moreover, it is important to examine how bottom-up effects on induced resistance influence plant interactions with multiple herbivores and their natural enemies (Strapasson et al. 2014; Becker et al. 2021). Our study was conducted in the greenhouse under controlled conditions; therefore, further studies are needed to determine if the bottom-up effects we observed here are consistent under field conditions. Improving our understanding on methods to manipulate plant nutrient and defense levels can help develop novel IPM strategies against herbivores, such as *T. absoluta*, in agroecosystems.

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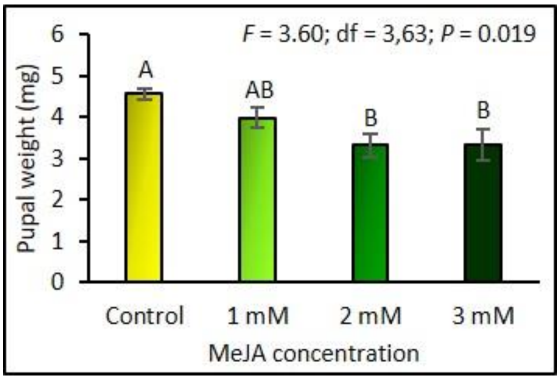
Supplemental Figure 1. Pupal weights (mean mg ± SE) of *Tuta absoluta* feeding on plants exposed to different induction levels by methyl jasmonate (MeJA). Different uppercase letters indicate significant differences among treatments (one-way analysis of variance, $P < 0.05$). n = 16.



5. FINAL REMARKS

Wild tomatoes are a promising genetic source for improving plant defenses against *Tuta absoluta*. In this study, we found out that the wild *Solanum habrochaites*, which exhibits high concentrations of chemical and morphological defensive mechanisms, significantly influenced *T. absoluta* resistance compared to the cultivated *S. lycopersicum*. Therefore, the introgression of traits from wild tomatoes into modern varieties that express constitutive resistance through high glandular trichome densities and elevated phenolic contents can effectively impair the growth and survival of *T. absoluta* larvae. Additionally, the high emission of methyl ketones from *S. habrochaites* can be employed as a strategic tool to deter *T. absoluta* moths from laying eggs on tomato crops.

On the other hand, managing fertilizer inputs can be a viable strategy for controlling *T. absoluta* in tomato crops. As expected, reducing fertilizer inputs in plants resulted in decreased concentration of nitrogen, phosphorus, and potassium in tomato leaves. This reduction was correlated with a decline in the number of eggs laid by moths and a decrease in larval performance, highlighting the crucial role of foliar macronutrients in *T. absoluta* performance. It is important to note that our study focused on the vegetative stage, thus further studies are needed to determine an optimal balance for reducing fertilizer inputs without compromising tomato crop yields or increasing susceptibility to *T. absoluta* infestation. In addition, it is essential to investigate the impact of plant fertilization on the natural enemies of *T. absoluta,* as this aspect remains relatively unexplored and holds signigficance in developing comprehensive pest management strategies.

Additionaly, induced defenses are an important tool for activating resistance mechanisms in tomato plants against *T. absoluta*. Our findings highlight that the exogenous application of MeJA alters tomato chemistry by increasing total phenolic concentrations and volatile emissions. This alteration results in decreased larval performance and a reduction in moth oviposition preference. We also demonstrated that both wild and cultivated tomato plants inoculated with *M. robertsii* alone or in combination with *Bacillus amyloliquefaciens* show induced defensive responses, including increased emission of terpenes in *S. lycopersicum* and *S. pimpinellifolium,* as well as methyl ketones in *S. habrochaites*. These emissions not only reduce oviposition by *T. absoluta*, but also act as an olfactory cue, attracting the predator *M. basicornis*. These results indicate that microbes can serve as a novel and efficient stategy for activating defensive mechanisms in tomato plants, which directly affect *T. absoluta* moths while enhancing biological control measures.

In conclusion, the research presented in this thesis significantly enhaces our understanding of tomato plant defenses, shedding light on their modulation by domestication, nutrient availability, and induced responses. These defense mechanisms play a crucial role in impeding the progress of *Tuta absoluta,* a highly destructive tomato pest native to South America that has caused extensive damage on global scale. The insights gained from our findings have far-reaching implications and will greatly contribute to the development of novel and sustainable management strategies aimed at minimizing the damages caused by this pest in tomato crops.