VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

PhD Thesis

Doctorado en Biomedicina y Biotecnología

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September 2024

A delicate balance:

Unraveling the interplay between tomato defenses and beneficial microorganisms in the control of *Spodoptera exigua*

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Informan:

Que la Sra. Ada Frattini Llorens, Graduada en Biología, ha realizado bajo su dirección el trabajo de investigación original recogido en esta tesis doctoral que lleva por título: "A delicate balance: Unraveling the interplay between tomato defenses and beneficial microorganisms in the control of *Spodoptera exigua*", para optar al Grado de Doctor por la Universitat de València.

Y, para hacer constancia, de acuerdo con la legislación vigente, firman la presente en Burjassot, a 10 de Septiembre de 2024.

Dr. Salvador Herrero Sendra

Dra. Maria Cristina Crava

Agradecimientos

En primer lugar, quiero agradecer a Salva y a Cris el haber confiado en mi y apoyarme en esta etapa tan intensa y enriquecedora. Me habéis enseñado mucho, tanto a nivel profesional como personal, gracias por haber sido mis padres científicos.

Durante este tiempo he tenido la suerte de trabajar con unos compañeros de laboratorio maravillosos: Alexandra, Ascen, Ayda, Balta, Burcu, Ceci, Dafne, Elvira, Hanane, Jesica, Joel, Juan, Julia, Leo, Lim, Luis H., María L., María M., Miguel, Mona, Noe, Óscar, Patri, Quan, Raquel, Rosi, Rosy, Salomé, Sandy, Sara H., Sara M., Sergio, Stefano, Vicky, Yolanda. Gracias a cada uno de vosotros por haber formado parte de este viaje.

A Leila, por pasarme el testigo e introducirme en el mundo de las interacciones multitróficas, gracias por tu paciencia y ayuda.

A Ángel, por haberme apoyado todo este tiempo y motivarme a seguir siempre hacia delante, has sido un apoyo fundamental.

A Adrià, porque no podría haber tenido mejor compañero de tesis. Hemos pasado por todo juntos, buenos y malos ratos, pero has hecho que este camino sea mucho más llevadero.

A Dani, Anabel y Luis B., por todos los momentos vividos juntos, tanto en el laboratorio como fuera. Muchas gracias por vuestro apoyo y consejos.

A Ana y Pablo, por haber sido un pilar imprescindible. Gracias por transmitir tanta empatía y sensibilidad, el final del doctorado no hubiera sido lo mismo sin vosotros.

A todos los compañeros de la EEZ-CSIC y de la UJI por haberme ayudado cuando lo he necesitado, ha sido un placer colaborar con vosotros.

A mis amigas de la universidad (Sandra, Estefanía, Rita, Elena), por estar a pesar de la distancia.

A mis amigos de Benidorm, los caratteristicos, por crecer y mejorar juntos. A Ricar, mil gracias por haberme diseñado la portada de la tesis. Tamara y Mari, gracias por vuestro apoyo incondicional.

A mis compañeras de APAX, por todo lo aprendido y vivido con vosotras. Siempre valdrá la pena luchar por ellos.

A todos los animales de acogida que hemos tenido, habéis llenado de alegría nuestro hogar.

A Bimba, Tommy, Tarantino, Lisa y Kika, por enseñarnos lo que es el amor puro e incondicional.

A la clínica Eurokine, por darme las herramientas para superar el dolor crónico, gracias de corazón (know pain, no pain).

A mi familia, por acompañarme en todo momento.

A mi hermana, porque juntas podemos con todo. A mi padre, por sostenerme siempre. Os quiero mucho.

A Pedro, Pepi y Marta, por cuidarme y escucharme.

A Hugo, porque sin ti hubiera sido imposible. Gracias por apoyarme y confiar siempre en mi, esta tesis es tan tuya como mía.

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List of abbreviations

Abbreviation	Description
ABC	Ammonium bicarbonate
AcMNPV	Autographa californica multiple nucleopolyhedrovirus
ACN	Acetonitrile
AD	Artificial diet
ALDH	Aldehyde dehydrogenase
AMF	Arbuscular mycorrhizal fungi
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
Bt	*Bacillus thuringiensis*
BV	Baculovirus
CCA	Canonical correspondence analysis
COI1	F-box protein coronatine insensitive 1
CP	Cysteine protease
Ct	Threshold cycle
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
EFN	Extrafloral nectar
ESI	Electrospray ionization
FAC	Fatty acid-amino acid conjugate
Fm	*Funneliformis mosseae*
GOX	Glucose oxidase
HAMP	Herbivore-associated molecular pattern
HARP1	Helicoverpa armigera R-like protein 1
HAS1	Highly accumulated secretory protein 1
HIPV	Herbivore-induced plant volatile
hpi	Hours post-infection
ICS	Isochorismate synthase
IJ	Infective juveniles
IPM	Integrated pest management
ISR	Induced systemic resistance
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucine
JAs	Jasmonates

JAZ	Jasmonate ZIM-domain
LA	Linolenic acid
LC-MS/MS	Liquid chromatography coupled to mass spectrometry
LefSE	linear discriminant analysis effect size
LOX	13-lipooxygenase
MAPK	Mitogen-activated protein kinase
MeJA	Methyljasmonate
MIR	Mycorrhiza-induced resistance
MM	Moneymaker
Nm	Non-mycorrhizal
OBs	Occlusion bodies
ODV	Occlusion-derived viruses
OPC:8	3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoic acid
OPDA	12-oxo-phytodienoic acid
OS	Oral secretion
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDV	Polydnavirus
PGPF	Plant growth-promoting fungi
PGPR	Plant growth-promoting rhizobacteria
PIN2	Protease inhibitor II
PLA1	Phospholipase A1
PLC	Phospholipase C
PM	Peritrophic membrane
PO	Phenoloxidase
POD	Peroxidase
PPO	Polyphenol oxidase
PR1	Pathogenesis-related protein 1
qPCR	Quantitative PCR
REPAT	Response to pathogens
RG	Relative growth
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	Salicylic acid glucoside

SB	Soyasapogenol B base + O-HexA, Hex, dHex, 1 malonyl
SeMNPV	Spodoptera exigua multiple nucleopolyhedrovirus
SfMNPV	Spodoptera frugiperda multiple nucleopolyhedrovirus
SGE	Salicylic acid glucose ester
SLEF	Solanum lycopersicum elongation factor 1α
SM	Secondary metabolite
sPLSDA	Sparse partial least squares discriminant analysis
SWATH-MS	Sequential window acquisition of all theorical fragment ion mass spectra
TFA	Trifluoro-acetic acid
TYLCCNV	Tomato yellow leaf curl China virus
TYLCV	Tomato yellow leaf curl virus
VOC	Volatile organic compound

SUMMARY

Plants live in a complex environment where trophic relationships with other organisms have significantly influenced their genetics. For instance, continuous attacks by insect herbivores have driven plants to develop a wide range of antiherbivore defenses, such as physical barriers and chemical compounds, to protect themselves from biotic threats. However, plants may also form relationships with other organisms that help them fight against insect herbivores. Among these beneficial organisms are entomopathogens, such as *Bacillus thuringiensis* (Bt) and baculoviruses, which can strongly reduce insect populations. These entomopathogens are currently used as biological control agents in sustainable pest management programs due to their high specificity and lack of toxicity to non-target organisms. Another group of beneficial microorganisms that plants closely associate with are arbuscular mycorrhizal fungi (AMF). The symbiosis between plant roots and AMF enhances plant nutrition, growth, and resilience to both abiotic and biotic stresses. These multitrophic interactions can significantly shape the outcomes of plant-insect-entomopathogen interactions. While entomopathogens exert top-down effects by enhancing plant growth and fitness through the reduction of herbivores, plants and other players like AMF may exert bottom-up effects on the effectiveness of entomopathogens. Therefore, the main purpose of this thesis is to provide insights into the multitrophic interactions among tomato plants, the lepidopteran pest *Spodoptera exigua*, mycorrhizae, and both bacterial and viral entomopathogens, to elucidate how these complex relationships influence plant defense and pest management strategies.

In the **first chapter**, we analyzed the influence of constitutive and inducible defenses on the lethality of Bt against *S. exigua.* To achieve this, the leaves of fifteen wild species and forty tomato varieties were lyophilized to obtain leaf powder, which was then used to prepare leaf-based artificial diets.

This approach allowed us to evaluate the effects of chemical compounds produced by the plant at the time of harvesting on the insects, without any further metabolic changes due to herbivory. Growth bioassays showed that constitutive defenses from cultivated varieties had a lesser effect on the growth of *S. exigua* larvae compared to wild species. This may be a consequence of tomato domestication, which has prioritized commercial traits, such as fruit size and yield, at the expense of defensive properties against insect pests. In contrast, bioassays in which Bt-challenged larvae were fed on different plant diets showed a similar mortality rates among both wild species and cultivated varieties, which were higher than the mortality observed in Bt-challenged larvae fed with artificial diet. This suggests that the constitutive defenses that potentially enhance the action of Bt remained unaffected by domestication.

We then examined the contribution of inducible defenses to Bt-triggered mortality. To do this, we selected six cultivated varieties that in the previous experiment showed contrasting levels of resistance against *S. exigua.* We induced tomato defenses with methyljasmonate (MeJA), an elicitor of jasmonic acid (JA) pathway, and performed growth and Bt-mortality bioassays with leaf-based artificial diets produced from induced and non-induced plants. The results showed a positive correlation among the negative effects of both constitutive and inducible defenses on the insect growth and the increase in mortality triggered by Bt, suggesting that plant compounds may contribute to the effectiveness of Bt against *S. exigua*. We speculate that the enhanced efficacy of Bt may be modulated by specific antiherbivore defenses that target insect digestion, peritrophic membrane integrity, or immune status of the insect. Finally, we delved into the metabolic changes occurred in the leaves of the induced varieties, which may be responsible for the increased action of Bt. After confirming the rearrangement of the leaf metabolome, we found that the triterpenoid saponin

soyasapogenol B base + O-HexA, Hex, dHex, 1 malonyl (SB) was overaccumulated in the six varieties. The detrimental effects of saponins are consistent with the impairment in larval growth, and may explain the enhanced lethality of Bt against *S. exigua.*

In the **second chapter**, we investigated whether mycorrhization and/or herbivory could influence the susceptibility of *S. exigua* to Bt and baculovirus in tomato plants. To achieve this, plants were previously exposed to herbivory by *S. exigua* larvae and/or associated with the AMF *Funneliformis mosseae*. The plants were then lyophilized, and the effects of the different treatments were tested on *S. exigua* larvae using leaf-based artificial diets. Growth bioassays showed a significant decrease in larval development due to prior herbivory, which may be explained by the activation of jasmonate pathway after insect feeding and the subsequent synthesis of toxic compounds. However, this effect was not observed with the AMF treatment alone. Interestingly, a significant interaction between herbivory and mycorrhiza was detected, suggesting that mycorrhizal symbiosis may prime plants for enhanced antiherbivore responses against *S. exigua.*

Regarding larval susceptibility to Bt, we found that both mycorrhization and herbivory, whether individually or in combination, resulted in a 20 % increase in the lethality of the entomopathogen. Moreover, the efficacy of baculovirus was enhanced only when larvae fed on mycorrhizal plants that had not been previously exposed to herbivory. The increased susceptibility of *S. exigua* to both entomopathogens may be attributed to alterations in larval physiology caused by the production of toxic metabolites or defense proteins, and/or changes in plant nutrition. Finally, we investigated whether plant compounds may modulate larval susceptibility by interfering with immunity or gut microbiota. Enzymatic assays showed that the activity of phenoloxidase, a

marker of insect immune status, was impaired only by herbivore-induced compounds. This suggests that chemical defenses produced after *S. exigua* feeding may interfere with the insect's immunity, reducing its ability to combat the pathogen and thereby increasing susceptibility to Bt. However, no changes were observed in gut microbiota composition, load, or diversity with any of the treatments, thus indicating that the enhanced entomopathogenic action is unlikely to be mediated by alteration in gut microbiota.

After demonstrating the contribution of tomato plant defenses to the efficacy of entomopathogens, the **third chapter** investigates whether baculovirus infection can modulate tomato responses to *S. exigua* herbivory. To address this, tomato plants were exposed to herbivory by mock- or Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV)-infected larvae for 3 hours. Gene expression and phytohormone level analyses confirmed the induction of both local and systemic responses associated with the JA pathway in response to *S. exigua* herbivory. However, SeMNPV-infected larvae elicited a reduced expression of two JA-related genes and a lower accumulation of the oxylipin 12-oxo-phytodienoic acid (OPDA), a precursor of JA. No differences in tissue damage levels were detected between mock- and SeMNPV-infected larvae, suggesting that the suppression of JA-related responses were not due to changes in the feeding rate of infected larvae. Another possible explanation is that viral infection may modulate the herbivory-associated molecular patterns (HAMPs) present in insect secretions. To investigate this, we collected the oral secretion (OS) from mock- and SeMNPV-infected larvae and performed proteomic characterization. The results revealed a reduction in a lipase domain-containing protein in OS samples from infected larvae compared to controls. Since lipases are known to participate in the activation of JA-dependent defenses, this reduction may explain the decrease of JA-response after herbivory by larvae

infected with baculovirus. Additionally, six proteins potentially associated with detoxification processes were overexpressed in infected larvae, suggesting that SeMNPV-infected larvae may have higher detoxification rates that non-infected conspecifics. We propose that the reduction in the activation of JA pathway, combined with increased detoxification, may improve the suitability of the tomato plant for *S. exigua*, thereby promoting larval growth. This, in turn, would allow to a higher production of virions, benefiting the dispersal and transmission of the virus.

The results obtained from this doctoral thesis significantly enhance our understanding of multitrophic interactions among insects, plants, and associated beneficial microorganisms. This research provides novel insights into how plant defenses contribute to the efficacy of bacterial and viral entomopathogens and how baculovirus infection can modulate tomato antiherbivore responses. These findings have important implications for the design of effective biological pest control programs. By elucidating the role of plant defenses in enhancing the action of entomopathogens, and understanding how baculovirus infection influences plant defense mechanisms, this research could inform strategies that optimize the use of entomopathogens in sustainable agriculture. Furthermore, the interaction between plant defenses, mycorrhization, and entomopathogens highlights the potential for integrating multiple biological control agents to improve pest management and crop protection. Ultimately, these insights could lead to more targeted and efficient pest control approaches, contributing to the development of more resilient and sustainable agricultural systems.

RESUMEN

Las plantas habitan en un entorno complejo donde establecen relaciones tróficas con otros organismos, lo cual ha ejercido una gran influencia sobre su genética. Por ejemplo, el ataque continuo de insectos herbívoros ha llevado a las plantas a desarrollar una amplia gama de defensas, como barreras físicas y compuestos químicos, para protegerse de amenazas bióticas. En este contexto, las plantas también pueden recurrir a otros organismos que les ayuden a combatir dichos insectos. Entre estos organismos beneficiosos se encuentran los entomopatógenos, como *Bacillus thuringiensis* (Bt) y baculovirus, con capacidad para controlar de manera eficiente las poblaciones de insectos. Estos entomopatógenos se utilizan actualmente como agentes de control biológico en programas de manejo sostenible de plagas debido a su alta especificidad y falta de toxicidad para el ambiente u otros organismos. Otro grupo de microorganismos beneficiosos con los que las plantas se asocian estrechamente son los hongos micorrízicos arbusculares (HMA). La simbiosis entre las raíces de las plantas y los HMA mejora la nutrición, el crecimiento y la resistencia de las plantas frente a estrés abiótico y biótico. Estas interacciones multitróficas pueden influir de forma significativa en las interacciones planta-insecto-entomopatógeno. Mientras que los entomopatógenos pueden mejorar el crecimiento y desarrollo de las plantas mediante el control efectivo del insecto, las plantas y otros organismos como los HMA pueden llegar a modular la eficacia de los entomopatógenos. Por lo tanto, el principal objetivo de esta tesis es profundizar en las interacciones multitróficas entre la planta de tomate, el insecto plaga *Spodoptera exigua*, la micorriza, y los entomopatógenos Bt y baculovirus para dilucidar cómo influyen estas complejas interacciones en los mecanismos de defensa de las plantas y las estrategias de manejo de plagas.

En el **primer capítulo**, analizamos la influencia de las defensas constitutivas e inducibles de la planta sobre la letalidad de Bt contra *S.*

exigua. Para ello, se liofilizaron las hojas procedentes de quince especies silvestres y cuarenta variedades de tomate hasta obtener un polvo de hoja, el cual se utilizó para preparar dietas artificiales con las que alimentar a *S. exigua.* Este enfoque nos permitió evaluar los efectos de los compuestos foliares sobre el insecto producidos por la planta en el momento de la cosecha, evitando así cualquier cambio metabólico debido a la herbivoría. Los bioensayos de crecimiento mostraron que las defensas constitutivas de las variedades cultivadas tuvieron un menor efecto sobre el crecimiento de las larvas de *S. exigua* en comparación con las especies silvestres. Esto podría deberse a la domesticación del tomate, proceso en el que se han priorizado rasgos comerciales, como el tamaño del fruto o rendimiento, a expensas de las propiedades defensivas frente a insectos. Por el contrario, los bioensayos de susceptibilidad en los que larvas infectadas con Bt se alimentaban de dichas dietas vegetales mostraron tasas de mortalidad larvaria similares entre las especies silvestres y las variedades cultivadas, siendo esta mortalidad mayor que la observada en larvas infectadas alimentadas únicamente con dieta artificial. Esto sugiere que las defensas constitutivas que potencialmente mejoran la acción de Bt no han sido afectadas por la domesticación.

A continuación, estudiamos la contribución de las defensas inducibles a la mortalidad inducida por Bt. Para ello, seleccionamos seis variedades cultivadas que en el experimento anterior mostraron niveles opuestos de resistencia frente a *S. exigua.* Las defensas de la planta se indujeron con metiljasmonato (MeJA), un elicitor de la vía del ácido jasmónico (JA), y se realizaron bioensayos de crecimiento y susceptibilidad a Bt con dietas artificiales a base de hojas procedentes de plantas inducidas y no inducidas. Los resultados mostraron una correlación positiva entre los efectos negativos de las defensas constitutivas e inducibles sobre el crecimiento y mortalidad larvaria y el incremento de la

mortalidad causada por Bt, lo cual sugiere que los compuestos de la planta podrían contribuir a la eficacia del entomopatógeno bacteriano frente a *S. exigua.* Especulamos que la mejora en la eficacia de Bt podría deberse a la acción de defensas específicas que interfieran con la integridad de la membrana peritrófica, la digestión o el estado inmunitario del insecto. Finalmente, profundizamos en los cambio metabólicos producidos en las hojas de las variedades inducidas, los cuales podrían ser responsables de la acción mejorada de Bt. Una vez confirmada la reorganización del metaboloma foliar tras el tratamiento con MeJA, encontramos que la saponina triterpenoide soyasapogenol B base + O-HexA, Hex, dHex, 1 malonilo estaba sobreproducida en las seis variedades. Los efectos perjudiciales de las saponinas son consistentes con el impacto negativo observado sobre el crecimiento larvario y podrían explicar la mayor letalidad de Bt frente a *S. exigua.*

En el **segundo capítulo**, investigamos si la micorrización y/o la herbivoría podrían influir en la susceptibilidad de *S. exigua* a Bt y baculovirus. Para ello, las plantas de tomate fueron expuestas a la herbivoría ejercida por larvas de *S. exigua* y/o asociadas con el HMA *Funneliformis mosseae.* Después, se liofilizaron las hojas y se analizaron los efectos de los distintos tratamientos sobre el insecto mediante el uso de las dietas vegetales preparadas a partir de las hojas. Los bioensayos de crecimiento mostraron una disminución significativa en el desarrollo larvario debido a la herbivoría, lo que puede explicarse por la activación de la vía del jasmonato a causa de la alimentación del insecto y la posterior síntesis de compuestos tóxicos. Sin embargo, este efecto no se observó con el tratamiento individual con HMA. Curiosamente, se detectó una interacción significativa entre la herbivoría y la micorrización, lo que sugiere que esta simbiosis podría preparar a la planta para responder de forma más eficiente frente a *S. exigua.*

Respecto a la susceptibilidad de las larvas a Bt, observamos que tanto la micorrización como la herbivoría, ya sea individualmente o en combinación, dieron como resultado un aumento del 20 % en la letalidad del entomopatógeno bacteriano. Además, la eficacia de baculovirus mejoró únicamente cuando las larvas se alimentaron de plantas micorrizadas no expuestas a herbivoría. El aumento de la susceptibilidad de *S. exigua* a ambos entomopatógenos podría atribuirse a alteraciones en la fisiología del insecto causadas por la producción de metabolitos tóxicos o proteínas de defensa y/o cambios en el estado nutricional de la planta. Finalmente, investigamos si los compuestos vegetales podrían modular la susceptibilidad de las larvas mediante la interferencia con su inmunidad o microbiota intestinal. Los ensayos enzimáticos mostraron que la actividad de la enzima profenoloxidasa, un marcador del estado inmunitario del insecto, se vio afectada únicamente por los compuestos inducidos por herbivoría. Esto sugiere que las defensas químicas producidas por la planta tras la herbivoría de *S. exigua* podrían interferir con la inmunidad del insecto, reduciendo su capacidad para combatir el patógeno y aumentando así la susceptibilidad a Bt. Sin embargo, no se observaron cambios en la composición, carga o diversidad de la microbiota intestinal con ninguno de los tratamientos, lo que sugiere que la acción mejorada del patógeno no está mediada por cambios en la microbiota intestinal.

Una vez demostrada la contribución de las defensas de la planta de tomate a la eficacia de los entomopatógenos, decidimos investigar en el **tercer capítulo** si la infección por baculovirus podría modular las respuestas defensivas del tomate frente a la herbivoría ejercida por *S. exigua*. Para abordar esto, las plantas de tomate fueron sometidas a la herbivoría de larvas control o infectadas con el nucleopoliedrovirus múltiple de *Spodoptera exigua* (SeMNPV) durante 3 horas. Los análisis de expresión génica y fitohormonas confirmaron la inducción de

respuestas asociadas a la vía de jasmonato tanto a nivel local como sistémico en respuesta a la herbivoría. Sin embargo, las larvas infectadas con SeMNPV indujeron una menor expresión de dos genes marcadores de la ruta de jasmonato así como una menor acumulación del ácido 12-oxo-fitodienoico (OPDA), precursor de JA. No se detectaron diferencias en la cantidad de tejido foliar ingerido por las larvas control e infectadas con baculovirus, lo que sugiere que la supresión de las respuestas relacionadas con JA no se debe a cambios en la tasa de alimentación de las larvas infectadas. Una posible explicación a la supresión observada es que la infección viral module los patrones moleculares asociados a herbivoría presentes en las secreciones orales (OS) del insecto. Para evaluar esto, se recogió la OS de las larvas control e infectadas y se realizó una caracterización proteómica. El análisis reveló una reducción en el contenido de una proteína que contenía un dominio lipasa en muestras de OS de larvas infectadas respecto a los controles. Dado que las lipasas han sido reconocidas como elicitores de la ruta de jasmonato, la reducción en el contenido de este compuesto podría explicar la menor activación de las respuestas defensivas dependientes de JA tras la herbivoría por larvas infectadas con baculovirus. Además, en las larvas infectadas se detectó la sobreexpresión de seis proteínas potencialmente asociadas con procesos de detoxificación, lo que sugiere que las larvas infectadas con SeMNPV podrían tener tasas de detoxificación más altas que las conspecíficas no infectadas**.** Proponemos que la reducción en la activación de la vía de jasmonato, combinada con el aumento de la detoxificación, podría mejorar la idoneidad de la planta de tomate para *S. exigua*, promoviendo así el crecimiento larvario. Esto a su vez permitiría una mayor producción de viriones, beneficiando la dispersión y transmisión del virus.

Los resultados obtenidos en esta tesis doctoral mejoran nuestra comprensión sobre las interacciones multitróficas entre insectos, plantas y microorganismos beneficiosos. Esta investigación proporciona nuevas perspectivas sobre cómo las defensas de las plantas contribuyen a la eficacia de los entomopatógenos bacterianos y virales, y cómo la infección por baculovirus puede modular las respuestas defensivas del tomate frente a herbivoría. Estos resultados tienen importantes implicaciones en el diseño de programas efectivos de control biológico de plagas. Al elucidar el papel de las defensas de las plantas en la mejora de la acción de los entomopatógenos y comprender cómo la infección por baculovirus influye en los mecanismos de defensa de las plantas, esta investigación podría proporcionar herramientas que optimicen el uso de entomopatógenos en la agricultura sostenible. Además, la interacción entre las defensas de las plantas, la micorriza y los entomopatógenos destaca el potencial de integrar múltiples agentes de control biológico para mejorar el manejo de plagas y la protección de los cultivos. En última instancia, estos conocimientos podrían conducir a enfoques de control de plagas más específicos y eficientes, contribuyendo al desarrollo de sistemas agrícolas más resilientes y sostenibles.

INTRODUCTION

1. Plant-insect interaction

Insects are the largest and most diverse group of organisms on Earth, belonging to the subphylum Hexapoda within the phylum Arthropoda (Chapman et al., 2013; Volkoff et al., 2020). The latest estimates suggest there are around 5.5 million insect species, of which approximately 80 % are currently undescribed (Stork, 2018). There are approximately 30 orders of insects, of which Coleoptera, Lepidoptera, Diptera, Hymenoptera, and Hemiptera are the dominant in terms of named species (Wilson, 2010).

Figure 1. Examples of plant-insect interactions.
A) A *Pseudomyrmex peperi* ant feeds on a Beltian body, a source of nutrients produced by certain *Acacia* species to feed protective ant colonies (Alex Wild®, www.alexanderwild.com). B) An *Halictus ligatus* sweat bee covered in pollen from a *Rudbeckia* flower while feeding on nectar (Alex Wild®, www.alexanderwild.com). C) The fruit capsule of the plant *Aquilaria sinensis* and the hornet seed disperser *Vespa vultina*, about to excise the diaspore (Peakall & Bohman, 2022). (D) A small cluster of leaf beetles from the genus *Xanthonia* feed on leaves of a lacey oak (Alex Wild®, www.alexanderwild.com). (E) An *Helicoverpa armigera* caterpillar, a serious pest of agricultural crops like cotton (www.andermattiberia.com). (F) An adult female of the hemipteran mealybug *Planococcus citri*, a sap-sucking insect of citrus plants (T. Masten Milek).

Many insects rely on plants for food and habitats, while flowering plants depend on insects for pollination. Studies of fossils suggests that plants and insects began to interact around 400 million years ago (Labandeira, 2013). Since then, different types of associations have been established between both groups, most notably mutualism and antagonism (Fig. 1). Mutualistic interactions involve beneficial insects, such as pollinators or seed dispersers, where both the plant and insect benefit (Bronstein et al., 2006). In contrast, antagonistic relationships benefit only the insect, while the plant is negatively affected by the interaction (Giron et al., 2018). These antagonistic relationships have raised special interest in the scientific community due to its negative impact on plant fitness and food supply.

Herbivorous insects, also called phytophagous insects, benefit from plants by feeding on their tissues - such as leaves, roots, and stems - or products like ripe fruits, seeds, or nectar (Del-Claro & Torezan-Silingardi, 2021). They have traditionally been classified by their feeding styles as generalists (or polyphagous), which are able to feed on many plant families; and specialists (or monophagous/oligophagous), which feed only on a limited range of host plants (Ali & Agrawal, 2012). Overall, herbivorous insects constitute approximately 50 % of the all insect species, and a small subset of these, known as agricultural pest, are responsible for 15-20 % of annual crop losses worldwide (Jankielsohn, 2018; van der Meijden, 2015).

In order to meet the needs of the global population, chemical pesticides have been applied in agricultural practices to control herbivore attack over the years, despite their well-documented detrimental effects on human health, non-target organisms, and the environment (Pathak et al., 2022). Moreover, their extensive use has led to the appearance of resistance in target pests, which has prompted the search for sustainable alternatives for plant protection (Hawkins et

al., 2019; Nicolopoulou-Stamati et al., 2016). As a result, integrated pest management (IPM) programs - combining biological, chemical, physical, and cultural management strategies for pest control - are being increasingly applied in crops worldwide (Dara, 2019; Chandler et al., 2011).

2. Plant defenses against herbivory

The interaction between insects and plants is one of the most complex and dynamic associations in nature. A coevolutive process has taken place over time as a result of the selective pressures exerted by both groups (Ehrlich & Raven, 1964; Bruce, 2015). Since plants are sessile organisms, they cannot physically escape herbivore damage, which has forced them to develop sophisticated strategies to overcome insect attack (Mithöfer & Boland, 2012). Among these strategies are physical barriers, such as trichomes or spines, and chemical defenses, such as secondary metabolites or proteins with toxic, repellent or antinutritive properties against insects (War et al., 2018). These defenses can be either constitutive, *i.e.* permanently present in the plant, or inducible by herbivory damage or associated elicitors (Karban, 2011; Chen, 2008). In response to plant defenses, insects have evolved adaptive mechanisms to counteract them, such as detoxification or sequestration of phytochemicals, target site insensitivity, or the development of avoiding behaviors (Heidel-Fischer & Vogel, 2015; Dussourd, 2017). Besides directly affecting herbivores, plants can also indirectly control insect populations by recruiting the natural enemies of the herbivores, such as predators or parasitoids, through the emission of volatiles (Aljbory & Chen, 2018).

2.1. Direct defenses

Plants have developed a wide array of morphological and biochemical defenses that directly affect the feeding, growth, survival, or reproductive success of the insect pests. Structural defenses confer physical protection to the plant by deterring herbivores from feeding, constituting the first line of defense. Among these are spines, thorns, and prickles (spinescence), although they are mainly effective in restricting the feeding rates of vertebrates due to their size relative with the attacker (Hanley et al., 2007). Other mechanical defenses include the epicuticular wax, which increases the slipperiness of leaf surfaces in most vascular plants, hindering insect attachment (Howe & Schaller, 2008; Prüm et al., 2012). Leaf toughness (sclerophylly), resulting from the deposition of macromolecules like lignin or suberin, impedes the penetration of tissue by piercing-sucking insects and accelerate mandibular wear in chewing herbivores (Schoonhoven et al., 2005). Feeding deterrence is also induced by raphides, which are needle-shaped crystals of calcium oxalate found in several plant tissues (Konno et al., 2014; Korth et al., 2006). Pubescence, another physical barrier in plants, consists of the presence of a layer of hairs in leaves, stems, and fruits, that limits insect movement and feeding (non-glandular trichomes) (Kaur et al., 2022). However, trichomes can also function as chemical defense since some types, known as glandular trichomes, can store and secrete toxic secondary metabolites (Tian et al., 2012b; Blanco-Sánchez et al., 2021).

Besides morphological traits, plants also withstand insect attack through the production of a large repertoire of specialized compounds. Secondary metabolites (SMs) are small organic molecules that are not required for basic physiological processes (unlike primary metabolites), but they play a major role in defense against herbivores and pathogens (Wink, 2010). Based on their biosynthetic origins, SMs can be classified into three major groups: terpenoids

or isoprenoids, which are classified according to the number of carbons; alkaloids, which are nitrogen-containing compounds including caffeine and nicotine; and phenols, such as flavonoids and tannins, that consist of aromatic acid compounds with antioxidant properties (Reshi et al., 2023). These chemicals, which are widespread in vascular plants, help control insect pests by acting as deterrents or being directly toxic to the herbivores, impairing their growth, development, survival, or reproduction (War et al., 2012; Mithöfer & Boland, 2012). In addition to SMs, plants also produce defensive proteins with antinutritional properties, such as peroxidases and phenoloxidases (Barbehenn et al., 2010; Felton et al., 1989), or with toxic properties, such as lectins, chitinases, and proteases, which are harmful to insects (Vandenborre et al., 2011; Harrison & Bonning, 2010; Cohen, 1993). To further interfere with the absorption of nutrients, plant also synthetizes proteinase inhibitors, which in addition disrupt the digestion process in herbivores (Dunse et al., 2010; Saikia & Kumar, 2021).

2.2. Indirect defenses

Plants' defensive strategies also rely on the recruitment of higher trophic levels, such as the natural enemies of herbivores. The most common bodyguards attracted by plants are parasitoids and predators, although entomopathogenic nematodes and insectivorous birds can also be involved (Mumm & Dicke, 2010). Indirect defenses may consist of the production and/or release of plant rewards, such as extrafloral nectar (EFN), food bodies, or domatia (structures used as refuges or nesting space), as well as root exudates or specific volatile organic compounds (VOCs) (Heil, 2008). These indirect defenses can be constitutively produced by plants; however, most of them, are induced after herbivory damage or perception.

VOCs are low molecular weight molecules that form odor blends composed mainly of terpenoids (abundant in glandular trichomes), phenylpropanoids/benzenoids, fatty acid derivatives (also called green leaf volatiles), and amino acid derivatives (Dudareva et al., 2013). After herbivory damage or perception, plants release VOCs that differ in quantity or quality from those emitted by non-infested plants (Silva et al., 2017; Ahmed et al., 2022). In fact, herbivore-infested plants can emit odor blends usually composed of more than 200 different compounds (Dicke, 2015). The herbivore-induced plant volatiles (HIPVs) can directly repel herbivores but also attract natural enemies of the insect pest, a strategy commonly referred to as a 'cry for help' (Dicke, 2009). While the abundance and diversity of HIPV vary depending on the plant species, the feeding guild of the attacker, and the host plant's age or genotype, HIPVs serve as reliable cues used by natural enemies to locate their prey (Clavijo McCormick et al., 2012). Additionally, these volatiles can prime neighboring plants to respond more efficiently to a herbivore attack (Paudel Timilsena et al., 2020; Engelberth et al., 2004). The emission of HIPVs occurs not only in the aboveground parts of the plant, like leaves, flowers, or fruits, but also belowground from roots (Rasmann et al., 2005; Grunseich et al., 2020). Roots release exudates into the rhizosphere with high metabolic diversity, including nutrients, primary metabolites, and secondary metabolites (Vives-Peris et al., 2020). These secretions, whose composition can change under biotic stress, can influence the dynamics of soil microbial communities and even recruit beneficial microorganisms to alleviate plant stress (Rolfe et al., 2019).

2.3. Mechanisms of defense induction

The expression of defenses against herbivory is known to be costly for the plant because it reduces the resources available for growth and development (Monson et al., 2022; Züst & Agrawal, 2017). Consequently, plants have evolved inducible defenses as a cost-saving strategy that activates only after wounding and herbivore attack (Karban et al., 1997). The ability of plants to induce defenses relies on the perception of damage-associated molecular patterns (DAMPs) and herbivore-associated molecular patterns (HAMPs).

DAMPs are endogenous danger signals released upon cellular stress or wounding, such as systemin, oligogalacturonides, and extracellular ATP (Tanaka & Heil, 2021; Hou et al., 2019). HAMPs are molecular patterns associated to herbivore feeding, primarily found in oral secretions (OS). The enzyme β-glucosidase was the first reported HAMP elicitor, which was isolated from the OS of the butterfly *Pieris brassicae* (Mattiacci et al., 1995; Bonaventure, 2012). Other types of HAMPs include proteolytic fragments derived from digested plant proteins, like inceptins (Schmelz et al., 2007; Carroll et al., 2008), and modified forms of lipids, such as fatty acid-amino acid conjugates (FACs) and sulfur-containing fatty acids (caeliferins) (Alborn et al., 2007). To mitigate the elicitor-mediated responses, some insects release effectors in their OS - specific proteins and chemicals able to suppress plant defensive mechanisms (Felton et al., 2014; Chen et al., 2019). An example is the enzyme glucose oxidase (GOX), which can act as an elicitor or effector depending on the insect or host plant species (Tian et al., 2012a; Musser et al., 2002). HAMPs and effectors can also arise from ovipositional fluids (Bruessow et al., 2010; Hilker & Meiners, 2006), digestive waste products (Ray et al., 2016b,a), and herbivore-associated endosymbionts (Acevedo et al., 2017; Chung et al., 2013).

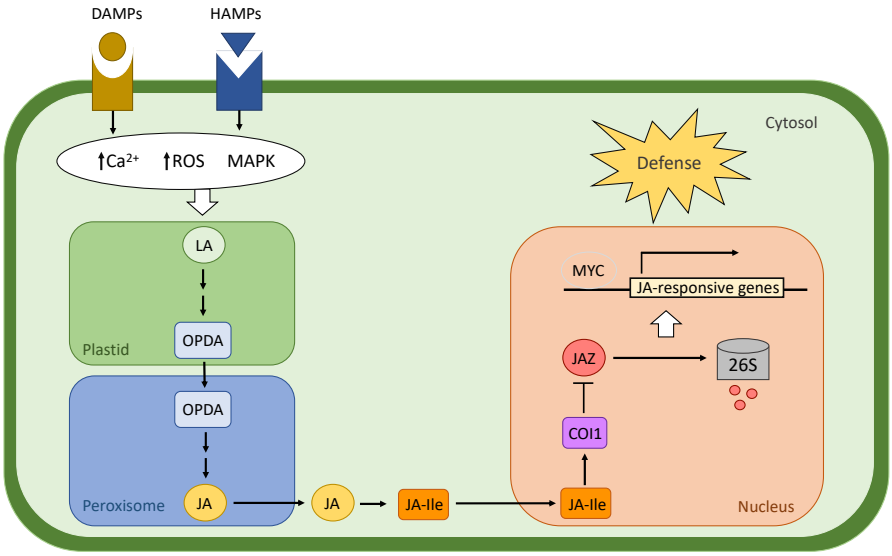

Figure 2. Jasmonic acid signaling pathway.
DAMPs: damage-associated molecular patterns. HAMPs: herbivore-associated molecular patterns. ROS: reactive oxygen species. MAPK: mitogen-activated protein kinases. LA: linolenic acid. OPDA: 12-oxo-phytodienoic acid. JA: jasmonic acid. JA-Ile: jasmonoyl-isoleucine. COI1: coronatine insensitive 1. JAZ: jasmonate ZIM-domain. 26S: proteasome. MYC: transcription factor.

Both DAMPs and HAMPs act coordinately to trigger the activation of the jasmonic acid (JA) pathway, which plays a key role in plant resistance against insect herbivores through the induction of both direct and indirect defenses (Erb et al., 2012; Howe & Jander, 2008) (Fig. 2). The activation of the JA pathway starts with the recognition of HAMPs and DAMPs by receptors on the plasma membrane of plant cells, which initiates early signaling events, including changes in the cytosolic calcium content, the production of reactive oxygen species, and the activation of mitogen-activated protein kinases (MAPK) (Wang et al., 2021b). As a result, α-linolenic acid (18:3) (LA) is released from galactolipids in the chloroplast thylakoid membrane through the action of the

enzyme phospholipase A1 (PLA1). Next, the plastidial enzymes 13-lipooxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) sequentially convert the substrate LA into the oxylipin 12-oxo-phytodienoic acid (OPDA) (Li et al., 2022a). OPDA is then transferred to the peroxisome, where it is reduced into the cyclopentanone 3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) and then converted into the phytohormone JA through several steps of β-oxidation. Finally, JA is transported to the cytoplasm and conjugated with isoleucine to form the jasmonoyl-isoleucine (JA-Ile), which is the biologically active form of jasmonate (Schuman et al., 2018).

After being transferred to the nucleus, JA-Ile is recognized by the F-box protein coronatine insensitive 1 (COI1), part of the Skp1/Cullin/F-box SFC^{COI1} ubiquitin E3 ligase complex, which induces the degradation of JAZ (jasmonate ZIM-domain) repressor proteins (Pauwels & Goossens, 2011). This degradation releases the transcription factor MYC2, leading to the expression of downstream defensive genes involved in the production and accumulation of secondary metabolites and toxic proteins that act against herbivores (Luo et al., 2023). Generally, this resistance is not only induced in the wounded regions but also systematically throughout the plant, via the transport of mobile signals through the phloem to distal intact tissues (Li et al., 2002; Schulze et al., 2019).

3. Induced resistance as a tool for crop protection

Besides the induction of defense after herbivory perception, plants can also develop a long-lasting induced state of resistance triggered by previous exposition of certain biotic stressors, chemicals, or association with beneficial microorganisms in the rhizosphere (Pieterse et al., 2014). This phenomenon, known as induced systemic resistance (ISR), involves the potentiation of innate defenses against subsequent biotic challenges. While ISR has been extensively studied in response to plant pathogens, an increasing number of studies demonstrate its role in herbivore defense as well (Choudhary et al., 2007; Yu et al., 2022). For instance, inoculation of tomato roots with *Trichoderma harzianum* reduced leaf damage caused by the necrotrophic pathogen *Botrytis cinerea* (Martínez-Medina et al., 2013), while association with *Pseudomonas fluorescens SS101* enhanced resistance in *Arabidopsis thaliana* against the insect pest *Spodoptera exigua* (van de Mortel et al., 2012).

Among the different agents that can promote ISR in plants, beneficial microorganisms colonizing roots play a primary role. These beneficial microorganisms include plant growth-promoting rhizobacteria (PGPR), such as *Agrobacteria*, *Pseudomonas*, and several Gram+ *Bacillus*; plant growth-promoting fungi (PGPF), such as *Trichoderma*; nitrogen-fixing rhizobia; root fungal endophytes; and arbuscular mycorrhizal fungi (AMF) (Pineda et al., 2010). In these interactions, plants provide photosynthetic carbon to the associated microorganisms while the microorganisms enhance plant health by increasing nutrient intake, promoting growth, and improving abiotic stress tolerance (Jacoby et al., 2017; Das et al., 2022). Due to their relevance to this thesis, special mention must be made of AMF. These fungi have been reported to provide protection against a wide range of attackers, including insects and

pathogens (Pozo & Azcón-Aguilar, 2007; Rivero et al., 2021; Schouteden et al., 2015). This topic will be discussed in greater detail in the next section.

3.1. Mycorrhiza-induced resistance

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs from the phylum Glomeromycota that establish associations with about 80 % of vascular plants, including agricultural and horticultural crops (Smith & Smith, 2011). This interaction, which began around 400 million years ago, benefits both partners (Strullu-Derrien et al., 2018). In this symbiosis, the fungus penetrates the cortical cells of the root to form intracellular structures called arbuscules, where a bidirectional nutrient transfer occurs. AMF can extend an extracellular hyphal network into the soil, providing the host plant with water and many inorganic nutrients, particularly nitrate and phosphate, in exchange of photosynthates (Bonfante & Genre, 2010). The enhanced water and nutrient uptake by the plant leads to improved overall growth and a greater ability to withstand environmental abiotic stresses, such as salinity (Aroca et al., 2013; Porcel et al., 2012), drought (Li et al., 2019a; Rapparini & Peñuelas, 2014), or presence of heavy metals (Dhalaria et al., 2020; González-Guerrero et al., 2008).

Mycorrhizal plants have also been reported to exhibit greater resistant to biotic attack, a phenomenon known as mycorrhiza-induced resistance (MIR) (Pozo & Azcón-Aguilar, 2007). During arbuscular mycorrhizal (AM) symbiosis, there is an initial increase in salicylic acid (SA) in root cells following the first contact with the fungus to control its proliferation (Jung et al., 2012). However, the establishment of a functional interaction between both partners requires the repression of SA-dependent defensive responses by AMF, which takes place during its colonization of the root cortex. This decrease in SA content is followed by a local increase in JA endogenous levels within arbuscule-containing cells

(Jung et al., 2012). The modulation of the plant immune system during AM symbiosis leads to a primed state of the tissues, allowing for a stronger and/or faster response to a subsequent biotic attack (Pozo & Azcón-Aguilar, 2007; Martinez-Medina et al., 2016a; Mauch-Mani et al., 2017). In addition, the priming of JA-dependent defensive responses has been proposed as the mechanism underlying MIR (Song et al., 2013, 2015; Jung et al., 2009).

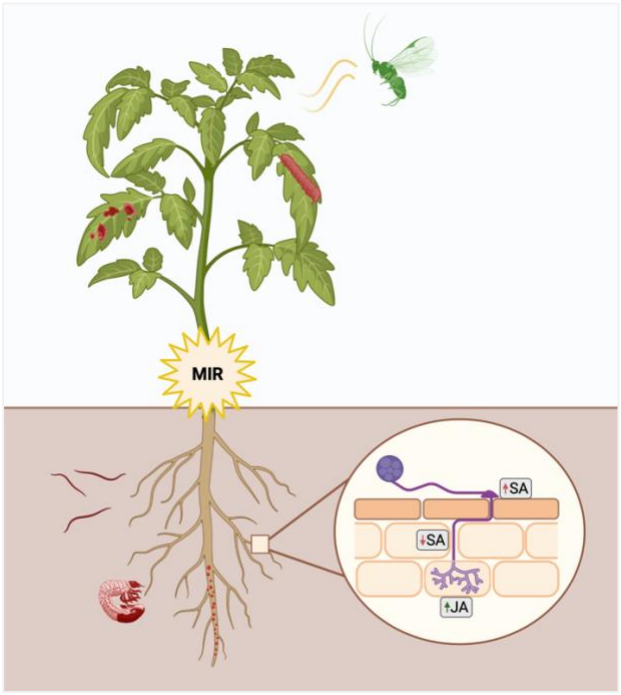

Figure 3. Spectrum of efficiency of mycorrhiza-induced resistance.
In red: insects and pathogens negatively affected by MIR. In green: parasitoids attracted by volatiles emitted from mycorrhizal plants. SA: salicylic acid. JA: jasmonic acid.

Mycorrhizal plants have shown an increased resistance not only against soil-borne fungal and bacterial pathogens, nematodes, or root-chewing insects at the belowground level (Vos et al., 2013; Gange, 2001; Pozo et al., 2002), but also against necrotrophic pathogens and generalist insects in aboveground parts (Ramírez-Serrano et al., 2022; Schoenherr et al., 2019; Sanmartín et al., 2020; Manresa-Grao et al., 2024) (Fig. 3). This resistance may be due to the accumulation of primary and secondary metabolites in the roots of mycorrhizal plants exhibiting MIR, as highlighted by previous transcriptional and metabolic studies (Schliemann et al., 2008; Laparre et al., 2014; Rivero et al., 2015). Additionally, some primed compounds have been also found in the leaves of mycorrhizal plants showing resistance against a biotic challenge (Sanchez-Bel et al., 2016; Rivero et al., 2021). Because of their biostimulant and biopesticide roles, mycorrhizal inoculants – whether based on a single AMF species, a consortium including two or more AMF genotypes/species, or a combination with beneficial bacteria - are receiving increasing interest in agricultural practices and are being commercialized worldwide (Pozo et al., 2021; Basiru et al., 2021).

4. Entomopathogenic allies: microbial defense against insects

A key component of plant defense strategies is the production of indirect defenses that recruit natural enemies of the herbivore pest, such as predators or parasitoids. Among these natural enemies, there is a large group capable of causing disease in insects, which has been often overlooked for its interactions with plants: the microbial entomopathogens. These pathogens are naturally present in a wide variety of ecosystems, are highly specific to insect hosts, and do not cause toxicity to the environment, humans, or beneficial organisms (Lacey et al., 2015). Certain entomopathogens are being commercialized as biopesticides in IPM programs as an alternative to the use of broad-spectrum synthetic pesticides (Irsad et al., 2023). They include protozoa, nematodes, fungi, virus, and bacteria.

4.1. Protozoa

Entomopathogenic protozoa are unicellular eukaryotes found in most habitats and they can be either free-living or parasitic (Sinden, 1977). These protozoa can infect a wide range of insect orders, such as Diptera, Lepidoptera, and Coleoptera (Sarwar et al., 2021). The infection process typically starts with the oral ingestion of the pathogen, which usually cause chronic infections in the host, resulting in a reduction of fitness parameters such as fertility or lifespan. In some cases, the infection can overwhelm the insect's immune system and lead to death (Yadav et al., 2022). An example of entomopathogenic protozoa is the neogregarine *Mattesia oryzaephili*, which is pathogenic for several stored-grain insect pests like the beetle *Oryzaephilus surinamensis* (Lord, 2003).

4.2. Nematodes

Entomopathogenic nematodes are present in the soil and are known to be effective against several insects orders, such as Lepidoptera, Diptera, and Hemiptera (Labaude & Griffin, 2018). The families Steinernematidae and Heterorhabditidae are the most relevant due to their symbiotic association with the bacteria genus *Xenorhabdus* and *Photorhabdus*, respectively (Forst & Clarke, 2002). Infective juveniles (IJ), the only free-living stage of nematodes, penetrate the insect host and release their bacterial symbionts into the hemolymph, causing septicemia and death (Stuart et al., 2006). In addition, these bacteria produce a cocktail of toxic and immunosuppressive compounds that further incapacitate the host (Dillman & Sternberg, 2012). The IJs feed on the cadaver and mature into adults, producing successive generations until depletion of resources. Eventually, new IJs emerge from the cadaver and search for new hosts to infect (Dillman et al., 2012).

4.3. Fungi

Entomopathogenic fungi are present in diverse habitats, although they are most commonly isolated from soil (Sharma & Sharma, 2021). They are able to infect a broad range of insects, including Lepidoptera, as well as different stages of the life cycle (Barra-Bucarei et al., 2019). Many commercial formulations are composed by fungi from the division Ascomycota, particularly the species *Beauveria bassiana* and *Metarhizium anisopliae* (Jaronski, 2010; Quesada-Moraga et al., 2024). The infection process begins when the fungal spores adhere to the insect cuticle, followed by spore germination and penetration into the hemocoel (Ortiz-Urquiza & Keyhani, 2013). Inside the host, fungal hyphae grow, leading to tissue disruption and nutrient depletion, which, together with the production of toxic metabolites, causes the insect's death. Lastly, the hyphae

emerge from the cadaver and produce new spores for fungal dissemination (Litwin et al., 2020).

4.4. Bacteria

Entomopathogenic bacteria are the most widely commercialized group of microbial pathogens for insect control, primarily due to the spore-forming bacterium *Bacillus thuringiensis* (Bt). This gram-positive species from the family *Bacillaceae* alone accounts for approximately 75-95 % of the microbial biopesticide market (Jurat-Fuentes et al., 2021). Bt has been isolated from several ecological niches, such as water, soil, plants, and dead insects, and can infect a wide range of insect orders, like Lepidoptera, Coleoptera, and Diptera (De Maagd et al., 2003). The insecticidal activity of Bt is largely attributed to the synthesis of insecticidal proteins: Cry and Cyt toxins, which are δ-endotoxins stored in parasporal crystalline inclusions during the sporulation phase, and Vip and Sip toxins, which are non-crystalline and produced in the vegetative phase of growth (Palma et al., 2014). In addition to these toxins, other virulence factors in Bt have also been discovered, such as chitinases, which facilitate the permeabilization of the peritrophic membrane (Martínez-Zavala et al., 2020); metalloproteases, which are involved in the evasion of immune responses and mucin layers degradation (Dammak et al., 2015; Fang et al., 2009); and β-exotoxins, which cause their toxic effect by inhibition of DNA-dependent RNA polymerase activity (Malovichko et al., 2019). The specific combination of these virulence factors in each Bt strain defines the bacterium's host range.

Among the proteins produced by Bt, Cry toxins deserve special attention due to their high effectiveness and widespread use, which has led to numerous studies resulting in a well-described mode of action (Fig. 4). The process starts with the oral ingestion of the bacterial spores and their associated crystalline

inclusions by the insect. These crystals solubilize in the alkaline environment of the insect gut, leading to the activation of Cry toxins by proteases and their binding to midgut receptors. This binding induces pore formation in the membrane of midgut cells, causing septicemia and eventually leading to the insect's death (Bravo et al., 2007; Pinos et al., 2021). After the disintegration of the insect's body, the bacteria are released into the environment, facilitating the dispersion of the pathogen. Due to the effectiveness of Bt, sprayable formulations containing spores and crystals from different Bt species, such as Xentari® (Bt subsp. *aizawai*) or Dipel® (Bt subsp. *kurstaki*), have been commercialized worldwide for insect control (Sanahuja et al., 2011). In addition, Cry and Vip3 genes have been cloned and transformed into plants, like cotton, maize, or rice, giving rise to transgenic *Bt* crops that provide resistance against insect pests (Romeis et al., 2006; Koch et al., 2015).

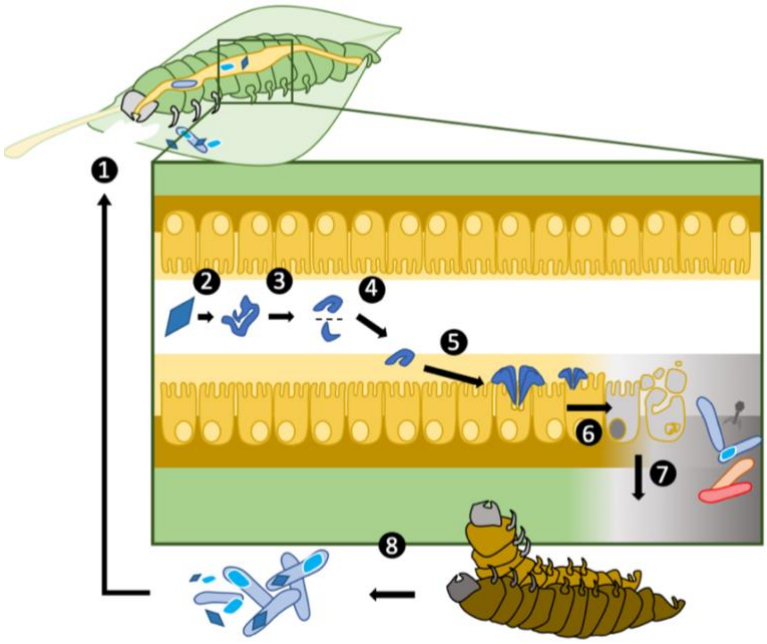

Figure 4. Schematic representation of the mode of action of *Bacillus thuringiensis* pore-forming toxins.

1) Ingestion, (2) Solubilization, (3) Activation, (4) Crossing the peritrophic matrix, (5) Interaction with midgut cells, (6) Oligomerization, pore formation and lysis, (7) Septicemia and death, (8) Dispersion. Pinos and Hernández-Martínez (2019).

4.5. Viruses

Entomopathogenic viruses that are commercialized as biological control agents primarily belong to the family *Baculoviridae*, the largest group of insect-attacking viruses (Valicente, 2019). This family is classified into four genera: *Alphabaculovirus* and *Betabaculovirus* (targeting Lepidoptera), *Gammabaculovirus* (targeting Hymenoptera), and *Deltabaculovirus* (targeting Diptera) (Thézé et al., 2018).

Baculoviruses (BV) are circular double-stranded DNA viruses with a genome ranging from 80 to 180 kpb, packaged in enveloped, rod-shaped nucleocapsides (van Oers & Vlak, 2007). These virions are occluded either individually (as granulovirus) or in groups (as nucleopolyhedrovirus) within proteinaceous occlusion bodies (OBs), which allow them to persist in the external environment, such as the plant surfaces or the soil (Cory & Myers, 2003). The life cycle of baculovirus begins when an insect ingests food contaminated with OBs, which then solubilize in the alkaline environment of the insect midgut, releasing the occlusion-derived viruses (ODV) (Fig. 5). These ODVs enter the epithelial cells of the midgut, initiating replication (primary infection), and produce another form of virions, the budded virus. The latter are responsible for spreading the infection throughout the insect's body (secondary infection), where new ODVs are produced and eventually occluded in new OBs. After the larva dies and undergoes liquefaction, a massive number of OBs are released to the environment, allowing the infection of new susceptible hosts (horizontal transmission) (Clem & Passarelli, 2013).

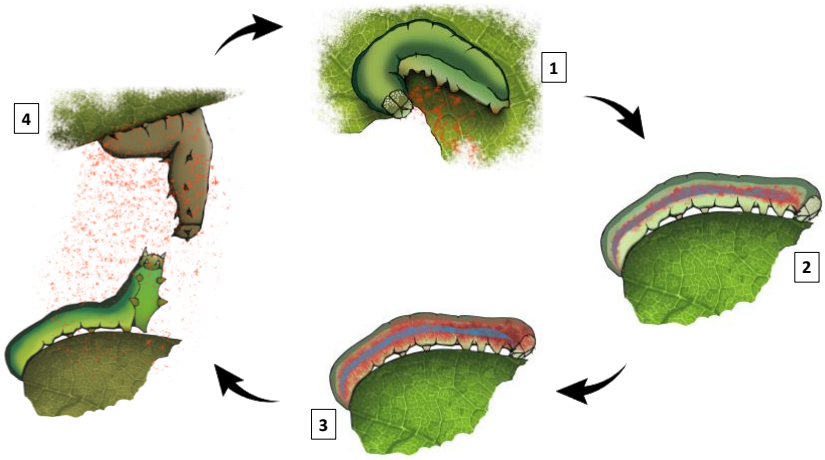

Figure 5. Schematic representation of baculovirus infection cycle.
(1) Ingestion of OBs, (2) Primary infection, (3) Secondary infection, (4) Larval death, liquefaction and viral dispersion.

Baculovirus can also be transmitted from infected adults to their progeny when present as covert infections (vertical transmission) (Cory, 2015). These viruses are known to manipulate the behavior of their hosts by inducing hyperactivity and/or causing them to climb to elevated positions before death (a phenomenon known as tree-top disease), which aids in viral dispersion (Gasque et al., 2019). The insecticidal effects of baculoviruses have led to the development of commercial formulations targeting several insect species, such as SPOD-X™, which is used to control populations of *Spodoptera exigua* (Moscardi et al., 2011).

5. Plant-insect-entomopathogen interaction

The interplay between plants, insects, and entomopathogens is dynamic and complex (Fig. 6). Due to their high effectiveness in decimating insect populations, it is believed that entomopathogens may benefit plants through top-down effects, such as directly infecting insect herbivores. Conversely, plants may enhance the dispersion, persistence, and effectiveness of these pathogens through bottom-up effects, such as by modulating plant chemistry and structure to favor these allies (Cory & Hoover, 2006; Shikano, 2017). For example, plant architecture, such as canopy structure, leaf shape, and color, can diminish the harmful effects of ultraviolet light on OBs from baculoviruses or Bt spores (Elliot et al., 2000). In contrast, alkaline exudates produced by some plants, like cotton and soybean, can inactivate OBs, which is likely due to their premature dissolution (Young et al., 1977). Herbivore-induced plant volatiles can either enhance (Lin et al., 2016; Hountondji et al., 2005) or inhibit (Brown et al., 1995) the conidia germination of entomopathogenic fungi. In addition, the belowground plant volatile (E)-β-caryophyllene has been involved in the attraction of the entomopathogenic nematode *Heterorhabditis megidis* to maize roots in response to feeding by weevil larvae (Rasmann et al., 2005). Plant can also act as vectors of insect viruses; for example Myzus persicae densovirus is transported through the phloem of *Brassica oleraceae* attacked by infected aphids, leading to the infection of healthy aphids (Van Munster et al., 2005).

Plants can also indirectly influence the efficacy of entomopathogens by modulating insect susceptibility to them (Shikano, 2017; Cory & Hoover, 2006). Studies have shown that the ingestion of entomopathogens, especially baculovirus and bacteria, on different host plants can greatly impact on insect mortality (Kouassi et al., 2001; Ali et al., 2004, 1998; Wan et al., 2016). Plant-mediated effects on larval susceptibility are probably due to the alteration of

some insect parameters such as the immunity system, the peritrophic membrane, or the gut microbiota.

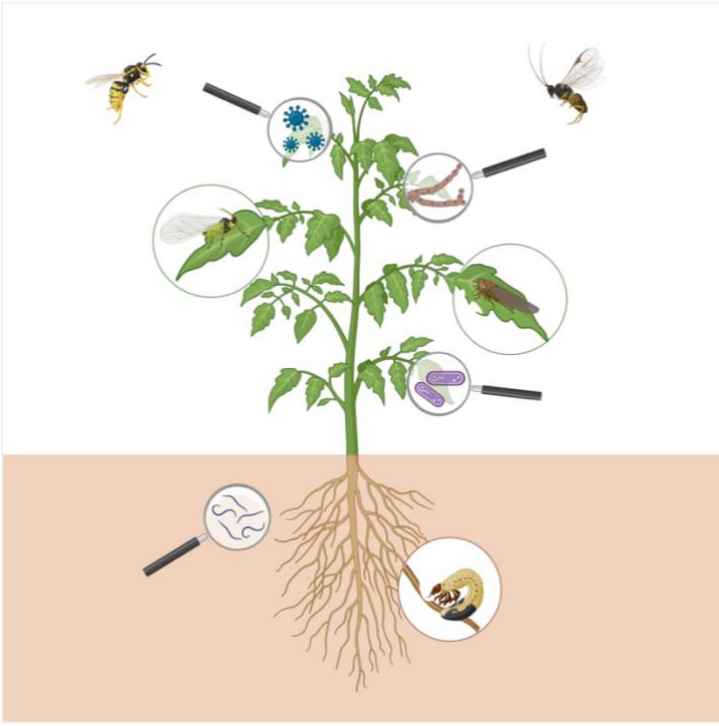

Figure 6. Representation of multritrophic interactions between plants, insects, and microbial entomopathogens.

Invertebrates have an innate immune system comprising both humoral and cellular responses, which are triggered upon pathogen recognition, as well as antiviral activity from small RNAs (Kingsolver et al., 2013). Humoral responses involve the production of proteins and small antimicrobial peptides released into the hemolymph, while cellular responses encompass phagocytosis of invading microorganisms and encapsulation of larger invaders, such as parasitoid eggs (Strand, 2008). Phytochemicals and diet quality can mediate insect-pathogen

relationships by changes in insect immune responses. Diets with a higher protein-to-carbohydrate ratio have been associated with increased survival of lepidopteran larvae when challenged with baculovirus, Bt, and *Bacillus subtilis*, likely due to enhanced immune activity (Lee et al., 2006; Shikano & Cory, 2014, 2015; Povey et al., 2009). Another study found that *Trichoplusia ni* larvae fed on broccoli were less susceptible to BV infection and had a higher number of hemocytes (Shikano et al., 2010). Conversely, a poor-quality of the diet has also been linked to increased susceptibility of lepidopteran insects, such as *S. exigua* and *Helicoverpa zea*, to the bacterium *Serratia*, suggesting that nutrition plays a relevant role into the transition from commensal bacteria to pathogens (Mason et al., 2022). Other studies have investigated the effects of some HIPVs on the immunity of *Spodoptera* caterpillars. For example, pre-exposure to the terpenoid linalool increased the activity of an insect immune marker, the phenoloxidase (PO), in *S. litura* larvae and reduced their mortality after infection with Bt (Ghosh & Venkatesan, 2019). However, Gasmi et al. (2019) found that pre-exposure to linalool and indole increased the susceptibility of *S. exigua* to Bt and baculovirus, with no changes in PO activity detected.

Another strategy used by plants to mediate insect-pathogen interaction involves the disruption of the peritrophic membrane (PM), a physical barrier composed by chitin, glycoproteins, and proteoglycans that covers the midgut epithelium of insects (Wang & Granados, 2001; Barbehenn, 2001). The PM is essential for digestion, protection against pathogen infection, mechanical damage, and acts as a functional antioxidant (Erlandson et al., 2019). Its integrity can be altered by structural plant defenses, such as trichomes (Kariyat et al., 2017), and chemical plant compounds, including lectins (Vandenborre et al., 2011), proteases (Pechan et al., 2002), and chitinases (Konno & Mitsuhashi, 2019). Alterations in PM integrity can affect insect susceptibility to

entomopathogens. For example, maize exposed to caterpillar feeding produced a 33-kDa cysteine protease (Mir1-CP), which impaired nutrient absorption and growth of *S. frugiperda* larvae and severely damaged insect PM (Pechan et al., 2002). Another study found that combining Mir1-CP with the insecticidal protein Cry2A from Bt had a synergistic effect on the mortality of different lepidopteran species (Mohan et al., 2008). Regarding viral infections, adding chitinase to the diet enhanced the activity of baculovirus against the gypsy moth *Lymantria dispar* (Shapiro et al., 1987). Furthermore, Plymale (2008) demonstrated that feeding on different plant species influence PM thickness and the susceptibility of *Heliothis virescens* larvae to baculovirus infection.

Finally, the gut microbiota of herbivorous insects plays a key role in regulating some host processes such as metabolism, development, and immunity (Voirol et al., 2018; Engel & Moran, 2013). Previous studies have reported that diet can influence the composition, diversity or abundance of the gut microbiota in lepidopteran insects (Hammer et al. 2017; Martínez-Solís et al. 2020), and host plants have been shown to shape the gut microbial communities of some insect pests (Tang et al., 2012; Chung et al., 2017; Mason & Raffa, 2014). Alterations in gut microbiota have been linked with changes in the host susceptibility to entomopathogens (Hernández-Martínez et al., 2010a; Jakubowska et al., 2013; Caccia et al., 2016). For instance, Mason et al. (2019) reported that plant defenses disrupting PM integrity, such as trichomes and chitinases, facilitated opportunistic colonization of resident gut bacteria in the body cavity of *S. frugiperda*, thereby potentiating the negative impact of plant defenses in herbivore performance. Taking this into account, plants may indirectly contribute or interfere with the efficacy of entomopathogens against pests through the induction of changes in the insect gut microbiota. In fact, it has been shown that the pre-exposure of *S. exigua* larvae to the plant volatile indole was

related to changes in the composition, load, and diversity of insect gut microbiota, which was in turn correlated with an increase on larval susceptibility to Bt and baculovirus (Gasmi et al., 2019b).

In this work, we used as a model system to evaluate multitrophic interactions among insects, plants, entomopathogens, and soil beneficial microbes the following organisms: the tomato plant, the insect pest *Spodoptera exigua*, the arbuscular mycorrhizal fungus *Funneliformis mosseae*, and the microbial entomopathogens *Bacillus thuringiensis* and Baculovirus.

5.1. The plant: tomato

Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family and the *Solanum* genus, which includes several wild tomato species like *S. pimpinellifolium*, *S. peruvianum*, and *S. cheesmaniae*, as well as a diverse range of cultivated varieties derived from *S. lycopersicum* L. (Knapp & Peralta, 2016). This dycotiledonous plant, originated in the Andean region of South America, has a perennial vegetative cycle (Bergougnoux, 2014). Tomato is the second most produced vegetable globally, after potato, and is valued for its high nutritive value and antioxidant properties (Quinet et al., 2019). It is commonly used as a model system for studying fruit development, plant stress response, and functional genomics, making it a valuable resource for both basic and applied research (Matsukura et al., 2008; Liu et al., 2022).

Tomato is susceptible to a variety of diseases and pests, which can significantly impact crop productivity. The main microbial pathogens affecting tomato are viruses (*e.g.*, tomato yellow leaf curl virus), bacteria (*e.g.*, *Pseudomonas syringae* pv. *tomato*, *Xanthomonas* spp.), fungi (*e.g.*, *Botrytis cinerea*, *Fusarium oxysporum*), or nematodes (*e.g.*, *Meloidogyne* spp.) (Blancard, 2012). In addition to microbial pathogens, tomato face challenges

from various arthropod pests, including caterpillars such as *S. exigua* (Lepidoptera: Noctuidae) and *Tuta absoluta* (Lepidoptera: Gelechiidae), beetles such as *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), mites such as *Tetranynchus urticae* (Trombidiformes: Tetranychidae), and whiteflies such as *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Wakil et al., 2017).

5.2. The insect: *Spodoptera exigua*

Spodoptera exigua (Lepidoptera: Noctuidae) (Hübner, 1808), commonly known as beet armyworm, is a polyphagous chewing caterpillar. Originating in Southeast Asia, it has spread globally over the past two centuries and was first described in North America in 1876 (Greenberg et al., 2001). Today, it is distributed worldwide, especially in warm climates, and causes significant damage to the leaves and fruits of a wide range of ornamental and crop plants, including tomato, pepper, cabbage, and cotton (Maharjan et al., 2022; Zheng et al., 2011). The lifecycle of this insect pest includes several stages: eggs, which are laid as clusters of 50-150; larvae, which go through five instars; pupae, typically formed in the soil; and adults, which require specific photoperiod for mating and oviposition (Capinera, 1999) (Fig. 7). Traditional control methods for *S. exigua* have been relied heavily on chemical pesticides such as pyrethroids, organophosphates, and carbamates, leading to issues with development of resistance (Hafeez et al., 2022). Consequently, alternative organic control strategies are being explored, including the use of natural enemies. Parasitoids such as the wasps *Chelonus insularis* and *Cotesia marginiventris* (Hymenoptera: Braconidae), or predators, like the bugs *Orius* spp. (Hemiptera: Anthocoridae) and *Geocoris* spp. (Hemiptera: Geocoridae) are commonly used (Ehler, 2007). Additionally, biopesticides based on microbial entomopathogens, including Bt (*e.g.*, XenTari®, DiPel®), baculoviruses (*e.g.*, Spod-X®, Spexit®), nematodes

(*e.g.*, CAPSANEM®), and fungi (*e.g.*, BIOSOFT®) are also being commercialized to reduce plant infestation by *S. exigua* larvae (Irsad et al., 2023).

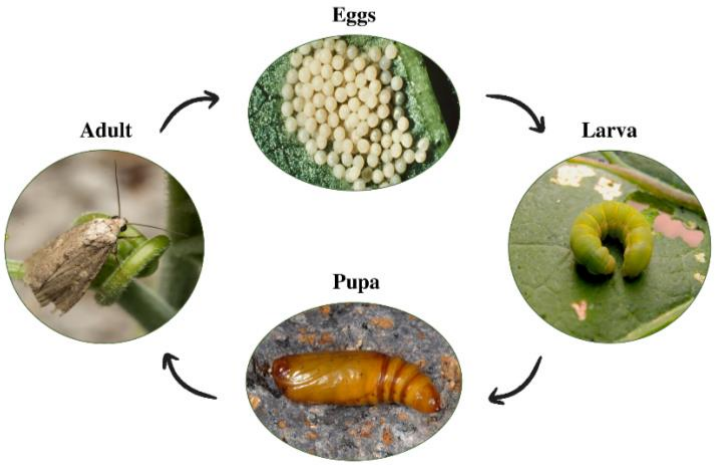

Figure 7. Life cycle of the beet armyworm *S. exigua*.

5.3. The microbes

5.3.1. Entomopathogens

In this research, both bacterial and viral microbial entomopathogens were used. The bacterial entomopathogen used was *B. thuringiensis* subspecies *aizawai*, present in the commercial sprayable formulation Xentari®, which contains a mixture of spores, toxins, and cells from this bacteria. From now on, the bacterial entomopathogen is referred throughout the text as Xentari, for the sake of brevity. This product has demonstrated proven efficacy against *S. exigua* (Yee & Toscano, 1998; Hernández-Martínez et al., 2010b; Martínez et al., 2004). The viral entomopathogen employed was Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV), a specialist baculovirus highly infectious to its host. Specifically, we selected the native Spanish isolate SeMNPV-SP2,

which has been widely used in our laboratory to study insect-virus interactions (Llopis-Giménez et al., 2021; Jakubowska et al., 2013; Gasmi et al., 2019a).

5.3.2. Arbuscular mycorrhizal fungi

The soil beneficial microorganism used in this work was the AMF, *Funneliformis mosseae* (Fm). This fungus belongs to the family *Glomeraceae* and it is widely used in commercial mycorrhizal inoculants for crops, gardens, and landscapes, due to its benefits for plant health, soil fertility, and sustainable agricultural practices (Ullah et al., 2023; Basiru & Hijri, 2022; Řezáčová et al., 2023; Huang et al., 2019). In addition, previous studies have demonstrated Fm's ability to induce MIR in tomato against some biotic stressors such as the chewing insect *S. exigua* or the fungal pathogens *Alternaria solani* and *Beauveria bassiana* (Dejana et al., 2022; Song et al., 2015; Rivero et al., 2021).

OBJECTIVES

Continuous insect herbivory threatens plant health and fitness. This pressure has driven plants to evolve a wide arsenal of defenses, which can harm directly the herbivore or indirectly by attracting natural enemies. In this multitrophic context, plants also live in close association with beneficial microorganisms such as arbuscular mycorrhizal fungi or entomopathogens, which can aid plants in defending themselves. However, how these microbes contribute to the plant's defensive strategies against insects or, in turn, how defenses might affect the effectiveness of entomopathogens, still remains unclear. This research aims to shed light on multitrophic interactions between tomato plants, the lepidopteran pest *Spodoptera exigua*, the arbuscular mycorrhizal fungus *Funneliformis mosseae*, and the entomopathogens *Bacillus thuringiensis* and baculovirus. To address this, the following specific objectives have been established:

1. Evaluating the effect of constitutive and inducible tomato defenses on *B. thuringiensis* efficacy against *S. exigua*, and search for potential bioactive compounds.

2. Exploring the impact of mycorrhizal symbiosis in tomato on *S. exigua* fitness and susceptibility to the entomopathogens *B. thuringiensis* and baculovirus.

3. Unraveling whether baculovirus infection modulates the tomato antiherbivore response against *S. exigua.*

METHODOLOGY

This section provides a concise overview of the methodologies and materials used throughout the thesis. More detailed descriptions can be found in the 'Material and Methods' sections of each chapter.

To achieve the various experimental objectives, a laboratory *Spodoptera exigua* colony was first established. This colony was initiated from eggs supplied by Andermatt Biocontrol AG (Grossdietwil, Switzerland) and maintained in our laboratory at the Universitat de València (Spain) for over 10 years. The colony was kept in a growth chamber under controlled conditions: a temperature of 25 ± 3 °C, relative humidity of 70 ± 5 %, and a photoperiod of 16 h light: 8 h dark. While larvae were continuously reared on artificial diet supplemented with 0.05 % of tetracycline, adults were provided *ad libitum* access to a 10 % sugar solution. The microbial entomopathogens used in this research were the bacteria *Bacillus thuringiensis* subsp. *aizawai* (XenTari®) and the Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV; SP2 strain).

In the **first chapter**, titled 'Influence of constitutive and inducible defenses on the susceptibility of *Spodoptera exigua* to *Bacillus thruringiensis*', we selected a total of fifteen wild tomato species and forty cultivated varieties based on their diverse fruit metabolic composition. The seeds for these plants were provided by the Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV) of the Universitat Politècnica de València (Spain). The plants were then grown in a controlled greenhouse at Universitat de València with temperatures ranging from 20 to 26 ºC and a photoperiod of 16h light: 8h dark.

To disentangle the effects of constitutive and inducible defenses on larval performance and susceptibility to Bt, we developed a bioassay method using a leaf-based artificial diet. This diet was elaborated with lyophilized (freeze-dried)

leaf powder. The advantage of this approach, compared to using fresh leaves or conducting on-plant bioassays, is that it preserves the exact metabolic composition of the leaves at the time of the sampling, preventing any further activation of metabolic pathways related to the herbivory caused by the larvae during the bioassay. Additionally, using lyophilized leaves allowed us to focus solely on the effects of chemical defenses by eliminating the influence of physical defenses.

To assess the impact of constitutive defenses on larval development, we performed bioassays in which newly molted second instar *S. exigua* larvae were individually placed into wells of a bioassay tray and fed the different leaf-based artificial diets for 48 hours. After this period, the leaf-based diets were replaced with an artificial diet until the end of the experiment. Larval relative growth was determined by calculating the biomass gained (in milligrams) per milligram of initial body weight after 48 hours of feeding.

To study the influence of constitutive defenses on the susceptibility of *S. exigua* to *B. thuringiensis*, newly molted second instar larvae were infected using the droplet feeding method. Specifically, larvae were placed in Petri dishes where they were allowed to fed four μL droplets of a phosphate-buffered saline (PBS) solution containing 10 % sucrose, 10 % (v/v) phenol red, and a sublethal concentration of XenTari®, a commercial product composed of a mixture of toxins and spores of *B. thuringiensis* var. *aizawai*. After selecting the red colored larvae, which had ingested the entomopathogen, they were fed plant diets for 48 hours, after which they were switched to an artificial diet until the end of the bioassay. Control larvae fed the same solution without the entomopathogen, and mortality rates and relative larval growth of both mock- and Bt-infected larvae were recorded.

To evaluate the effects of induced tomato defenses on *S. exigua*, six cultivated varieties with contrasting levels of constitutive resistance were selected from the first assay and grown in a greenhouse. Then, they were sprayed with a solution of the phytohormone methyljasmonate (MeJA) in order to elicit jasmonic acid-inducible defenses. Control plants were treated with a solution containing only the solvent. Leaves were harvested 72 hours after the treatment to ensure the accumulation of inducible compounds and then processed to prepare leaf-based artificial diets. Growth and Bt susceptibility bioassays were conducted as previously described, and relative larval growth and mortality rates were recorded.

To identify metabolites overaccumulated after MeJA treatment, an untargeted metabolomic analysis using liquid chromatography coupled with mass spectrometry (LC/MS-MS) was performed on one leaflet from each treatment and variety. An Acquity UPLC system (Waters, Mildford, MA, USA) coupled with a hybrid quadrupole time-of-flight equipment (QTOF MS Premier) was used to obtain the mass-to-charge ratios (m/z) of metabolites. Raw data were obtained using Masslynx v.4.2 software (Waters, USA) and processed with R software v.4.3.2. Signal comparisons among treatments were conducted using MarVis Suite 2.0 software, and metabolite identification was achieved through the utilization of online metabolomic databases. MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca) was used to generate heatmap plots.

In the **second chapter**, titled 'Compatibility of mycorrhiza-induced resistance with viral and bacterial entomopathogens in the control of *Spodoptera exigua* in tomato', various approaches were employed to explore this topic. *Solanum lycopersicum* cv. MoneyMaker (MM) plants were grown in a controlled greenhouse at the Zaidín Experimental Station (CSIC, Granada) under conditions of 24/16 ºC and a 16h:8h light/dark photoperiod. Plants were

subjected to herbivory by *S. exigua* larvae and/or mycorrhization with the arbuscular mycorrhizal fungus (AMF) *Funneliformis mosseae*, resulting in four groups of plants: control (Nm-), herbivory only (Nm+), mycorrhization only (Fm-), and both herbivory and mycorrhization (Fm+). Plants were harvested 8 weeks after mycorrhiza inoculation and 15 days after starting of herbivory, and leaflets were lyophilized to prepare leaf-based artificial diets.

To assess the impact of mycorrhization and herbivory on larval development, newly molted fourth instar *S. exigua* larvae were fed the different plant diets for 48 h, after which larval relative growth was recorded.

To examine the influence of both treatments on larval susceptibility to entomopathogens, newly molted second instar *S. exigua* larvae were infected with *B. thuringiensis* and baculovirus using the droplet feeding method. Bt infections were conducted using both sublethal and lethal concentrations of XenTari®, while viral infections were carried out using a sublethal concentration of SeMNPV. SeMNPV- and Bt-infected larvae, along with their respective control groups, were fed the different plant diets for 48 hours, and then reared exclusively on an artificial diet until days 7 and 8, respectively. Mortality rates were recorded at the end of the bioassay.

To evaluate whether mycorrhization and herbivory could impair the immune system of *S. exigua*, the phenoloxidase (PO) enzymatic activity in hemolymph samples from larvae used in the growth assay was measured. Hemolymph was extracted, hemocytes were removed by centrifugation, and the remaining hemolymph was used to determine the activity of this insect immune marker by measuring absorbance of the PO substrate (TECAN Group Ltd, Switzerland). Additionally, PO inhibition assays were performed to investigate whether plant compounds could interfere with PO activity. For this, plant

compounds were extracted from leaf powder from the different treatments, and incubated with larval hemolymph and PO substrate for 15 min at room temperature, after which PO activity was calculated (TECAN Group Ltd, Switzerland).

To assess the effect of mycorrhization and herbivory on larval gut microbiota composition, a 16s barcoding of the guts from larvae previously used for PO activity quantification was carried out. Total DNA was extracted from homogenized guts, followed by PCR amplification and sequencing of the 16S rRNA gene using Illumina (FISABIO, Valencia). The sequences obtained were processed with QIIME v.1.9, and then bacterial composition and diversity were analyzed. Additionally, bacterial load was calculated by amplifying total DNA with universal primers for the 16S rRNA gene using a quantitative PCR.

In the **third chapter**, titled 'Modulation of tomato defense responses against *Spodoptera exigua* herbivory by baculovirus infection', we delved into the mechanisms involved in this multitrophic interaction. To address this, newly molted fourth-instar *S. exigua* larvae were infected with a lethal concentration of SeMNPV using the droplet feeding method. At 72 hours post infection, mock- and SeMNPV-infected larvae were allowed to feed *S. lycopersicum* cv. Moneymaker tomato plants for 3 hours. After feeding, the larvae were removed, and the damaged leaflets were cut, frozen in liquid nitrogen, and stored at -80 ºC. At 24 hours post induction, both damaged leaflets and undamaged leaflets distal to the damaged ones were collected and processed as described above.

To assess the influence of baculovirus infection on larval feeding rate, the areas of consumed leaflets after 3 hours of herbivory were quantified using LeafByte, an open source mobile application for measuring herbivory.

To evaluate the plant transcriptional responses to herbivory by larvae infected with baculovirus, RNA was extracted from the frozen leaflets, and quantitative PCR (qPCR) was performed to quantify the expression of the JA biosynthesis genes *AOS* (allene oxide synthase) and *OPR3* (12-oxophytodienoate reductase 3), as well as the late JA responsive genes *PIN2* (protease inhibitor II) and *PPO* (polyphenol oxidase). Besides exploring the jasmonate pathway, the expression of salicylic acid-related genes *ICS* (isochorismate synthase) and *PR1* (pathogenesis-related protein 1) was also measured. The gene *SlEF* (*Solanum lycopersicum* elongation factor 1α) was used as housekeeping gene for expression normalization.

To assess the influence of herbivory by larvae infected with baculovirus on tomato phytohormone levels, leaf samples were subjected to LC-MS/MS analysis. An Acquity UPLC system liquid chromatography coupled with a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK) was used to quantify the levels of hormones such as OPDA (12-oxo-phytodienoic acid), JA (jasmonic acid), JA-Ile (jasmonic acid-isoleucine conjugate), SA (salicylic acid) and its conjugates SGE (salicylic acid glucose ester) and SAG (salicylic acid glucoside) in the damaged leaflets. External calibration curves for each tested compound were prepared using pure standards.

To investigate whether baculovirus infection could alter the composition of insect' oral secretion (OS), the protein composition of the OS from mock- and SeMNPV-infected larvae (after 3 hours of herbivory on tomato) was analyzed by LC-MS/MS. Proteomic analysis on the OS was carried out at the Central Service for Experimental Research (SCSIE) at the Universitat de València. A mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSCIEX) was used for peptide analysis. The raw data obtained were analyzed using DIA-NN 1.8

software, which generated the theorical spectra corresponding to the amino acid sequences of the insect of interest, as available in the Uniprot datatabase.

CHAPTER 1:

Influence of constitutive and inducible tomato defenses on the susceptibility of *Spodoptera exigua* to *Bacillus thuringiensis*

This chapter has been published as:

Frattini A., González-Martínez R.M., García J.M., Minchev Z., Pozo M.J., Flors V., Crava C.M., Herrero S. 2024. Constitutive and inducible tomato defenses contribute to *Bacillus thuringiensis* lethality against *Spodoptera exigua*. *Biological Control*, **198**:105624.

1.1. Introduction

The interaction between plants and herbivorous insects started around 400 million years ago, giving rise to a coevolutionary process wherein plants have been compelled to acquire different defensive strategies (Labandeira 2013). These strategies encompass the production of a wide array of direct defenses, such as physical barriers (*i.e.* trichomes, cuticle, etc…) or toxic and antinutritional compounds (*i.e.* secondary metabolites, proteinase inhibitors, etc…) (War et al. 2018; Fernández de Bobadilla et al. 2022). Plant defenses may be constitutively produced, or induced following herbivory damage or detection (Hilker and Meiners 2006; Felton et al. 2014; Watts et al. 2023). In response, insects have developed mechanisms to counteract plant defenses, including sequestration, detoxifying or avoiding behaviours (Heidel-Fischer and Vogel 2015; Dussourd 2017). Furthermore, plants encompass additional layers of defense, such as indirect defenses that involve the emission of volatiles acting as prey cues to attract natural enemies of the herbivores, including arthropod predators or parasitoids (Aljbory and Chen 2018). Plant inducible defenses against herbivores are quickly triggered upon herbivory and finely regulated by signaling pathways coordinated by jasmonic acid and related compounds, collectively known as jasmonates (JAs) (Turner et al. 2002; Howe and Jander 2008). Indeed, exogenous treatment with JAs induced proteinase inhibitors, alkaloids and other bioactive secondary metabolites (Thaler et al. 1996; War et al. 2015; Flores-Sanchez et al. 2016). For example, exogenous application of methyljasmonate (MeJA) is a common strategy used to trigger inducible plant responses against insect herbivores, such as chewing herbivores (Rodriguez-Saona et al. 2001; Tian et al. 2014; Senthil-Nathan 2019).

In nature, insect herbivores are exposed to a wide range of microbial entomopathogens. This term includes all the microorganisms that have the ability to infect and kill different species of arthropods (Ruiu 2018). Due to their host specificity and lack of toxicity to non-target organisms or the environment, microbial entomopathogens are widely used as control agents in integrated pest management programs (Lacey et al. 2015; Irsad et al. 2023). Among entomopathogens, bacteria, baculoviruses, fungi, and nematodes are the most applied as biopesticides. Currently, their commercial use accounts for about 5 % of the entire crop protection market with expectations that this percentage will rise from 10 % to 20 % in the coming years (Sabbahi et al. 2022). The bacterium *Bacillus thuringiensis* (Bt) is the most commonly used entomopathogen in pest control, with formulations based on it representing around 75-95 % of the microbial biopesticide market (Jurat-Fuentes et al. 2021). Bt is a gram-positive bacterium which is present in different ecological niches including soil, water, plants and dead insects, and it is effective against several insect orders such as Lepidoptera, Coleoptera and Diptera (Palma et al. 2014). The infection process begins with the oral ingestion of the bacterial sporulated cells, and/or their formulated spores and the associated insecticidal crystals, which are present in the soil or on the phylloplane. Once inside the insect's gut, the crystals are solubilized, leading to the activation of the insecticidal proteins that form the crystal (Cry toxin). These proteins bind to receptors in the midgut cell membrane, inducing pore formation and ultimately causing the death of the insect (Bravo et al. 2011). Entomopathogens may be targeted by the plant defensive strategies, which may promote the action of these herbivores' enemies for their own benefit (Rasmann et al. 2005; Mohan et al. 2008a; Lin et al. 2016; Hay et al. 2020). For example, maize defensive responses target the caterpillars' protective gut barrier. This accelerates the invasion of gut bacteria into the larval body cavity where

they exacerbate the negative impact of plant defenses on the insect (Mason et al. 2019).

It is known that the effectiveness of Bt formulations against insect pests can be influenced by various plant traits. For instance, nutrition quality of plants can impact Bt efficacy (Shikano and Cory 2014), as well as plant-produced compounds modulate the insect interaction with the bacterium (Macintosh et al. 1990; Mohan et al. 2008b; Gasmi et al. 2019). Additionally, certain plant defenses are known to interfere with the digestive process of the insect (Chen et al. 2005; Bhonwong et al. 2009), impact on its immune system (Shi et al. 2020; Chen et al. 2022) or disturb the integrity of the peritrophic membrane (Pechan et al. 2002; Kariyat et al. 2017). These alterations can increase the susceptibility of the target insect to *B. thuringiensis*, being the basis of the contribution of the host plant genotype in shaping insect-pathogen interaction (Janmaat and Myers 2005; Carrière et al. 2005; Bird and Akhurst 2007). In this context, Shikano et al. (2018) found that tomato induced defenses enhanced the efficacy of Bt against the polyphagous caterpillar *Helicoverpa zea*. They suggested that orthoquinones, resulting from the oxidation of chlorogenic acid by plant PPO enzymes, were responsible for alkylating Bt prototoxins, which increased their solubilization and/or proteolysis, thereby enhancing the effectiveness of the toxins (Ludlum et al. 1991).

Given this, we hypothesized that plant defenses could modulate insect-Bt interactions by increasing the susceptibility of the insect pest to the bacterial entomopathogen. In the present work, we investigated the interaction between constitutive and inducible foliar defenses in tomato (*Solanum lycopersicum*) and Bt infection in the polyphagous caterpillar *Spodoptera exigua*. To explore the diversity of constitutive chemical defenses, we compared 55 tomato genotypes,

including fifteen wild tomato species and forty cultivated tomato varieties. We established a bioassay system using leaf-based artificial diets to assess the impact of constitutive chemical defenses from tomato on *S. exigua* performance, in the presence or absence of Bt. We then selected six varieties showing contrasting levels of resistance to *S. exigua* herbivory and performed further experiments to assess the combined effects of constitutive and inducible defenses on Bt-infected larvae. To induce defenses, plants were treated with MeJA to activate JA-dependent responses, and the effects on larval growth and susceptibility to Bt were recorded. Finally, we characterized the chemical changes in elicited leaves that may have contributed to the observed effects. For that, we performed an untargeted metabolomic analysis to identify metabolites that accumulated after elicitation of plant defenses and that may play a role in defense against *S. exigua* and increase susceptibility to Bt. Overall, our findings show that both constitutive and herbivore-inducible tomato chemical defenses increase the action of Bt against *S. exigua*, and we identified key metabolites that may regulate this interaction.

1.2. Material and methods

1.2.1. Insects

The *Spodoptera exigua* caterpillars used in this study were sourced from a laboratory colony established over 10 years ago from eggs supplied by Andermatt Biocontrol AG (Grossdietwil, Switzerland). The colony was maintained in a growth chamber under controlled conditions, with a temperature of 25 ± 3 °C, relative humidity of 70 ± 5 % and a photoperiod of 16h light: 8h dark. Larvae were reared on artificial diet (Elvira et al. 2010), while adults were provided *ad libitum* access to a 10 % sugar solution.

1.2.2. Tomato varieties and leaf-based diet preparation

The wild tomato species and cultivated varieties were provided by the Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV) of the Universitat Politècnica de València (Spain). The selection of the varieties for this study (Table 1, Supplementary) was based on their diversity in fruit metabolic composition (Antonio Granell, Personal communication; Pons et al. 2022, 2023). Tomato seeds were sown in jiffys (0.4 cm), composed by pressed coconut fiber, inside seedbeds (6.5 x 6.5 cm). They were grown in a controlled greenhouse with a temperature ranging 20-26 ºC during the day and 19-22 ºC at night, and with a diurnal photoperiod of 16 h light and 8h dark (natural light supplemented with fluorescent tube lighting at dawn and dusk). Plant growth was monitored, no phytosanitary treatments were applied in the greenhouse, and no auxiliary insects were released to avoid untargeted effects on plant defenses. Tomato plants with 6-8 expanded true leaves were harvested after four weeks of growth. Only plants with lack of disease symptoms were employed. A minimum of 3 plants per variety were processed together, with an

average of 8 plants per sample. Leaves were immediately frozen in liquid nitrogen and stored at -80 °C. Leaf samples were grounded in a mortar with liquid nitrogen to obtain a fine leaf powder, which was then lyophilized in 10 mL tubes using an Alpha1-2 lyophilizer (Christ, Germany) for 48 hours (-52 °C, vacuum pressure 0.2 mm Hg), and stored at room temperature in the dark until their use for plant-based larval diet preparation.

Plant-based diets were formulated by mixing 0.23 g of lyophilized material with 4 mL of agar solution supplemented with 5 % of the compounds used for the standard artificial diet (without antibiotics) for rearing *S. exigua* larvae. To prevent heat inactivation of enzymes and metabolites, the leaf powder was mixed with the agar once it had cooled to approximately 35-40 °C.

1.2.3. *S. exigua* larval performance

Larval performance was assessed by measuring the relative growth of newly molted second instar *S. exigua* larvae (Herrero et al. 2002). The bioassay spanned 144 h, during which larvae were fed for the first 48 h on the plant-based diet, followed by a switch to artificial diet for the remainder of the experiment. Since the *S. exigua* larvae used in these bioassays were sourced from a laboratory colony reared exclusively on artificial diet, the feeding period on plant diets was restricted to 48 hours to avoid larval mortality due to a lack of dietary adaptation and to minimize the interference from other factors, such as the nutritional properties of the different plants. At the start of the experiment, larvae were weighed, individually placed in separate wells of a bioassay tray (product no. 9074; Frontier Agricultural Sciences), which was sealed with microperforated adhesive tape (product no. 9074-L; Frontier Agricultural Sciences). The trays were maintained in the growth chamber under the same conditions of the

laboratory colony mentioned above. After 144 hours, larvae were weighted again, and the relative growth was calculated as milligrams of biomass acquired per milligram of initial body weight. Larval weight was determined using a precision balance (Sartorius MC-1 Analytic AC 120S; Göttingen, Germany) with an accuracy of 0.1 mg. A total of 16 larvae were used per tomato species/variety, and two biological replicates of the experiment were performed at different periods.

1.2.4. *B. thuringiensis* susceptibility bioassays

Newly molted second instar *S. exigua* larvae were infected with the bacterial entomopathogen using the droplet feeding method. Four µL droplets containing 10 % sucrose, phosphate-buffered saline (PBS; pH 7.4), 10 % (v/v) tracking dye phenol red, and a sublethal concentration of *B. thuringiensis* subsp. *aizawai* (XenTari® GD, 15 million U.I./g; Kenogard S.A, Barcelona, Spain) were prepared. The sublethal concentration of 0.6 mg/mL was determined on the basis of previous bioassays conducted under the same experimental conditions (second instar *S. exigua* larvae, feeding on plant diet for 48 h). Control larvae received a mock treatment with the same procedure but without Bt. Larvae were allowed to feed on the droplets for 15 min, and after visually confirming ingestion, they were immediately placed individually in bioassay trays and provided with plant-based diets. The trays were kept for 48 h in a growth chamber under controlled conditions. After this period, the plant-based diet was replaced with artificial diet. Mortality was registered every 24 h for a period of 6 days. Larval relative growth of larvae at 144 hours was calculated and analyzed as described above. A total of 16 larvae were used per species/variety and treatment, and two biological replicates were performed at different time periods. For statistical analysis, differences in larval relative growth between wild tomato species and

cultivated varieties were calculated using Student's t-test. Mortality in control and Bt-infected larvae were compared using one-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Software Inc., San Diego, CA). The Spearman and Shapiro-Wilk tests were applied to determine homoscedasticity and normality of data, respectively. Additionally, the Pearson correlation coefficient was used to measure the linear correlation of growth and basal mortality values of non-infected larvae with the mortality of Bt-infected larvae.

1.2.5. Elicitation of MeJA-inducible plant defenses and evaluation of their effects on *S. exigua*

The results of the growth inhibition and Bt susceptibility bioassays with plant-based diet derived from the fifteen wild tomato species and forty cultivated tomato varieties, allowed us to selected six cultivated varieties with contrasting levels of constitutive resistance (i.e., leading to low or high relative larval growth and Bt-associated mortality in plant-based diets, Suppl. Table 1). These were chosen to further investigate the effect of JA-inducible defenses on larval growth and susceptibility to Bt (Table 1). To this purpose, tomato seeds were surface-sterilized by immersion in 4 % NaHClO (10 min), then thoroughly rinsed with sterile distilled water, and germinated in sterile vermiculite at 25 °C in a phytotron for 10 days. Seedlings at two-cotyledon stage were then transplanted to 250 mL pots containing a sterile sand: vermiculite (1:1) mixture (Rivero et al. 2021). Plants were randomly distributed on greenhouse benches and grown in a greenhouse at 24 ºC/16 ºC with a 16h/8h diurnal photoperiod and 70 % humidity. After 6 weeks of growth, half of the plants were treated by spraying plant shoots with a solution of 100 μM MeJA (Sigma-Aldrich) in water, which was prepared from a 100 mM MeJA stock solution in 96 % (v/v) ethanol. To ensure uniform

application, Silwett 77 (0.02 % v/v) was added as a surfactant, and leaves were sprayed until run off using an aerograph. Control plants were similarly sprayed with a mock solution containing only the solvent and surfactant. This exogenous application of 100 μM MeJA has been shown to efficiently elicit plant defenses in various species, including tomato (Martínez-Medina et al. 2017). Five plants were treated per variety. Leaves were harvested 72 h after MeJA or mock application to ensure the accumulation of the JA-inducible compounds. The harvested leaves were immediately frozen with liquid nitrogen and stored at -80 ºC.

For the *S. exigua* bioassays, frozen leaves were pooled by treatment and variety (5 plants), grounded, and lyophilized as described previously to prepare the plant-based diets. Bioassays to evaluate larval growth and susceptibility to Bt were conducted as described above, with the only modification being the use of a sublethal concentration of XenTari® at 1 mg/mL. Three independent biological replicates were performed, with 16 larvae used per treatment, variety and replicate. Relative growth was calculated after 48 h of feeding on plant-based diet and mortality was recorded every 24 h for a period of 7 days. Statistical differences in relative growth and mortality after Bt infection between treatments were analyzed using two-way ANOVA, with MeJA application and constitutive resistance level as factors (GraphPad Software Inc., San Diego, CA). The Spearman and Shapiro-Wilk tests were applied to determine homoscedasticity and normality of data, respectively. The Pearson correlation coefficient was used to measure the linear correlation of growth and basal mortality values of non-infected larvae with mortality values of Bt-infected larvae.

1.2.6. Untargeted metabolomic analysis

A comparative metabolomic analysis was conducted to identify metabolites that were overaccumulated in the selected varieties after MeJA treatment. For this purpose, the apical leaflet of the fourth true leaf (young, fully expanded leaf) from each plant sprayed with either MeJA or the solvent was harvested and kept at -80 ºC for molecular analysis. In total, there were five biological replicates per treatment (control or MeJA application) for each of the six selected varieties, resulting in 60 samples in total. Each leaflet was individually ground with liquid nitrogen, lyophilized, and 10 mg of powder were resuspended in 1 mL of the extraction buffer (MeOH:H_2O 30:70 containing 0.01 % of HCOOH). After incubating on ice for 40 min, the homogenate was centrifuged at 15,000 × *g* for 15 min at 4 ºC. The supernatant was filtered with 0.2 μm cellulose filters (Regenerated Cellulose Filter, 0.20 μm, 13mmØ. Pk/100; Teknokroma, St Cugat, Spain). The filtered supernatants (50 μL) were diluted 1:3 using the extraction buffer, and an aliquot of 20 μL of each sample was resolved by an Acquity UPLC system (Waters, Mildford, MA, USA) in positive (ESI+) and negative (ESI-) ion modes for electrospray ionization, coupled to a hybrid quadrupole time-of-flight equipment (QTOF MS Premier, Waters, Mildford, MA, USA) for detection of metabolites. Identification of the signals was performed by introducing a second fragmentation function into the TOF analyzer in a t-wave ranging from 5 to 45 eV.

Raw data were obtained from Masslynx v.4.2 software (Waters, USA) and transformed into .cdf files using the DataBridge tool. Chromatographic signals from positive and negative ESI were processed separately with R software v.4.3.2 (https://cran.r-project.org), and the XCMS algorithm was used for filtering and peak identification, grouping, and signal corrections. Metabolite

amounts were quantified by normalizing peak area units to the dry weight of each sample. MarVis Suite 2.0 software allowed signal comparations between treatments using Kruskal-Wallis test ($P < 0.05$) followed by adduct and isotope correction, and clustering. Only peaks with signal-to-noise ratio > 10 were included in the analysis. Compounds whose signal intensity was significantly increased a minimum of 2-fold after MeJA treatment and were shared by at least four tomato varieties were selected. Identification of metabolites was based on their exact mass, retention time, and spectrum fragmentation (Gamir et al. 2014; Schymanski et al. 2014). Signals were identified with the use of different online databases such as Massbank, MassBank of North America, PubChem and Human Metabolome databases (www.massbank.jp; https://mona.fiehnlab.ucdavis.edu; https://pubchem.ncbi.nlm.nih.gov; https://hmdb.ca). In addition, data from positive and negative ESI were combined and normalized by median, transformed by cube root and scaled by Pareto method using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca) to obtain sparse partial least squares discriminant analysis (sPLSDA) plots and heatmaps for each variety. Heatmaps were generated with those metabolites which differed significantly ($P < 0.05$, Kruskal-Wallis test) in signal intensity after MeJA induction compared to the control.

1.3. Results

1.3.1. Effect of tomato genotypes on *S: exigua* performance and susceptibility to *B. thuringiensis*

To explore the effects of natural diversity in constitutive chemical defenses in tomato on *S. exigua* performance and mortality by Bt, we performed a plant-based screening. Specifically, we assessed both direct effects by measuring the *S. exigua* relative growth and mortality, and indirect effects by measuring the susceptibility of *S. exigua* larvae to Bt when feeding on different tomato genotypes, including wild species and cultivated varieties.

We measured the relative growth of *S. exigua* larvae after 48 hours of continuous feeding on plant-based diet, followed by feeding on artificial diet until 144 h. When larvae were fed exclusively on the artificial diet, their relative growth at 144 h averaged 130. In contrast, feeding on plant-based diets resulted in noticeably reduced larval relative growth, ranging from 10 to 110 with a median of 32.1 (Suppl. Table 1). A significant influence of plant genotype on *S. exigua* growth was detected (ANOVA, $F_{54,55} = 1.7$, $P = 0.026$). The mean relative growth recorded from larvae fed on wild species was lower compared to that of larvae fed on cultivated varieties (Student's t-test, $P = 0.0383$) (Suppl. Table 1, Fig. 8A).

We next evaluated the effect of plant genotypes on Bt performance. Feeding mock-infected larvae on the different plant diets resulted in around 20 % mortality. This increased up to 40 % after Bt infection in both wild (Tukey HSD test, $P = 0.0003$) and cultivated (Tukey HSD test, $P < 0.0001$) varieties (Suppl. Table 1, Fig. 8B), confirming the efficacy of Bt. However, no significant differences were observed between wild species and cultivated varieties in terms

of larval mortality, either with (Tukey HSD test, $P = 0.7282$) or without Bt (Tukey HSD test, $P = 0.9982$) (Suppl. Table 1, Fig. 8B).

In addition, we found a clear increase in the mortality of larvae fed on plant-based artificial diets compared with those exclusively reared on artificial diet, either with or without Bt infection (mock-infected: 0 – 5 %, Bt-infected: 5 – 10 %). The latter mortality values were obtained from previous assays conducted multiple times, being these data consistent among the bioassays.

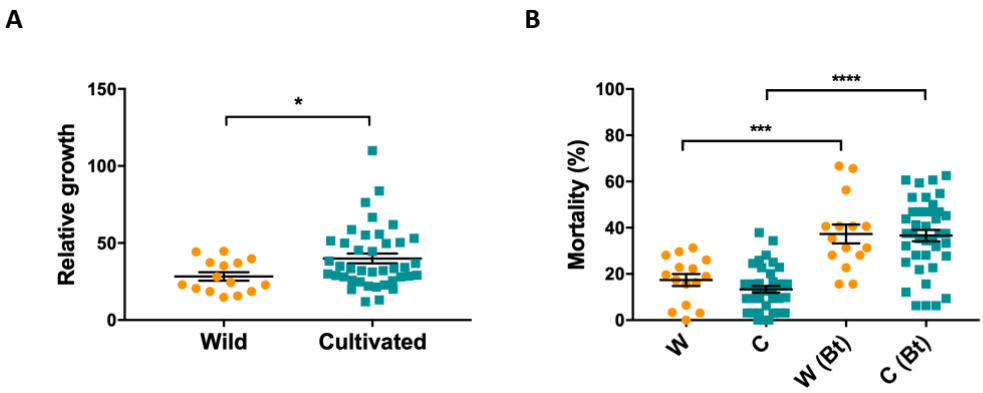

Figure 8. Effect of constitutive chemical defenses from wild species and tomato cultivated varieties on *S. exigua* growth and mortality.
(A) Relative growth of larvae fed on plant-based diets for 48 h and replaced by artificial diet until 144 h. Mean values were analyzed with Student´s t-test. Whiskers plot represent the average and standard error of the mean (SEM). (B) Mortality of larvae reared on plant-based diets and infected or not with *B. thuringiensis* (144 h post infection). One-way ANOVA followed by Tukey's multiple comparisons test was used to analyze differences. Whiskers plot represent the average and SEM. Asterisks indicate significant differences between groups (*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$). Color coding and abbreviations: W, wild species, are depicted in orange; C, cultivated varieties are depicted in aquamarine.

Analyzing the combined data, we found that both larval relative growth and basal mortality triggered by each genotype correlated with susceptibility to Bt (Fig. 9). Specifically, relative growth and susceptibility to Bt showed an inverse correlation (r = -0.6137, $P < 0.0001$, Fig. 9A), whereas mortality induced by the plant diet alone and mortality triggered by Bt exhibited a positive correlation (r = 0.5207, $P < 0.0001$, Fig. 9B). These results suggest that constitutive chemical defenses in tomato, which negatively affect larval development and survival, also contribute to exacerbate the effect of Bt.

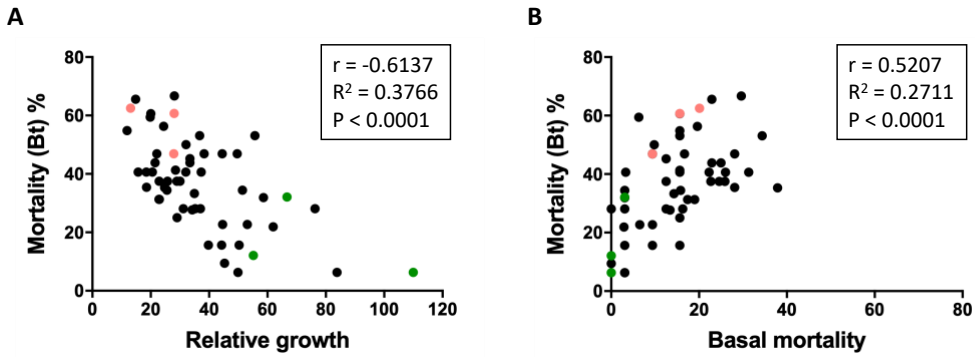

Figure 9. Correlation between impact of plant compounds on *S. exigua* and its susceptibility to *B. thuringiensis*.
Scattered plot and Pearson correlation analysis between relative growth of non-infected larvae (A) or basal mortality (B) and mortality values of Bt-infected larvae. Highlighted points correspond to values corresponding to the selected cultivated varieties (red: high constitutive resistance, varieties 1 - 3; green: low constitutive resistance, varieties 4 - 6).

1.3.2. Effect of inducible defenses on larval performance and susceptibility to *B. thuringiensis*

We further investigated how the inducible defenses in six different cultivated tomato varieties affected larval growth and influenced susceptibility to Bt. The six tomato varieties selected for this set of experiments were grouped based on their constitutive resistance levels observed in the previous screening results. Varieties exhibiting low larval growth and high mortality in response to Bt were classified as having high constitutive resistance (varieties 1, 2, 3), while those showing high larval growth and low mortality to Bt were classified as having low constitutive resistance (varieties 4, 5, 6) (Table 1).

Table 1. Effect of the selected cultivated varieties on *S. exigua* growth and susceptibility to Bt.

Constitutive resistance [1]	Internal code	Selected variety	Relative growth ± SEM	Bt mortality ± SEM (%)
High	20T	1	27.9 ± 3.2	46.9 ± 15.6
High	35T	2	13.1 ± 3.9	62.5 ± 6.3
High	39T	3	28.0 ± 13.7	60.7 ± 1.9
Low	3T	4	55.1 ± 15.5	12.1 ± 0.4
Low	25T	5	66.7 ± 28.6	32.1 ± 10.7
Low	16T	6	109.9 ± 35.0	6.3 ± 0.0

[1]High group corresponds to cultivated tomato varieties (1, 2, 3) with a high constitutive resistance against *S. exigua* (low larval growth and high mortality with Bt). Low group corresponds to cultivated tomato varieties (4, 5, 6) with a low constitutive resistance against *S. exigua* (high larval growth and low mortality with Bt).

Inducible defenses were triggered by the application of MeJA, the active form of JA, which is well known for inducing plant defenses (Turner et al. 2002). As expected, MeJA application significantly reduced larval relative growth in the absence of Bt, with no observed differences between the high and low constitutive resistance groups (MeJA: $F_{1,24} = 17.64$, $P = 0.0003$; resistance level: $F_{5,24} = 1.663$, $P = 0.1819$; interaction: $F_{5,24} = 0.4834$, $P = 0.7851$; Fig. 10A). Regarding the mortality of Bt-infected larvae, we observed that the MeJA treatment led to an overall increase of approximately 15 % compared to larvae raised on non-induced plant-based diets (Fig. 10B), revealing the contribution of induced defenses to higher susceptibility to Bt. The combined effect of the plant constitutive resistance group (high or low) and MeJA application on Bt susceptibility was not significant (MeJA: $F_{1,24} = 12.13$, $P = 0.0019$; resistance level: $F_{5,24} = 4.193$, $P = 0.007$; interaction: $F_{5,24} = 0.1260$, $P = 0.9851$; Fig. 10B), indicating that inducible defenses had an effect regardless the constitutive defenses. Hence, combining both factors (constitutive resistance and induced defense) had an additive, but not synergistic effect on larval susceptibility to Bt. Once again, we found that lower relative growth and higher basal mortality, triggered by the effects of MeJA-elicited chemical defenses, correlated with higher susceptibility of larvae to Bt ($r = -0.8808$, $P = 0.0002$, Fig. 10C; and $r = 0.5876$, $P = 0.045$, Fig. 10D), highlighting that inducible tomato defenses that directly impair larval growth and survival also increase Bt toxicity against *S. exigua*.

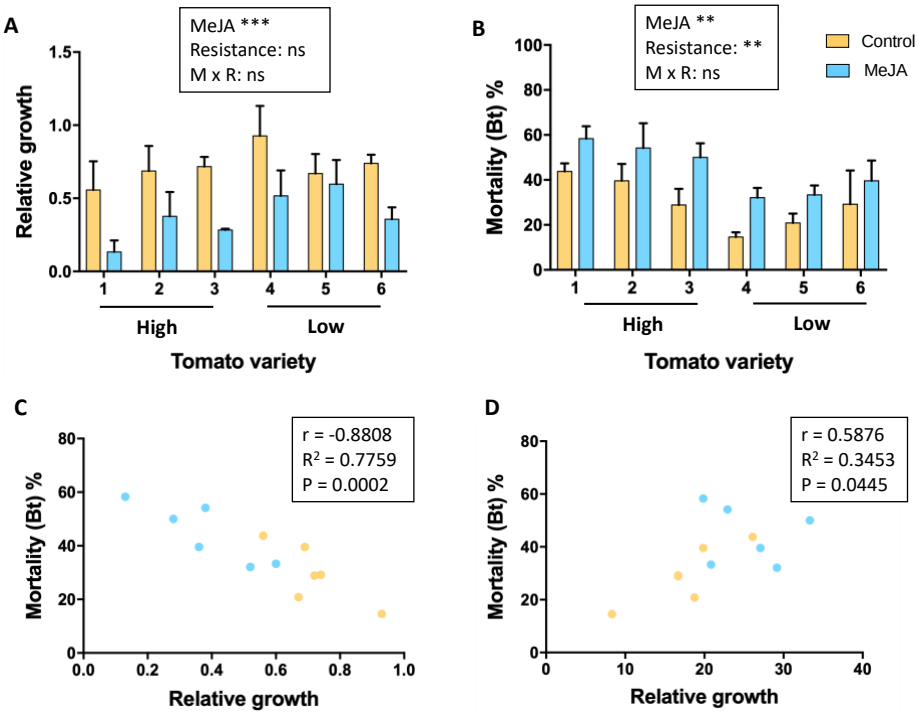

Figure 10. Effect of inducible chemical defenses from the selected tomato varieties on *S. exigua* growth and susceptibility to Bt.

(A) Relative growth of larvae fed on plant-based diets for 48 h. (B) Percentage of mortality of larvae reared on plant-based diets and infected with *B. thuringiensis* (168 h post infection). In both growth and mortality plots, mean values were analyzed with two-way ANOVA using MeJA and constitutive resistance as factors. Error bars depict standard error of the mean (SEM). Scattered plot and Pearson correlation analysis between relative growth (C) and basal mortality (D) of non-infected larvae and percentage of mortality of Bt-infected larvae. Asterisks show significant differences (**$P < 0.01$; ***$P < 0.001$).

1.3.3. Impact of MeJA elicitation on foliar metabolic profile

As our data show a clear negative effect of JA-inducible defenses on larval performance, we aimed to explore the changes in the leaf chemical composition triggered by the MeJA treatment potentially responsible for the observed phenotypes. For that, we analyzed the metabolomic profiles by LC-MS/MS. MeJA application led to a rearrangement of the leaf metabolome in all the 6 varieties, as indicated by supervised PCA analysis (Fig. 11A). Heatmap plots were generated to visualize differentially abundant signals between the control and MeJA treatment groups. Depending on the variety, the number of signals significantly differentially accumulated ranged from 100 to 300, and the clustering confirmed the impact of MeJA treatment on the tomato metabolic profile (Fig. 11B).

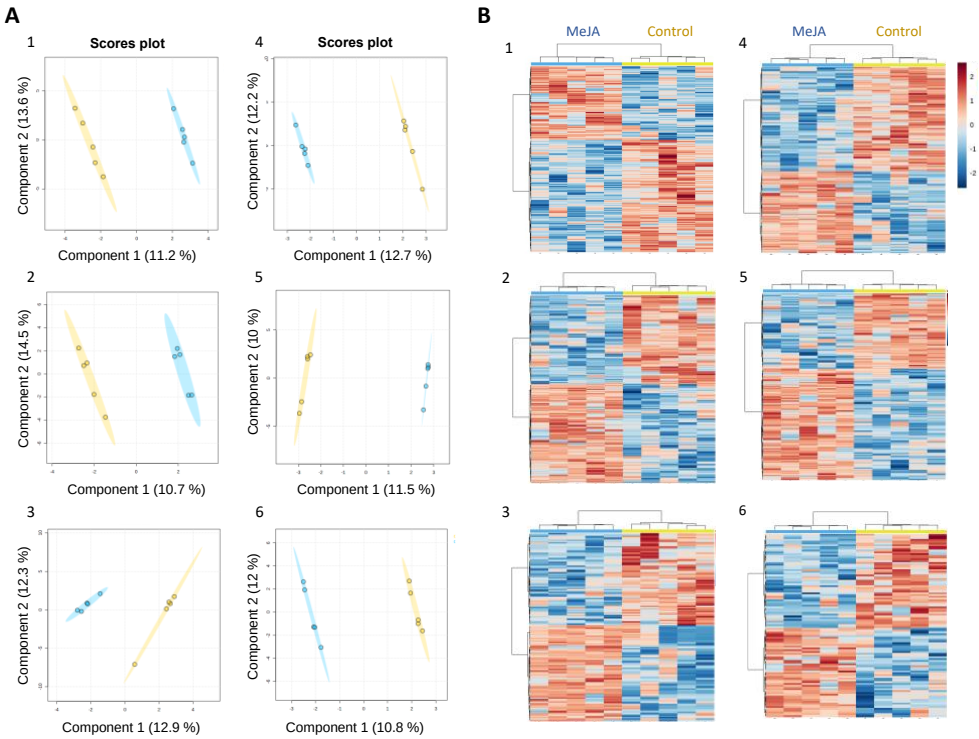

Figure 11. Metabolomic profile of MeJA-elicited tomato plants from the selected varieties.

A non-targeted metabolic analysis of the leaves was performed to assess metabolic changes after treatment of plant shoots with MeJA. Signals from positive and negative electrospray ionization were combined. A) Sparse partial least squares discriminant analysis (sPLSDA) representation of the major sources of variability from the obtained signals. B) Heatmap representing metabolites that significantly differed in signal intensity after MeJA induction compared to the control ($P < 0.05$, Kurskal-Wallis test). Yellow: control plants; blue: MeJA-elicited plants. Varieties 1-3 (high constitutive resistance), varieties 4-6 (low constitutive resistance).

A more detailed analysis of the metabolomic profiles led to the identification of six compounds which were significantly overaccumulated (over 2-fold) after MeJA treatment in at least four out of the six selected tomato varieties (Fig. 12 and Table 2). Among them, four compounds were identified in the positive ionization mode: 5-hydroxyindole-3-acetic acid, (+)-catechin, an unidentified compound (m/z 826.10) and soyasapogenol B base + O-HexA, Hex, dHex, 1malonyl (SB). In the negative ionization mode, the metabolites syringaldehyde and phosphatidylglycerol 42:10 were found. Remarkably, SB was the only one significantly overaccumulated in the six cultivated varieties.

Table 2. Metabolites with significant induction following MeJA application across the six selected tomato varieties.

Ion mode	Rt (s)[1]	Exact mass	Parent ion	Major fragments	Compound identity	Varieties[2]	Induction level[3]
Positive	4.81	1029.14	1030.02	528.95 > 145.07	Soyasapogenol B base + O-HexA, Hex, dHex, 1malonyl	1 – 6	3.5 – 14.9
Positive	2.66	191.03	192.10	147.04 > 117.03	5-Hydroxyindole-3-acetic acid	3 – 6	2.2 – 9.5
Positive	3.73	290.09	291.08	207.08 > 123.09	(+) – Catechin	1, 3, 5, 6	2.2 – 7.8
Positive	4.81	826.10	827.35		Unknown	1, 2, 4, 5	2.2 – 4.1
Negative	2.64	182.05	181.04	123.03 > 151.01	Syringaldehyde	1, 3 – 6	2.8 – 23.5
Negative	2.41	843.31	842.24	153.01 > 327.01	Phosphatidylglycerol 42:10	1, 3 – 6	3 – 8.6

[1]Rt: retention time

[2] *S. lycopersicum* cultivated varieties grouped as high (1 – 3) or low (4 – 6) constitutive resistance based on their impact on *S. exigua* performance and mortality.

[3]Relative abundances of MeJA-elicited metabolites were compared with those from control treatment to obtain the induction level values (Kruskal-Wallis test, $P < 0.05$).

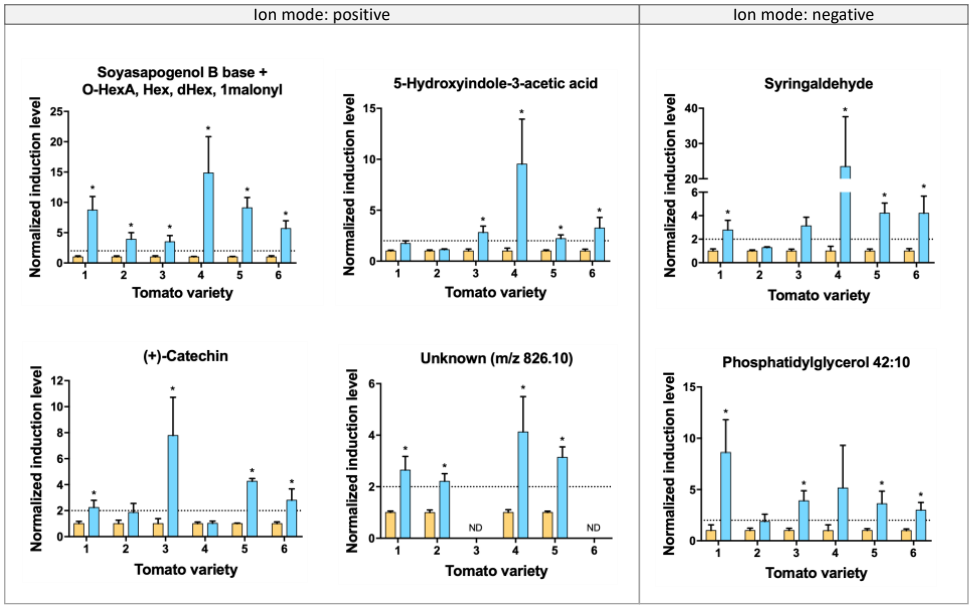

Figure 12. Relative abundance of tomato metabolites elicited across selected tomato cultivated varieties.

Relative abundances are shown as normalized signal intensities of MeJA-elicited metabolites compared to the control within each variety. Four compounds were identified in positive mode ionization (left), while two others were identified in negative mode ionization (right). Error bars depict standard error of the mean (SEM). The color code indicates control plants in yellow and MeJA-elicited plants in blue. Asterisks indicate significant differences between MeJA-elicited and the respective control plants (Kruskal-Wallis test, $P < 0.05$, FC ≥ 2, n = 5).

1.4. Discussion

Plants rely on constitutive and inducible defenses to protect themselves from herbivorous insects, such as caterpillars. These defenses serve various functions, including deterrence, antifeedant properties, or toxicity against the herbivore itself, as well as attraction of natural enemies, like predators and parasitoids (Mithöfer and Boland 2012). However, their interaction with microbial entomopathogens, another major group of insect natural enemies, has been poorly addressed. In this study, we demonstrate that both constitutive and inducible defenses in tomato increase the lethality of the bacterial entomopathogen *Bacillus thuringiensis* against the caterpillar *Spodoptera exigua*.

Unlike previous studies that focused solely on the effects of individual plant defensive compounds on Bt activity (Felton and Dahlman 1984; Krischik et al. 1988; Ludlum et al. 1991; Sivamani et al. 1992; Appel and Schultz 1994), our approach involves the use of chemical elicitation of plant defenses and lyophilized leaf material to assess the influence of overall chemical defenses, including the contribution of both constitutive and inducible components. Physical defenses such as leaf thickness, shape, or hardness were excluded, as the leaf material was grounded into powder. Moreover, by comparing different plant genotypes, we demonstrate that the effect on larval growth is genotype-dependent. Our results reveal the positive contribution of tomato defenses to the effectiveness of the entomopathogen *B. thuringiensis* against a generalist caterpillar.

Tomato plants synthesize a plethora of chemical compounds to defend against insect herbivores, such as alkaloids, phenolic compounds, or triterpenoids, which possess antifeedant properties and direct toxicity against

herbivores (Howe and Jander 2008; War et al. 2012; Paudel et al. 2019). However, many of these compounds are not produced constitutively due to their high cost to the plant (Karban and Baldwin 1997). Instead, their accumulation is induced upon detection of herbivory. The balance between constitutive and induced defenses within a plant is often influenced by the domestication process, which can either enhance or reduce the rates of inducibility in response to herbivore attack (Whitehead et al. 2017; Ferrero et al. 2020; Szymański et al. 2020). For instance, many ornamental plant species exhibit lower constitutive and higher induced resistance in domesticated species (Kempel et al. 2011). The findings of our study, which assessed the effect of constitutive chemical defenses on the relative growth of *S. exigua* using plant-based diet bioassays, corroborated this trend. Indeed, non-induced cultivated tomato varieties allowed for a greater relative growth of *S. exigua* compared to wild species. This may be a consequence of selective breeding over the years selecting for yield and fruit characteristics, but not for resistance (Bergougnoux 2014). In contrast, the mortality of Bt-challenged larvae fed on different plant diets was similar, regardless of whether the plants were wild or cultivated. This suggests that while domestication altered the levels of constitutive chemical defenses against the caterpillars, it did not affect those defenses that could increase the efficacy of Bt.

The clear correlation between the mortality produced by Bt and the detrimental effects on larval performance (growth decrease and mortality) from non-induced plant-based diets reveals that constitutive tomato defenses enhance larval susceptibility to Bt. This finding was further corroborated by observations from MeJA-elicited plants, which exhibited an analogous pattern. A similar trend was observed in another pathosystem: a tomato-based diet exacerbated the effect of the opportunistic pathogen *Serratia marcescens* in *Helicoverpa zea*, another

generalist lepidopteran from the Noctuidae family (Mason et al. 2023). Based on these data, we hypothesized that the enhanced action of Bt provides a protective advantage to the plant, allowing for more effective control of insect herbivores.

Plants may use different strategies to weaken insects and enhance Bt activity. *B. thuringiensis* kills its hosts through the production of pore-forming toxins, namely Vip and Cry proteins (Bravo et al. 2011). These toxins bind to the gut epithelium of susceptible insects, where they insert into the brush border membrane of columnar cells, creating an osmotic imbalance that leads to gut destruction. The death of the insect may be further facilitated by the action of opportunistic gut bacteria, which benefit from the gut destruction produced by Bt to invade the host body and cause septicaemia (Mason et al. 2011; Caccia et al. 2016). We hypothesize that plant defenses may contribute to Bt action in several ways. First, several plant phytochemicals directly target the digestion process, jeopardizing the insect's ability to obtain nutritional resources. For example, phenolics are oxidized by polyphenol oxidases (PPOs) resulting in the formation of orthoquinones that alkylate aminoacids and proteins (Felton et al. 1992; Constabel and Barbehenn 2008). Lectins bind to glycosyl groups and damage the luminal epithelial membrane (Vandenborre et al. 2011), and proteinase inhibitors obstruct the activity of digestive enzymes (Zhu-Salzman and Zeng 2015). The interaction between these molecules, which limit the bioavailability of nutrients, and the destruction of the gut epithelial cells prompted by Bt, may further diminish the insect's ability to obtain nutritional resources, thus accelerating their death. In line with this, a previous observation related the Bt-mortality of *H. zea* in JA-induced tomato with the plant PPO activity (Shikano et al. 2018b). Secondly, other plant compounds like triterpenoids, alkaloids, flavonoids or lectins may directly weaken the immune

system of the insect, interfering with its ability to remove the pathogen and eventually enhancing the larval susceptibility to Bt due to their inherent toxicity (War et al. 2012). In this regard, we previously showed that *S. exigua* raised on plant-based diet from plants already exposed to herbivory impaired caterpillar growth, increased susceptibility to Bt, and decreased larval PPO activity in the hemolymph, a key marker of the insect immune status (Frattini et al. 2022). Thirdly, some phytochemicals and defensive proteins like tannins, chitinases or proteases disrupt the peritrophic membrane (Mason et al. 2019), a physical barrier against pathogens and mechanical damage that covers the entire larval gut (Barbehenn and Peter Constabel 2011; Konno and Mitsuhashi 2019), whose absence increases the susceptibility of caterpillars to Bt (Guo et al. 2019; Güney et al. 2024).

Although we observed an increase in the susceptibility of *S. exigua* larvae to Bt after feeding on the tomato plants, other factors, such as larval instar, body size, or timing of exposure to Bt (before or after plant diet feeding), should be considered for a more complete understanding of the effects of plant defense compounds on the activity of Bt. Previous studies have shown that smaller larval body size or slower larval growth is associated to a higher susceptibility to pathogens (Vogelweith et al. 2013; Shikano et al. 2018a), likely due to a lower allocation of resources to combat infection and/or a prolonged period of vulnerability to the natural enemies. Based on this, it is plausible that infecting younger *S. exigua* larvae (neonates or first instars) with Bt would result in a higher efficacy of the bacterial entomopathogen against the caterpillar.

Timing of pathogen challenge is a critical factor when evaluating the role of plants in the interaction between insects and pathogens (Cory and Hoover 2006; Shikano 2017). Depending on the timing of pathogen ingestion and plant

material ingestion, we may encounter different scenarios; (1) If plant material is ingested well in advance of pathogen-challenge, this may result in plant-mediated variation in host physiology, for example the weakening of peritrophic membrane may facilitate the further action of the entomopathogen; (2) If plant material and the pathogen are co-ingested, direct interactions between plant chemicals and the pathogen may occur in the insect's gut, for example in the case of the potential alkylation of Bt protoxins triggered by quinones produced by tomato defensive enzyme PPO; and (3) If plant material is ingested after pathogen-challenge, the plant quality may affect the insect's immune function. In our study, we infected larvae with Bt prior to feeding on plant diets, with a 15-minute time window between the treatments. After 48 h of feeding on the plant diets, larvae were shifted to an artificial diet. We hypothesize that the enhanced Bt mortality related to the ingestion of plant material, such as that observed in MeJA-induced plants, is likely related to changes in the host physiology. For example, it could be associated with the opening of pores in the insect gut by Bt pore-forming toxins, which may allow plant chemicals to enter the hemocoel and have greater toxic effect. However, future research should assess the effect of leaf compounds on Bt lethality when plant diets are ingested simultaneously with, or prior to, pathogen challenge.

To date, only few individual defensive plant compounds have demonstrated to enhance Bt action (Felton and Dahlman 1984; Krischik et al. 1988; Sivamani et al. 1992; Appel and Schultz 1994). To identify bioactive molecules potentially responsible for both detrimental effects on larval growth and mortality and Bt susceptibility, we compared the profiles of the six MeJA-elicited tomato varieties. Our results revealed a substantial rearrangement of the leaf metabolic profile in all varieties after MeJA treatment, regardless of their

constitutive resistance classification. This metabolic shift in leaves after exogenous application of MeJA is a well-documented phenomenon in various plants (Flores-Sanchez et al. 2016; Papazian et al. 2019; Ramabulana et al. 2020), attributed to JA-mediated induction of secondary metabolite biosynthesis (Wasternack and Hause 2013; Wasternack 2014; Yu et al. 2019). We looked for metabolites consistently overaccumulated in elicited plants across the tested varieties. Among them, we identified six metabolites overaccumulated across varieties, five of them with significant increases in abundance in at least four out of the six tested tomato varieties: 5-hydroxyindole-3-acetic acid, (+)-catechin, syringaldehyde, phosphatidylglycerol 42:10 and an unidentified compound with a m/z of 826.10. Among these, the flavonoid catechin showed an acute toxicity against *Spodoptera litura* larvae (Ruttanaphan et al. 2023), and reduced the larval growth of the lepidopteran pest *Ectropis grisescens* (Li et al. 2022) when supplemented in artificial diet. Interestingly, the compound flavone, belonging to the flavonoid family, has been reported to interact in a synergistic way with the Bt toxin Cry1Ac when inducing mortality against *Helicoverpa armigera* (Wang et al. 2021). Similarly, the polyphenol syringaldehyde negatively affected the mobility of the bean weevil *Acanthoscelides obtectus* and increased its mortality (Regnault-Roger et al. 2004).

A single compound was significantly overaccumulated in all six tomato varieties: soyasapogenol B base + O-HexA, Hex, dHex, 1 malonyl. This compound corresponds to a triterpenoid saponin, a secondary metabolite widely distributed in plants and whose production is influenced by biotic environmental factors (Szakiel et al. 2011; de Costa et al. 2013). Saponins play a role in plant defenses against herbivores, particularly due to their amphipathic properties, which lead to the formation of strong complexes with cholesterol, triggering

cellular toxicity and altering insect molting (Weng et al. 2011). Moreover, saponins disturb insect digestion by inactivating digestive enzymes and altering insect gut microbiota (Singh and Kaur 2018). Previous studies where saponins were added to the artificial diet have shown a negative effect on growth and/or mortality of other lepidopteran insects such as *Spodoptera littoralis* (De Geyter et al. 2007), *Ostrinia nubilalis* (Nozzolillo et al. 1997) and *Helicoverpa zea* (Dowd et al. 2011). Moreover, a synergistic interaction between a tea saponin, a mycotoxin from *Metarhizium anisopliae* and Bt var. *kurstaki* was detected against *S. exigua* in terms of mortality (Rizwan-Ul-Haq et al. 2009). The reported antiherbivory effects of these compounds support their role in the growth reduction observed in larvae fed on MeJA-induced plants and may be responsible of the increased mortality upon Bt infection.

In conclusion, our study demonstrated that both constitutive and inducible tomato defenses increase the susceptibility of the generalist caterpillar *S. exigua* to *B. thuringiensis.* We identified some compounds which may contribute to these toxic effects. These results strongly support the notion that tomato defenses, which directly affect the insect herbivore, may also indirectly enhance the action of a third trophic level, the entomopathogen group. This finding has positive implications for sustainable crop management programs, which may combine the selection of plant defense traits with the use of entomopathogens.

CHAPTER 2:

Compatibility of mycorrhiza-induced resistance with viral and bacterial entomopathogens in the control of *Spodoptera exigua* in tomato

This chapter has been published as:

Frattini A., Martínez-Solís M., Llopis-Giménez Á., Pozo M.J., Rivero J., Crava C.M., Herrero S. 2022. Compatibility of mycorrhiza-induced resistance with viral and bacterial entomopathogens in the control of *Spodoptera exigua* in tomato. *Pest Management Science*, **78**:4388–4396.

2.1. Introduction

Plants must constantly cope with adverse environmental factors, consequently they have evolved several strategies to face them (Erb & Reymond, 2019; Ben Rejeb et al., 2014). They display multiple defense mechanisms to deal with diverse stressors (War et al., 2018). The plant microbiota is known to modulate such defense mechanisms, and beneficial soil microorganisms can increase plant stress resistance/tolerance (Berendsen et al., 2012; Pozo et al., 2021). In this context, the association with arbuscular mycorrhizal fungi (AMF) deserve special attention. These obligate biotrophs belong to the phylum *Glomeromycota* and form symbiotic interactions with more than 80 % of land plants, including most agricultural crops (Smith & Read, 2008). In this interaction, AMF colonize the root cortex and develop an extraradical mycelium, increasing the acquisition of water and inorganic nutrients (mainly phosphate and ammonia) of the plant. In return, the fungus receives photosynthates for the maintenance of mycorrhizal structures (Wang et al., 2017). Besides improving plant nutritional status and growth, this symbiosis also improves the ability of the plant to overcome abiotic stresses such as salinity, drought, or presence of heavy metals (Cornejo et al., 2017; Begum et al., 2019).

Mycorrhization is also involved in enhancing plant defense against a broad spectrum of pathogens and pests, a phenomenon known as mycorrhiza-induced resistance (MIR) (Pozo & Azcón-Aguilar, 2007; Pozo et al., 2013; Rivero et al., 2021). The symbiosis with AMF stimulates the immune system of the plant, leading to a primed state that implies a more efficient activation of defensive responses after exposure to biotic attack (Mauch-Mani et al., 2017; Martinez-Medina et al., 2016b). In the absence of stress, the defenses of mycorrhizal plants are slightly activated, hence allowing the plants to redirect resources to other

biological functions (low-cost defensive strategy) (Pozo & Azcón-Aguilar, 2007; Jung et al., 2012). In the presence of stress, mycorrhizal plants can trigger a faster defense response, at both below- and aboveground (Pozo et al., 2010; Rivero et al., 2021). The protective role of mycorrhization has been proven at root level against soil-borne pathogens, nematodes, or root-chewing insects (Gange, 2001; Azcón-Aguilar et al., 2002; Vos et al., 2012) whereas in aboveground tissues MIR enhances resistance against necrotrophic pathogens and generalist chewing insects (Rivero et al., 2021). The mechanisms that drive MIR in aboveground tissues are still elusive, but there is emerging evidence showing transcriptional and metabolic reconfiguration in the leaves of mycorrhizal plants which led to MIR-primed responses (Gallou et al., 2012; Kaling et al., 2018; Schweiger et al., 2014; Campos-Soriano et al., 2012; Sanmartín et al., 2021). Besides defense priming, some basal changes occur in leaves of mycorrhizal plants. For example, mycorrhizal plants often contain higher amounts of bioactive phenolic metabolites in their leaves than nonmycorrhizal plants. However, these metabolic responses are highly specific to each AMF-plant species combination (Schweiger et al., 2014).

For orchestrating a full defense response against herbivores, plants rely on the jasmonic acid (JA) signalling pathway, which is a conserved core pathway that is activated after insect feeding and leads to the accumulation of defensive compounds such as secondary metabolites (*e.g.* terpenoids, phenolics, and alkaloids compounds) and proteins (*e.g.* protease inhibitors, polyphenol oxidases) (Erb & Reymond, 2019; Howe & Jander, 2008). These defensive molecules deter insect herbivory by directly impairing insect growth (Schuman & Baldwin, 2016). In addition, they interplay with the herbivore microbiome (Mason et al., 2020), indirectly altering insect fitness, and may make target

insects more susceptible to biotic stresses such as the entomopathogens (Cory & Hoover, 2006). These are a group of diverse microorganisms that are pathogenic to insects. They include bacteria, viruses, fungi, and nematodes that are widely used in pest control in organic farming or integrated pest management strategies (Ruiu, 2018; Singh et al., 2019; Senthil-Nathan, 2015). Among them, *Bacillus thuringiensis* and baculoviruses are two of the most successfully organisms used in the control of lepidopteran larvae (Kong et al., 2018; Bravo et al., 2011). Both infect target insects by ingestion and lead to insect death within few days (Palma et al., 2014; Jiang et al., 2021). Besides their direct mode of action (Pinos et al., 2021; Herrero et al., 2016; Harrison & Hoover, 2012), research in the last decades has revealed the existence of complex interactions among these two entomopathogens, plant defenses (Hoover et al., 1998; Shikano et al., 2018) and the gut microbiome (Martínez-Solís et al., 2020; Caccia et al., 2016; Broderick et al., 2006; Jakubowska et al., 2013) that can lead to a faster death of target insects, perhaps targeting the immune system or facilitating the weakening of insect protective barriers such as the peritrophic membrane, which can in turn facilitate the start of secondary infections (Mason et al., 2019).

How the metabolic reconfiguration undergone by herbivory and/or plant mycorrhization impacts susceptibility to entomopathogens of phytophagous larvae has not yet been thoroughly investigated. In the present work, we compared the effects of mycorrhizal and non-mycorrhizal *Solanum lycopersicum* tomato plants in the absence or in the presence of herbivory on the growth of larvae from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) and their susceptibility to *B. thuringiensis* subsp. *aizawai* (XenTari®) and the Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV). We also analyzed the changes in the phenoloxidase, a marker of the insect immune status, and

midgut microbiota. Lyophilized instead of fresh leaves were used to discriminate the effect of the herbivory-induced phytochemicals from the changes associated with plant mycorrhizal status exclusively. We used these data to address four major questions: Are plant defenses enhancing susceptibility to selected entomopathogens? Is this higher susceptibility increased when mycorrhization is present? Are plant defenses altering insect immune system and/or gut microbiota? Are these changes further modulated by the presence of AMF? Our results reveal that mycorrhization of tomato plants in combination with previous herbivory increases the susceptibility of *S. exigua* to the bacterial entomopathogen and does not interfere with the lethality of the viral entomopathogen, suggesting that the use of both methods in the field (AMF and use of *B. thuringiensis* or baculovirus) may be combined with promising results.

2.2. Material and methods

2.2.1. Insects

Spodoptera exigua eggs were provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland) and maintained in our laboratory (Valencia, Spain) by continuously rearing on artificial diet supplemented with 0.05 % of tetracycline (Elvira et al., 2010) at 25 ± 3 ºC with 70 ± 5 % relative humidity and a photoperiod of 16 h light: 8 h dark.

2.2.2. Tomato plants, mycorrhization and diet preparation

Solanum lycopersicum cv. Moneymaker (MM) plants were grown at the Zaidín Experimental Station (CSIC, Granada). Tomato seeds were surface sterilized and germinated in sterile vermiculite. Seedlings at two cotyledons stage were inoculated with the AMF *Funneliformis mosseae* (BEG 12) (Nicolson and Gerdemann) Gerdemann et Trappe (Banque Européenne des Glomales [BEG] code 12) at transplanting by mixing the AMF inocula with the growing substrate, as previously described in Rivero *et al.* (2021). Plants were randomly distributed and grown in a controlled greenhouse at 24 ºC/16 ºC with a 16 h/8 h diurnal photoperiod. After 6 weeks of growth to allow the establishment of the mycorrhizal symbiosis, herbivory was initiated by infesting plants with two second instar *S. exigua* larvae per plant which were placed on a leaflet from the third true leaf, using 30 mm Ø clip-cages to limit the feeding area and avoid their escape. Clip-cages were moved into new leaflets every two days to make sure they always had food available. All the plants showed significant damages in leaves. Accordingly, four groups of plants were generated depending on the presence or absence of mycorrhization and/or herbivory: control Nm- (absence of both factors) and treatments Nm+ (exposition to herbivory), Fm- (presence of

mycorrhization) and Fm+ (presence of both factors) (Fig 13). Each treatment consisted in 12 plants growing in a randomized position in the greenhouse. Plants were harvested 8 weeks after mycorrhiza inoculation (15 days after starting of herbivory). Upon harvesting, tomato leaves were immediately frozen with nitrogen liquid and stored at -80 ºC. An aliquot of each root system was reserved for mycorrhizal assessment. Mycorrhizal colonization was evaluated by ink staining fungal structures within the roots according to Vierheilig et al. (2005), and the percentage of root length colonized was quantified under a light microscope (Eclipse 50i microscope. Nikon, Japan) using the gridline intersection method (Giovannetti & Mosse, 1980). Mycorrhizal colonization was confirmed in all inoculated plants (Fm), with an average of 12 % of the root length colonized by the fungus. Absence of mycorrhizal colonization was confirmed in Nm plants. No significant differences in shoot nor root fresh weight were found between mycorrhizal and non-mycorrhizal plants (t-test, n=10, $P > 0.05$).

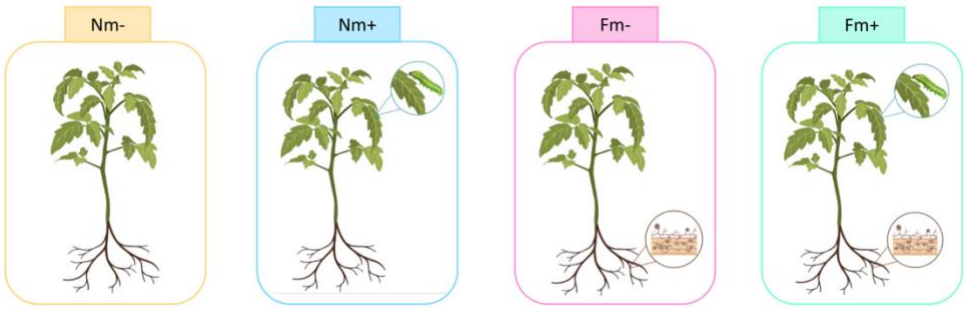

Figure 13. General diagram of the treatments applied to *S. lycopersicum* plants.
Four groups of tomato plants were classified according to the presence of mycorrhization and/or herbivory. Nm−, non-mycorrhizal plants without previous herbivory, used as control; Nm+, non-mycorrhizal plants exposed to herbivory by *S. exigua* larvae for 15 days; Fm−, mycorrhizal plants associated with the AMF *F. mosseae*; Fm+, mycorrhizal plants exposed to *S. exigua* herbivory.

The frozen leaf material was fully grinded in liquid nitrogen until obtaining leaf powder, which was lyophilized and stored until diet preparation. Plant-derived diets were prepared by mixing the lyophilized material in 1.8 % agar containing 5 % of the compounds used for the standard artificial diet (Hoffman & Lawson, 1964) in order to obtain a suitable diet for *S. exigua* larvae (Frattini et al. 2024).

2.2.3. Growth bioassay and larval development

With the aim of studying the effects of mycorrhization and previous herbivory on larval growth, newly molted fourth instar *S. exigua* larvae were reared on the different leaf-based diets for 48 hours. For that, disposable polypropylene boxes (11 cm x 8 cm x 5 cm) with the top cover replaced by paper (airflow window) were used. Each box contained four *S. exigua* larvae, previously weighed (in grams) in a precision balance (Sartorius MC-1 Analytic AC 120S, Göttingen, Germany) with an accuracy of 0.1 mg. The boxes were maintained in an insect chamber at 25 ± 1 ºC with a photoperiod of 16 h light: 8 h dark. To prevent the impact on the weight gain of larval molting, larvae were pooled per treatment and replicate, and weighed at 48 h. Weight increase was recorded and relative growth (RG) was estimated as grams of biomass acquired per gram of initial body weight (Herrero et al., 2002). Three independent biological replicates were performed. Statistical differences in relative growth between treatments were identified using two-way ANOVA with mycorrhization and herbivory as factors, followed by a Tukey HDS's multiple comparison test using R Statistical Software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). Before, Levene and Shapiro-Wilk tests were applied to determine homoscedasticity and normality of data, respectively.

2.2.4. Interaction with natural entomopathogens

The effect of mycorrhization and herbivory on the susceptibility of *S. exigua* larvae to *B. thuringiensis* and baculovirus (SeMNPV) was tested using the droplet feeding method in newly molted second instar larvae (Frattini et al. 2024). For Bt infections, we used *Bacillus thuringiensis* subsp. *aizawai* (XenTari®GD, Kenogard S.A, Spain), previously dissolved in water, at two concentrations (1 mg/mL and 3 mg/mL). For BV infections, we used a viral suspension containing $2x10^4$ occlusion bodies (OBs) per mL from SeMNPV (SP2 strain) (Caballero et al., 1992). We conducted previous assays to estimate the sublethal and lethal concentrations of the pathogens under our experimental conditions. After 20 min, larvae with red-colored body were selected for the next step, thus ensuring to select only larvae that had ingested the entomopathogen. Selected larvae were placed individually in a single well (2 cm x 2 cm x 2 cm) of a bioassay tray and fed with a piece of the different plant-based diets for 48 h at 25 ± 1 ºC. Each well was sealed with a microperforated adhesive tape (product no. 9074-L; Frontier Agricultural Sciences).

After that, plant-based diet was replaced by artificial diet (prepared without antibiotic addition) and mortality was recorded every 24 h for 7 and 8 days (Bt and SeMNPV, respectively) since the beginning of the bioassay. In Bt assays, four independent replicates were performed with the sublethal dose (1 mg/mL), whereas two were carried out with the lethal dose (3 mg/mL). In SeMNPV assays, three independent replicates were conducted. A total of 16 larvae were used per treatment and replicate. Survival curves were assessed using the Kaplan–Meier method and compared using the log-rank analysis (Mantel–Cox test) (GraphPad software Inc., San Diego, CA, USA).

2.2.5. Phenoloxidase enzymatic assays

In order to evaluate the effect of mycorrhization and herbivory on the *S. exigua* immune system, the hemolymph from the larvae used in the growth assay was extracted immediately after weighing to measure the phenoloxidase (PO) enzymatic activity, a marker of the insect immunity. In brief, the posterior proleg of each larva was cut, the hemolymph was collected with a micropipette and pooled by treatment (4 larvae each). Then, hemolymph was centrifuged at 500 x *g* for 2 min at 4 ºC to remove the hemocytes and kept on ice. Four µL of cell-free hemolymph, 46 µL of PBS and 50 µL of the substrate L-dopamine (100 µg/mL in PBS) were added to each well in a 96-well microtiter plate placed on ice. PO activity was determined by monitoring the increase of absorbance at 492 nm for 30 min using the Infinite 200 PRO multimode plate reader (TECAN Group Ltd., Switzerland). The activity of the enzyme was obtained as the initial velocity (Vo) of the reaction, measuring the change in absorbance per time.

Changes in PO activity in larval hemolymph could be due to the effect of the different treatments on insect immune status but also by direct interaction of plant metabolites on the PO enzymes. To test that, PO inhibition assay to assess the direct effect of the plant extract on these enzymes was also performed. For such purpose, leaf powder from the different treatments was mixed vigorously with methanol (50 mg/mL, respectively), incubated for 10 min and centrifuged at maximum speed for 10 min at 4 ºC. The supernatant was collected and used as plant metabolites source. A solution containing 2 µL of plant extract, 100 µL of L-dopamine (100 µg/mL in PBS) and 100 µL of larval hemolymph derived from fifth instar larvae feeding on artificial diet (20 mg of lyophilized hemolymph in 1 mL of PBS) was added to each well in a 96-well microtiter

plate. After incubating for 15 min at room temperature, PO activity was monitored and calculated as described above.

Statistical differences in PO activity were identified using two-way ANOVA with mycorrhization and herbivory as factors, followed by a Tukey HDS's multiple comparison test using R Statistical Software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). Normality of data sets was assessed by Shapiro-Wilk test and homoscedasticity by Levene's test.

2.2.6. Gut microbiota composition and diversity

To test if feeding on the different plant-based diets could influence the gut microbiota of *S. exigua*, we carried out metagenomic sequencing of the guts extracted from the larvae used in the growth bioassay and PO quantification. An additional treatment (AD) represented by larvae of the same instar raised side-by-side during 48 h in 100 % artificial diet without antibiotic was added. Each sequenced sample (AD, Nm-, Nm+, Fm-, Fm+) was composed by a pool of four larvae, and 3 independent biological replicates per treatment were analyzed. In brief, after 48 h of feeding, the whole gut of each larva was dissected with forceps, homogenized in Luria-Bertani medium supplemented with 10 % glycerol and kept at -80 ºC until DNA extraction. Total DNA extraction from the homogenized guts was carried out using the MasterPure™ DNA purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions, followed by PCR amplification and sequencing of the 16S rRNA (V3-V4 region). Sequencing was performed as previously described in Martínez-Solís *et al*. (2020) using a 2 x 300-pb paired-end run on a MiSeq sequencing platform (Illumina) at the Foundation for the Promotion of Health and Biomedical Research (FISABIO, Valencia). PRINSEQ-lite program (Schmieder & Edwards, 2011) was used to evaluate the quality of the obtained reads, setting the following

parameters: min_length, 50; trim_qual_right, 20; trim_qual_type, mean; trim_qual_window, 20. Paired reads were joined using *fastq-join* from the ea-tools suite (Aronesty, 2011). Then, filtered and demultiplexed sequences were processed with software QIIME v.1.9. (Caporaso et al., 2010) using default parameters. The sequences, from a total of 15 samples, were clustered in operational taxonomic units (OTUs) of 97 % sequence identity using *de novo* OTU picking based. After filtering the unassigned *Chloroflexi* and *Cyanobacteria* taxa, bacterial composition was determined for the 20 most abundant genera and represented in Excel software. In addition, the OTU table data was transformed and normalized (CSS (cumulative sum scaling) + log with total sum normalization) using the Calypso software (version 8.2) to generate a canonical correspondence analysis (CCA) plot showing the relationship among mycorrhization/herbivory (exposure factors) and larval gut microbial communities at genus level. Determination of alpha diversity (Shannon index) and linear discriminant analysis (LDA) effect size (LEfSE) was undertaken at genus level using mycorrhization and herbivory as factors.

To calculate the bacterial load in each sample, total DNA was amplified using universal primers for the 16S rRNA gene by performing a specific quantitative PCR (qPCR) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction was carried out using 5x HOT FIREpol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) in a total reaction volume of 20 µL. To estimate the bacterial concentration, the threshold cycle (Ct) values of our samples were compared with those from a standard curve of known *Escherichia coli* DNA concentrations. Statistical analysis was performed using one-way ANOVA with a Tukey's multiple comparison test (GraphPad software Inc., San Diego, CA, USA).

2.3. Results

2.3.1. Effect of mycorrhization and herbivory on larval development

To understand how mycorrhization (Fm-), herbivory (Nm+) or a combination of both (Fm+) impact on the development of fourth instar *S. exigua* larvae, we measured the relative growth of caterpillars after feeding on the different plant diets for 48 h (Fig. 14A). A significant decrease of about 2-fold in relative growth was observed after herbivory (Nm+ and Fm+) but not after mycorrhization, and both factors interacted in a significant way (mycorrhiza: $F_{1,8}$=3.727, P = 0.090; herbivory: $F_{1,8}$=21.939, P = 0.002; interaction: $F_{1,8}$=5.435, P = 0.048). No larval mortality was observed in either treatment.

2.3.2. Influence of mycorrhization and herbivory on larval susceptibility to entomopathogens

The effect of mycorrhization and herbivory on the susceptibility of *S. exigua* to two natural bacterial (*B. thuringiensis*) and viral (SeMNPV) pathogens was evaluated. Susceptibility of larvae to Bt was analyzed at sublethal and lethal concentrations. At the sublethal concentration (1 mg/mL), no mortality was observed for the insects feeding on diet based on control plants (Nm-) whereas about 20 % decrease in survival was observed when infected larvae were reared on either Nm+, Fm- or Fm+ diets (χ^2 = 12.31, df = 3, P = 0.0064; Fig 14B). There were no significant differences among these three treatments. In contrast, when larvae were exposed to a higher concentration of Bt (3 mg/mL), no differences on survival were observed between the larvae feeding on the different treated plant-based diets and the control Nm- (χ^2 = 2.114, df = 3, P = 0.5492; Fig 14C). Susceptibility to baculovirus was evaluated using a sublethal concentration of

SeMNPV suspension (2x10^4 OBs/mL). Only the feeding on mycorrhizal plant (Fm-) based diet caused a significant increase in mortality when compared to the control (Nm-) (χ^2 = 3.944, df = 1, *P* = 0.0470; Fig 14D).

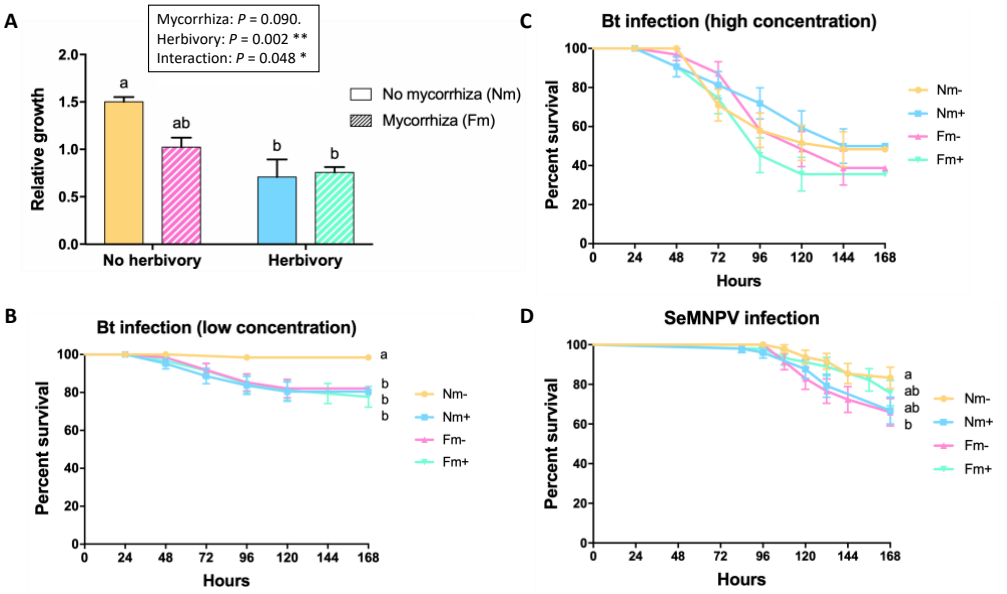

Figure 14. Effect of mycorrhization and herbivory on the development and susceptibility of *S. exigua* larvae to bacterial and viral pathogens.

(A) Larval development, represented as relative growth, after feeding on plant-based diets for 48 h. Mean values were analyzed with two-way ANOVA with mycorrhization and herbivory as factors followed by a Tukey HSD post hoc test. Error bars represent standard error of the mean (SEM). (B) Percentage of survival of larvae infected with XenTari® (*Bacillus thuringiensis* subsp. *aizawai*) at 1 mg/mL (sublethal) and (C) 3 mg/mL (lethal). (D) Percentage of survival of larvae infected with SeMNPV virus at a sublethal concentration (2 x 10^4 OBs/mL). Mortality curves were plotted using the Kaplan–Meier method and statistical analyses were performed using the log-rank (Mantel–Cox) test. Error bars depict standard error of the mean (SEM). Different letters denote significant differences among the treatments. Nm−, non-mycorrhizal plants without previous herbivory; Nm+, non-mycorrhizal plants exposed to herbivory; Fm−, mycorrhizal plants; Fm+, mycorrhizal plants exposed to herbivory.

2.3.3. Impact of treatments on larval immunity

Changes in the insect metabolism may weaken its immune status and in turn make insects more susceptible to pathogens. Hence, we asked whether feeding on plants that had been mycorrhized or previously exposed to herbivory may alter insect immune defenses. To test this, we focused on the analysis of one of the key components of the insect immune system, the phenoloxidase (PO), whose activity in the hemolymph is a widely used marker of the immune status. Two-way ANOVA showed that only previous herbivory has a significant effect on the activity of PO (mycorrhiza: $F_{1,8} = 1.361$, $P = 0.277$; herbivory: $F_{1,8} = 6.665$, $P = 0.033$; interaction: $F_{1,8} = 0.010$, $P = 0.924$). (Fig 15A). To verify that reduction in PO activity was not driven by the presence of PO inhibitors in the lyophilized leaves, we performed an inhibition assay that confirmed that none of the different treatments inhibited PO activity in insect hemolymph (mycorrhiza: $F_{1,8} = 2.061$, $P = 0.189$; herbivory: $F_{1,8} = 0.010$, $P = 0.924$; interaction: $F_{1,8} = 0.157$, $P = 0.702$) (Fig 15B).

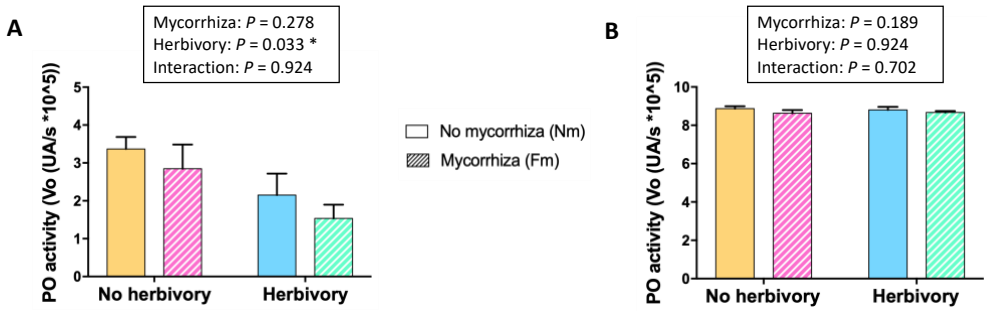

Figure 15. Effect of mycorrhization and herbivory on *S. exigua* phenoloxidase (PO) activity.

(A) Enzymatic activity of PO, represented as the initial velocity (Vo) of the reaction. (B) PO enzymatic inhibition assay using leaf extracts from the different treatments. Means were analyzed with two-way ANOVA using mycorrhization and herbivory as factors. Error bars represent standard error of the mean (SEM).

2.3.4. Gut microbiota changes after feeding on the different diets

Gut microorganisms are critical to the nutrition, physiology, and immune responses of many insect species, and have a complex interplay with both plant metabolites and entomopathogens. Thus, we studied the influence of the different plant-based diets to understand the impact of mycorrhization and herbivory on the gut bacterial communities of *S. exigua.*

Bacterial microbiota composition was determined in larval guts after 48 hours of feeding on artificial or the plant-based diets (Fig. 16A). Most abundant bacteria belonged to the *Delftia* genus, which was highly present in all the groups (with a relative abundance ranging from 10 % to 50 %), followed by unclassified *Oxalobacteraceae, Comamonadaceae* and *Enterobacteriaceae*. Canonical correspondence analyses (CCA) showed significant differences (P = 0.038; Fig 16B, upper panel) in larval gut microbiota composition at the genus level among the artificial and the plant-based diets. However, these differences disappeared when only plant diets were compared (P = 0.053; Fig 16B, bottom panel). Great variability and heterogeneity were observed among different diet groups but also among samples from the same group. No differences in the bacterial load (P = 0.407; Fig 16C) nor in the diversity (P = 0.37; Fig 16D) were observed among the different diets. LEfSE analysis revealed the existence of three genera with differential abundance according to the type of ingested diet. High abundance of unclassified *Caldilineaceae* and *Anaerobaculaceae* were detected in larvae fed on artificial diet when compared with the plant-based diet. Unclassified *Xanthomonadaceae* were found more abundant in the insects fed on the Fm+ plant diet when compared to the other diets.

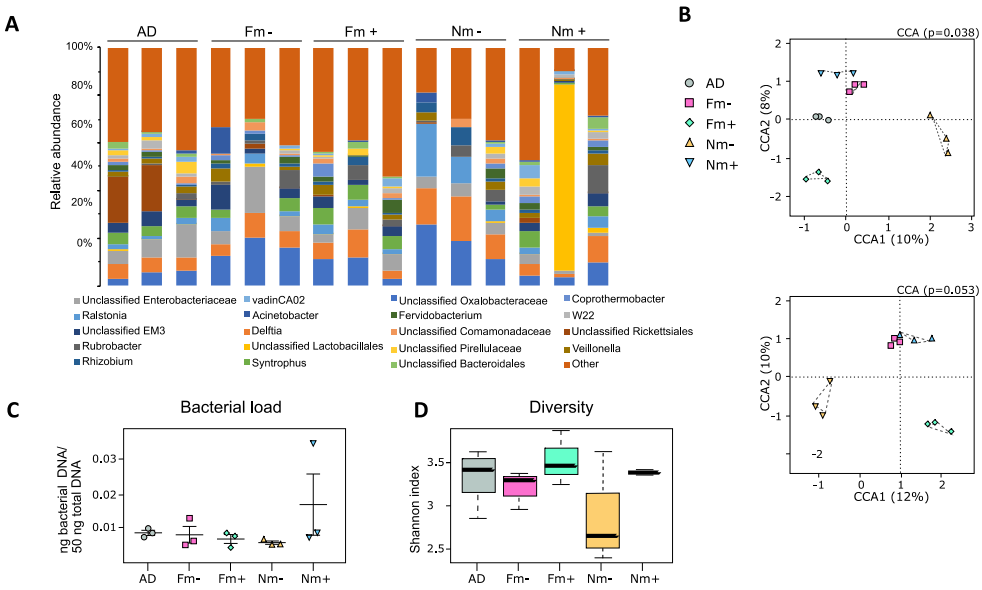

Figure 16. Effect of mycorrhization and herbivory on the gut microbiota of *S. exigua* larvae.

(A) Relative abundance (percentage) of the different genera of bacteria of guts from larvae fed on the different diets for 48 h. Each bar represents a pool of four larvae. (B) Canonical correspondence analysis (CCA) showing the relationship between the composition of larval gut microbiome at genus level and the diets tested: artificial diet (AD) and plant-based diets. (C) Bacterial load of larval guts calculated as nanogram of bacterial DNA per 50 ng of total DNA (means ± SEM). (D) Microbial diversity of larval guts represented using Shannon index. Nm−, non-mycorrhizal plants without previous herbivory; Nm+, non-mycorrhizal plants exposed to herbivory; Fm−, mycorrhizal plants; Fm+, mycorrhizal plants exposed to herbivory.

2.4. Discussion

Our results show that mycorrhizal colonization of tomato roots by *Funneliformis mosseae* coupled with previous herbivory decreases *S. exigua* growth, enhances its susceptibility to *Bacillus thuringiensis*, and has positive or neutral impact on the lethality of SeMNPV. These bacterial and viral entomopathogens are widely used for the control of folivorous *S. exigua* larvae (Bravo et al., 2011; Lacey et al., 2015; Beas-Catena et al., 2014), and our results support the compatibility of AMF application in tomato with the use of entomopathogens in pest management strategies.

AMF colonization is known to imply changes in plant physiology and metabolism that may enhance its resistance to foliar-feeding herbivores (Jung et al., 2012; Rapparini et al., 2008; Hause et al., 2007; He et al., 2017; Pozo et al., 2015). Our larval growth assays have revealed that *F. mossae* inoculation in combination with herbivory had a negative impact on the growth of *S. exigua*. This confirms previous observations with the same mycorrhiza-plant-insect combination (Rivero et al., 2021; He et al., 2017), with a related foliar-feeder, the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Song et al., 2013), with *S. exigua* feeding on *Plantago lanceolata* inoculated with *F. mossae* (Wang et al., 2015), and also with other *Spodoptera* spp.-plant-AMF combinations (Barber, 2013; Shrivastava et al., 2015; Rabin & Pacovsky, 1985; Formenti & Rasmann, 2019). Our approach differs from these previous studies because we used a plant-based artificial diet composed by lyophilized leaves from the inoculated and/or herbivory-exposed plants. This allows the dissociation between the effects of mycorrhization and herbivory, and to test separately their dietary outcomes on larval growth and susceptibility to entomopathogens. We observed that previous herbivory but not AMF

colonization reduced larval growth, and that both factors interacted in a significant way. The weight decrease of *S. exigua* larvae feeding on a diet based on herbivory-exposed plants, either with or without AMF inoculation, may be related to the JA-dependent defense response of the tomato plants, which accumulate secondary metabolites toxic to deter the herbivore (Erb & Reymond, 2019; Wu & Baldwin, 2009). In fact, upon *S. exigua* herbivory, tomato plants undergo a dramatic metabolic reconfiguration, which has been shown by previous studies to be primed by *F. mossae* mycorrhization (Rivero et al., 2021). In contrast, in the absence of herbivory, AMF colonization has very low impact on the foliar metabolic profile (Begum et al., 2019) although some transcriptional shifts in transcription in the levels of genes related to JA biosynthesis and response -even in the absence of any aggressor- have been reported (Cervantes-Gámez et al., 2016; Sanmartín et al., 2020). Thus, the significant interaction between herbivory and AMF colonization on *S. exigua* larval growth may be explained by the effect of AMF priming to the subsequent response to herbivory. However, we should be aware that in lack of metabolic data supporting the induction of plant defenses and their modulation by AMF priming, we cannot exclude other hypothesis explaining the reduced larval growth. For example, a drop in the nutritional quality of the attacked leaves might reduce the conversion efficiency of the ingested food by the larvae (Wang et al., 2015). Previous studies have shown that in tomato, *F. mossae* inoculation increased leaf photosynthesis, nutrient absorption, and altered foliar hormone homeostasis (He et al., 2017). Thus, additional experiments are needed to unravel the causes of decreased *S. exigua* growth.

All the plant diet based on either mycorrhizal (Fm-) and/or plants exposed to herbivory (Nm+ and Fm+) displayed an increased susceptibility to a sublethal

concentration of *B. thuringiensis*, with no additional effects when AMF and previous herbivory were combined. Equally, a general increase in mortality of SeMNPV-infected *S. exigua* larvae was observed when they were fed on the treated plant-based diets, although only Fm- diet was leading to a statistically significant difference. These observations suggest that mycorrhization and previous herbivory, individually or in combination, could enhance the susceptibility of *S. exigua* larvae to entomopathogens. This contrasts with the only previous study so far that evaluated the combination of AMF and an entomopathogen, namely baculovirus. García-Gómez et al. (2021) reported that inoculation of maize roots with a natural community of AMF (including *Glomus* spp., *Acaulospora* spp., *Gigaspora* spp. and *Intraspora* spp) increased growth of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) under certain nitrogen fertilization conditions, and reduced about 25 % larval mortality by its baculovirus, SfMNPV (García-Gómez et al., 2021).

The increased lethality of *B. thuringiensis* after previous herbivory or the combination of herbivory and AMF may be explained by the defense metabolites produced by the plant after attack, which have been previously shown to influence the outcome of insect-baculovirus interactions (Hoover et al., 1998; Ali et al., 1998; Shikano et al., 2017) as well as increase or decrease the lethality of *B. thuringiensis* at various extents. For instance, tannins and nicotine decreased mortality of *B. thuringiensis* against a variety of insects (Krischik et al., 1988; Appel & Schultz, 1994; Navon et al., 1993; Lüthy et al., 1985; Lord & Undeen, 1990) whereas protease inhibitors, L-canavanine, resorcinol, and gallic acid increased *B. thuringiensis* lethality (Sivamani et al., 1992; Macintosh et al., 1990; Felton & Dahlman, 1984). The induction of herbivory defenses in tomato increased susceptibility to *B. thuringiensis* in the lepidopteran *Helicoverpa zea*

(Lepidoptera: Noctuidae) (Shikano et al., 2018), in a similar fashion to what we observed for *S. exigua*. In the tomato-*Helicoverpa* system, the increased susceptibility to *B. thuringiensis* correlated with increased plant's polyphenol oxidase (PPO) and peroxidase (POD) activity, which are part of the inducible defensive armoury of tomato (Shikano et al., 2018). Interestingly, in our system we reported an increased susceptibility to *B. thuringiensis* also in mycorrhizal plants, which had not been induced by previous herbivory and thus, no major changes in these defense related enzyme activities are expected (Rivero et al., 2021; He et al., 2017). Therefore, the enhanced lethality of *B. thuringiensis* but also of SeMNPV in larvae reared on Fm- diets may be led by other causes. The effects of plant nutrients dictate herbivore physiology, which in turn could influence their susceptibility to the third trophic levels such as the entomopathogens (Bauce et al., 2002; Coley et al., 2006).

Susceptibility to pathogens is strongly correlated with the immune status of an organism (Jiang et al., 2010; Casanova-Torres & Goodrich-Blair, 2013). Thus, we sought to evaluate the effects of the plant-based diets on the larval immune system, specifically focusing on the activity of the insect's phenoloxidase (PO), due to its relevant function in cellular and humoral response in insects (Lu et al., 2014). PO enzyme is involved in the formation of melanin, which is deposited around the damaged tissue or foreign object, leading to the production of intermediate products with toxic effects against bacterial, fungal and viral agents (Kanost & Gorman, 2008). Enzymatic assays revealed that PO activity was significantly reduced by herbivory but not by AMF or the interaction of the two factors. Additionally, inhibition assays showed that the leaf extracts from the tested plants did not suppress PO activity, ruling out the possibility of direct interference with the enzymatic activity. Thus, the results suggest that

compounds induced during herbivory negatively influence the immune status of *S. exigua* larvae. The reduction in PO activity may be the outcome of direct toxic effects of leaf allelochemicals that debilitate the larval immune system, or indirect consequences of the altered herbivore performance and development of the insect (Cory & Hoover, 2006; Smilanich et al., 2009).

Finally, we asked whether the different plant-based diets had an impact on the gut microbiota, which is known to influence insect physiology by the modulation of caterpillar nutrition and metabolism, development and immune responses (Engel & Moran, 2013; Wu et al., 2016). Although we did not observe major influence of the diet on the bacterial load or alpha diversity of the gut microbiota, CCA analyses revealed significant differences in bacterial composition between larvae fed on artificial diet and tomato plant-based diets. Similar changes in the microbiota composition associated with the source of the ingested food (artificial diet, pepper and tomato) were previously observed in *S. exigua* larvae by Martínez-Solís et al. (2020). Nevertheless, when only plant-based diets were included in the multivariate canonical analysis, the differences disappeared. This may indicate that changes in the leaf nutritional quality or phytochemicals induced by mycorrhization and/or exposition to herbivory were not/or just slightly influencing microbiota gut composition of the caterpillar.

In conclusion, AMF colonization in tomato plants increase the susceptibility of the generalist pest *S. exigua* to the bacterial entomopathogen, *B. thuringiensis* and does not negatively affect the action of the viral entomopathogen, baculovirus. These findings support the compatibility of AMF inoculation with the use of bacterial and viral entomopathogens and supports the design of pest control strategies combining the effects of both treatments. Nevertheless, further studies using different combinations of plants, AMF,

herbivores, and entomopathogens are needed to extend these results to other crops and pest systems.

CHAPTER 3:

Modulation of tomato defense responses against *Spodoptera exigua* herbivory by baculovirus infection

3.1. Introduction

The persistent pressure caused by insect herbivory has forced plants to produce a wide arsenal of defenses to minimize or prevent tissue losses (Mitchell et al., 2016). The ability of plants to induce antiherbivore defenses requires the perception of the presence of the insect (Bonaventure, 2012). This is partially achieved by the detection of endogenous danger signals, known as damage-associated molecular patterns (DAMPs), which are released after the disruption of tissues occurred during herbivory (Quintana-Rodriguez et al., 2018; Tanaka & Heil, 2021). In addition, plant also perceive molecules specifically associated to insect feeding, known as herbivore-associated molecular patterns (HAMPs), which allow plants to discriminate between herbivory and mechanical wounding, and induce stronger plant resistance (Felton & Tumlinson, 2008; Erb & Reymond, 2019). These insect-produced cues have been identified in the regurgitant, saliva, ovipositional fluids, eggs, and digestive waste products (Jones et al., 2022; Basu et al., 2018; Malik et al., 2021). However, HAMPs are mostly found in oral secretions (OS), being referred in caterpillars as the combination of saliva, which is secreted from labial and mandibular glands, and regurgitant, which arises from the fore- and midgut and is mainly composed by gut contents (Chuang et al., 2014; Musser et al., 2006; Liu et al., 2004). OS, which are deposited in leaves during larval feeding, contain HAMPs comprising fatty acid-amino acid conjugates (FACs), digestive enzymes (*i.e.* β-glucosidase and glucose oxidase), and proteolytic fragments derived from digested plant proteins (*i.e.* inceptins), among others (Bonaventure et al., 2011b; Acevedo et al., 2015).

The perception of DAMPs and HAMPs generally induces the activation of antiherbivore responses which are triggered by the phytohormone jasmonic acid

(JA) and its derivatives (Duran-Flores & Heil, 2016; Heil & Land, 2014; Snoeck et al., 2022). The jasmonate pathway has an essential role in conferring plant protection against chewing insects, cell-content feeders, some phloem-sucking insects, and necrotrophic pathogens (Campos et al., 2014; Thaler et al., 2012; Zhang et al., 2017). In contrast, the salicylic acid (SA) pathway usually mediates resistance to (hemi)-biotrophic pathogens, viruses, and some phloem-sucking insects (Li et al., 2019b; Stroud et al., 2022; Spoel et al., 2007). Induction of JA-dependent defenses triggers the production of chemical compounds with toxic, repellent, or antinutritive properties against insects (*i.e.* secondary metabolites, proteinase inhibitors, etc…) as well as indirect defenses, like volatiles, that attract natural enemies (Mithöfer & Boland, 2012; Fürstenberg-Hägg et al., 2013).

To counteract plant defenses, insects have evolved effectors in their OS, which are specific molecules that interfere or suppress host plant defensive responses (Felton et al., 2014; Jones et al., 2022). A well-studied effector is the glucose oxidase (GOX), a salivary enzyme found in many caterpillars species which is able to modulate plant immunity in a context-dependent fashion (Musser et al., 2005; Lin et al., 2020; Eichenseer et al., 2010). GOX from *Helicoverpa zea* larvae suppressed inducible defenses in tobacco by reducing the production of foliar nicotine (Musser et al., 2002). However, this enzyme had an opposite effect in tomato since it increased JA levels and triggered a higher density of type VI glandular trichomes after *Helicoverpa zea* feeding (Tian et al., 2012a). Besides GOX, other effector compounds have been identified in the OS of caterpillars (Jones et al., 2022; Wang et al., 2023). For example, the Helicoverpa armigera R-like protein 1 (HARP1) and the Highly Accumulated Secretory Protein 1 (HAS1) are effectors found in the OS of the cotton bollworm

Helicoverpa armigera (Chen et al., 2019, 2023). It has been reported their role in the suppression of defensive responses in *Arabidopsis* plants but using different mechanisms: HARP1 interacts with JASMONATE-ZIM-domain (JAZ) repressors and prevent their degradation, thus attenuating JA signaling transduction (Chen et al., 2019), whereas HAS1 binds to specific transcription factors and block the expression of JA defense genes (Chen et al., 2023). In addition, a recent work in the leafminer *Phthorimaea absoluta* found that the protein REPAT 38, an ortholog of the HARP1 and HAS1 proteins, was responsible for inhibiting tomato plant defenses (Wang et al., 2024). These effectors belong to a superfamily of small glycosylated proteins named as REPAT (REsponse to PAThogens), which has at least 46 members in the beet armyworm *Spodoptera exigua* (Navarro-Cerrillo et al., 2013). These proteins are known to be upregulated in the insect midgut after exposition to entomopathogens such as *Bacillus thuringiensis* or baculovirus (Herrero et al., 2007; Navarro-Cerrillo et al., 2013).

Baculoviruses, which belong to the family Baculoviridae, possess a circular double-stranded DNA genome and are mainly effective against the genera Lepidoptera, Hymenoptera, and Diptera (van Oers & Vlak, 2007). Due to their high specificity and lack of toxicity to non-target organisms, they are commonly used as biological control agents in integrated pest management (IPM) programs (Landwehr, 2021). Baculoviruses persist in the environment, *i.e.* plant surface or soil, as virions occluded in the occlusions bodies (OBs), which are proteinaceous matrixes that protect infectious particles. Their life cycle begins after the ingestion by the target insects of food contaminated with OBs, which are solubilized in the midgut, and occlusion-derived viruses (ODV) are released to infect the epithelial cells of the insect midgut (Cory & Myers, 2003;

Williams, 2023). This primary infection leads to the production of budded virus that spread to other tissues, such as trachea, fat body, midgut, or nervous system, and cause a systemic secondary infection (Clem & Passarelli, 2013). At late infection stages, new occlusion-derived viruses (ODV) will be produced and finally occluded in new OBs, which will be released to the environment after larval death and liquefaction (Rohrmann GF, 2019).

Since previous reports showed that baculovirus infection induced the expression of several REPAT proteins in the midgut of lepidopteran larvae (Herrero et al., 2007; Navarro-Cerrillo et al., 2013), as well as some REPAT proteins present in caterpillar's OS specifically weaken host plant defenses (Chen et al., 2019, 2023; Wang et al., 2024), we hypothesize that infection with baculovirus may alter the composition of insect's OS and, consequently, interfere with the perception and/or response of plants to herbivory. To explore this hypothesis, we investigated the effect of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) infection of *Spodoptera exigua* larvae on the induction of tomato plant defenses. We subjected plants to herbivory by mock- or infected larvae, and subsequently we evaluated tomato defense responses in terms of gene expression and phytohormone abundance. In addition, OS protein composition of both groups of larvae was also analyzed. Overall, our results show a reduction in the activation of jasmonate pathway after feeding with SeMNPV-infected larvae, suggesting that baculovirus infection may interfere in the plant-insect relationship by modulating tomato defenses.

3.2. Material and methods

3.2.1. Insects

In this study, we used *S. exigua* caterpillars from a laboratory colony, which was established over 10 years ago using eggs provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland). The colony was kept in a growth chamber under controlled conditions: temperature of 25 ± 3 °C, relative humidity of 70 ± 5 %, and photoperiod of 16 h light: 8 h dark. Larvae were reared on artificial diet (Elvira et al., 2010), while adults had *ad libitum* access to a solution containing 10 % sugar.

3.2.2. Tomato plants

Solanum lycopersicum cv. Moneymaker (MM) seeds were sown in jiffys (0.4 cm), composed by pressed coconut fiber, inside seedbeds (6.5 x 6.5 cm). Seedlings at two cotyledons stage were transplanted to 12 cm diameter plastic pots containing brown peat: perlite (2:1) as a growing substrate. They were grown in a controlled greenhouse with a temperature ranging from 20-26 ºC during the day and 19-22 ºC at night, and with a 16 h:8 h diurnal photoperiod. Plants were grown in an ebb-and-flow subirrigation system with nutrient solution. No phytosanitary treatments were applied in the greenhouse, and no auxiliary insects were released.

3.2.3. Viral infections

Newly molted fourth instar *S. exigua* larvae were infected with the viral entomopathogen Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) SP2 strain (Caballero et al., 1992), using a concentration that killed about 90 % of larvae (5 x 10^7 OBs/mL). Larval infection was performed using the droplet

feeding method (Hughes & Wood, 1981). Specifically, larvae were placed in Petri dishes where previously 4 µL droplets were arranged in circle. Each droplet was composed by 10 % sucrose in phosphate-buffered saline (PBS; pH 7.4), 10 % (v/v) tracking dye phenol red, and the viral suspension. Once the ingestion of the droplets was visually confirmed, larvae were individually placed in bioassay trays, provided with artificial diet, and maintained for 72 h in a growth chamber under the controlled conditions mentioned above. Control larvae received a mock treatment, which consisted of the same procedure without the presence of the virus.

3.2.4. Plant exposure to herbivory

Healthy four-week-old tomato plants, each with 6-8 expanded true leaves, were moved from the greenhouse to a controlled climatic chamber (temperature of 25 ºC ± 1 ºC and a 16 h:8 h diurnal photoperiod) three days before the experiments and randomly distributed. To induce tomato defenses, plants were exposed to feeding by newly molted fifth instar *S. exigua* larvae, either mock- or SeMNPV-infected at 72 hours post infection (hpi), for 3 hours. Each plant was infested with two larvae, each placed on the terminal leaflet of the third or fourth true leaf, respectively, using 25 mm ∅ clip-cages to limit the feeding area. Plants that were mechanically wounded (tracing wheel + clip-cage) served as controls. Each plant was considered a biological replicate, and five plants were used per condition and assay. After 3 hours of induction, all the larvae and clip-cages were removed, and the terminal leaflets from the third leaves were immediately frozen in liquid nitrogen and stored at -80 ºC. Twenty-four hours after the start of the experiment, the terminal leaflets from the fourth leaves and the adjacent leaflets were collected in the same way. The removed larvae were kept in bioassay trays and provided with artificial diet in a growth chamber under controlled conditions

to confirm they were infected with SeMNPV. Consumed leaflet areas were quantified using the LeafByte mobile application (Getman-Pickering et al., 2020). Three independent experiments were performed at different times.

3.2.5. RNA extraction and gene expression analysis

Extraction of total RNA from leaflets was carried out by adding 0.6 mL of TRIdity G™ (AppliChem GmbH.; Darmstadt, Germany) to 50 mg of fresh ground leaflets corresponding to each replicate and condition. After gently mixing and incubating for 5 min at room temperature, samples were centrifugated at 12.000 × *g* for 5 min at 4 ºC. The supernatant was transferred into an Eppendorf tube containing 132 µL chloroform and centrifuged at 12.000 × *g* for 10 min at 4 ºC. The resultant supernatant was collected and mixed with 350 µL of isopropanol, 350 µL of a mix of 0.8 M citrate and 1.2 mM NaCl, and 4 µL of glycogen 1:10. Once centrifuged at 12.000 × *g* for 15 min at 4 ºC, the supernatant was discarded, and the pellet was rinsed twice with 500 µL of 75 % ethanol. Hereafter, the pellet was air-dried, resuspended in 20 µL of nuclease-free water and incubated at 55 ºC for 10 min. One microgram of each RNA sample was treated with DNAse I (Thermo Fisher Scientific; Vilnius, Lithuania) following manufacturer's protocol. Then, DNAse-treated RNA was converted into cDNA using PrimeScript™ RT Reagent (Takara Bio Inc.; Kusatsu, Japan) according to manufacturer's recommendations. Real-time quantitative PCR Kit (RT-qPCR) was performed in a StepOnePlus Real-time PCR System (Applied Biosystems; Massachusetts, United States) using 5x HOT FIREpol Eva Green qPCR Mix Plus (ROX) (Solis Biodyne; Tartu, Estonia) in a total reaction volume of 20 µL. Specific primers for amplifying *SlEF* (Solanum lycopersicum elongation factor 1-alpha), *PIN2* (wound-induced proteinase inhibitor 2), and

PR1 (pathogenesis-related protein 1) genes were provided by Martínez-Medina et al. (2013); whereas *AOS* (allene oxide synthase), *OPR3* (12-oxophytodienoate reductase 3), *PPO* (polyphenol oxidase) and *ICS* (isochorismate synthase) genes were amplified with specific primers designed with the online software tool Primer3Plus (Untergasser et al., 2007). The list of sequences is provided in the Supplementary Table S2. The housekeeping gene *SlEF* was used as endogenous control to normalize the expression data. The differences in gene expression between treatments (wounding, herbivory by mock- or infected-larvae) were calculated using the ΔΔCt method (Livak & Schmittgen, 2001). Differences in gene expression among treatments were assessed using one-way ANOVA followed by Tukey's multiple comparisons test (R Core Team, 2023).

3.2.6. Phytohormone analysis

For hormone quantification, ten milligrams of freeze-dried leaf powder corresponding to each replicate and condition were mixed with 1 mL of 10 % methanol supplemented with 0.01 % HCOOH containing a mixture of internal standards (jasmonic-2,4,4d3-(acetyl- 2,2-d2) acid (JA-d5), jasmonic acid isoleucine-13C6 (JA-Ile-13C6), and salicylic acid-d5 (SA-d5) to a final concentration of 5 ppb. Samples were centrifuged at 15.000 rpm for 15 min at 4 ºC, and the supernatant was transferred to a new tube and filtered. Then, 5 µL of a 1:3 dilution were injected into an Acquity UPLC system (Waters, Milford, MA, USA) coupled with a triple quadrupole mass spectrometer using a UPLC Kinetex 2.6 µm EVO C18 100 A, 2.1 Å~ 50 mm (Phenomenex) column in negative ionization mode (ESI−). External calibration curves for each tested compound were prepared with pure standards. The phytohormones OPDA, JA, JA-Ile, SA, and SGE+SAG were analyzed, and nanograms of each phytohormone were normalized by grams of dry weight. Differences in phytohormone levels among

treatments were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test (R Core Team, 2023).

3.2.7. Oral secretion proteomics

The composition of the OS was evaluated in both mock- and SeMPNV-infected *S. exigua* larvae after fed on tomato plants for 3 h. To obtain the OS, each larva was carefully immobilized by holding it between the thumb and the index finger, and the larval mouth was gently touched with a 0.1 – 1 µL pipette tip, which made the larva spit out the OS. As OS were collected, they were maintained in a tube on ice to avoid protein degradation. OS samples belonging to 10 larvae from the same treatment (control or SeMNPV) were pooled and centrifuged at 1.000 × *g* for 5 min at 4 ºC to remove the debris. Then, supernatants were collected, immediately frozen in liquid nitrogen and kept at -80 °C until being analyzed. Two technical and two biological replicates per treatment were conducted at different times.

Proteomic analysis was performed in the Central Service for Experimental Research (SCSIE) at the University of València. Precipitation of proteins was carried out using the 2-D Clean-Up Kit (Cytiva, Marlborough, USA) following the manufacturer's instructions. Pellets were resuspended in 25 µL of 1 X Laemmli's Buffer and denatured at 95 °C for 5 min. For individual sequential window acquisition of all theorical fragment ion mass spectra (SWATH-MS) analysis, 15 µg of protein extract were loaded in a 1D SDS-PAGE gel for sample cleaning. Gel fractions were cut and digested with 1 µg sequencing grade trypsin (Promega, Madison, USA) in 100 µL of ammonium bicarbonate (ABC) solution (Shevchenko et al., 1996). Digestion was stopped with trifluoro-acetic acid (TFA) (1 % final concentration), followed by a double extraction with

acetonitrile (ACN). The final peptide solutions were dried in a rotatory evaporator and resuspended with 45 µL of 2 % ACN and 0.1 % TFA.

For each mixture of digested peptide, 5 µL were loaded by a Ekspert nanoLC 425 (Eksigent Technologies LLC, Dublin, USA) onto a trap column (3 µ C18-CL, 350 µm x 0.5 mm, Eksigent Technologies LLC) and desalted with 0.1 % TFA at 5 µL/min for 3 min. The peptides were eluted onto an analytical column (3 µ C18-CL 120 Ă, 0.075 x 150 mm Eksigent Technologies LLC), equilibrated in 5 % ACN 0.1 % formic acid. Peptide elution was undertaken with a linear gradient of 7 to 40 % of solution B (ACN, 0.1 % formic acid) in solution A (0.1 % formic acid) for 60 min at a 300 nL/min flow rate. A mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSCIEX) was used for peptide analysis. The sample was ionized in a Source Type Optiflow < 1 µL Nano applying 3.0 kV to the spray emitter at 200 °C. The tripleTOF was operated in SWATH mode, in which a 0.050 s TOF MS scan from 350–1250 m/z was performed. Then, 0.080-s product ion scans in 100 variable windows from 400 to 1250 m/z were acquired throughout the experiment. The total cycle time was 2.79 s. The wiff files obtained from SWATH experiment were analyzed by DIA-NN 1.8 (Demichev et al., 2020). DIA-NN generated the spectral library from a FASTA file with all the Uniprot sequences for *S. exigua* and the virus SeMNPV. Normalized quantities for protein groups were filtered at 1 % FDR, using global q-values for protein groups and both global and run-specific q-values for precursors. The unique genes file (genes identified and quantified using only proteotypic peptides) was employed to analyze the OS compositions. Differences among treatments were analyzed with Student's t-test (R Core Team, 2023).

3.3. Results

In order to investigate whether baculovirus infection would interfere in the perception of damage and the induction of host plant defenses, we evaluated the influence of herbivory by SeMNPV-infected *S. exigua* larvae on the expression of tomato defense genes, as well as the impact of baculovirus on the protein composition of insect's OS.

3.3.1. Effect of SeMNPV infection on plant response

3.3.1.1. Leaflet damage

We initially examined whether SeMNPV infection in *S. exigua* larvae affected their feeding rate by recording the areas of leaflets exposed to mock- or SeMNPV-infected larvae after 3 hours of feeding. No differences were detected in the level of damage between both groups ($P > 0.05$, Fig. 17), suggesting that the infection did not affect the feeding rate of *S. exigua* larvae compared to non-infected ones.

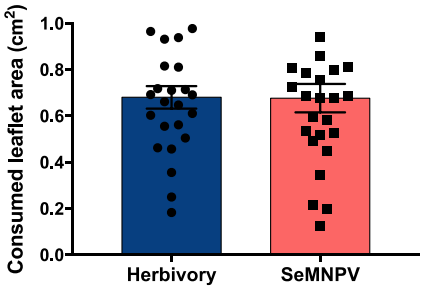

Figure 17. Damage level of tomato leaflets.
Consumed leaflet area after feeding for 3 hours by mock- or SeMNPV-infected *S. exigua* larvae. Mean values were analyzed with Student´s t-test. Whiskers plot represent the average and standard error of the mean (SEM).

3.3.1.2. Expression of tomato defense genes

After confirming the similar feeding among mock- and SeMNPV-infected larvae, the impact of SeMNPV infection on local plant response to herbivory was assessed. To do that, the expression of early and late JA-related genes of the damaged leaflets exposed to herbivory by mock- or infected-larvae was analyzed.

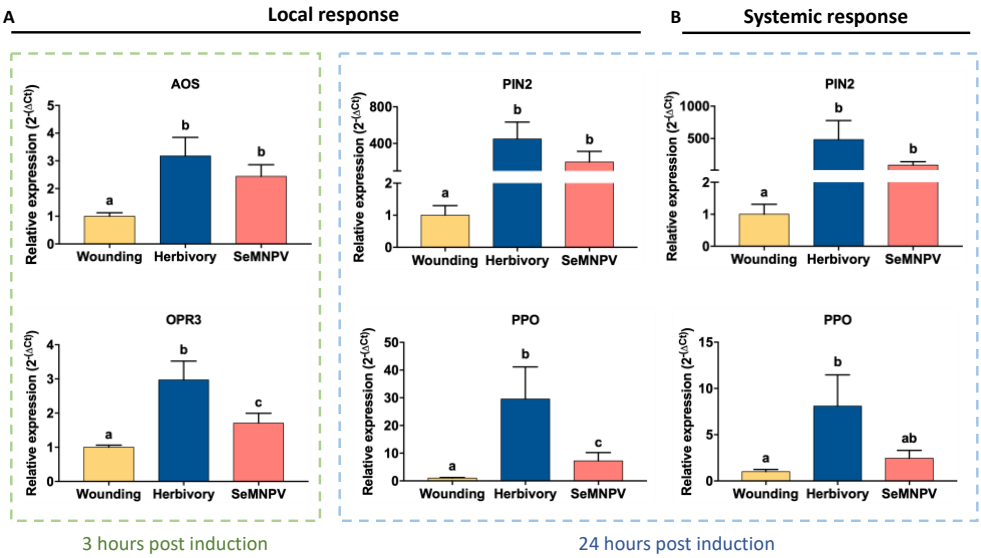

Figure 18. Gene expression of JA-related genes at local and systemic level.
Relative expression of tomato JA genes of leaflets exposed to 3 hours of damage by mechanical wounding or feeding by mock- or SeMNPV-infected larvae. Defense response was recorded at the local site of damage (A) and in the leaflet adjacent to that locally damaged (B). Expression of *AOS* and *OPR3* early genes was recorded after 3 hours of feeding, whereas *PIN2* and *PPO* expression was analyzed at 24 hours post induction. Mean values were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Error bars depict standard error of the mean (SEM). Different letters denote significant differences among the treatments. *AOS*: allene oxide synthase; *OPR3*: 12-oxophytodienoate reductase 3; *PIN2*: proteinase inhibitor II; *PPO*: polyphenol oxidase.

Leaves with mechanical damage were used as no-herbivory control. Feeding by mock-infected larvae significantly induced the expression of *AOS*, *OPR3*, *PIN2*, and *PPO* genes compared to the wounding treatment (Tukey HSD test, $P < 0.0001$; Fig. 18A), confirming the activation of JA signaling pathway. Instead, herbivory by SeMNPV-infected larvae revealed an overall decrease in the expression of JA-related genes compared to mock-infected larvae. In concrete, the herbivory by SeMNPV-infected larvae compared to the mock-infected ones showed a significantly reduced activation of *OPR3* and *PPO* genes (Tukey HSD test, *OPR3*: $P = 0.021$; *PPO*: $P = 0.016$; Fig. 18A). Taking together, these results show that herbivory by *S. exigua* larvae clearly induces local JA defenses in tomato, whereas baculovirus infection seems to modulate these antiherbivore responses, thus decreasing the activation of the JA signaling pathway and the expression of defense genes in the damaged tissue.

In order to assess the influence of SeMNPV infection on systemic plant response, expression of JA-related genes of adjacent leaflets to those that were locally damaged were analyzed. Again, herbivory by mock-infected *S. exigua* larvae significantly induced the expression of *PIN2* and *PPO* genes (Tukey HSD test, $P < 0.001$; Fig. 18B), suggesting that tomato herbivory responses were activated systemically. However, in this case the presence of the virus did not show so obvious reduction in the gene expression and no significantly reduction in the activation of *PIN2* and *PPO* genes at a systemic level was observed (Tukey HSD test, *PIN2*: $P = 0.3664$; *PPO*: $P = 0.0855$; Fig. 18B).

The expression of SA-related genes were also evaluated after mechanical damage or insect herbivory. Herbivory did not alter the expression of *ICS* and *PR1* neither at local site nor at the adjacent leaflet (Tukey HSD test, $P > 0.05$; Fig. 19), suggesting that this signaling pathway may be not required in tomato defense against *S. exigua* feeding. In addition, no differences between mock- and SeMNPV-infected herbivory were observed in the expression of SA-genes (Tukey HSD test, $P > 0.05$; Fig. 19), although a significant reduction in *ICS* expression was detected with SeMNPV infection compared to the wounding treatment that was not different from the mock-infected treatment (Tukey HSD test, $P < 0.001$; Fig. 19).

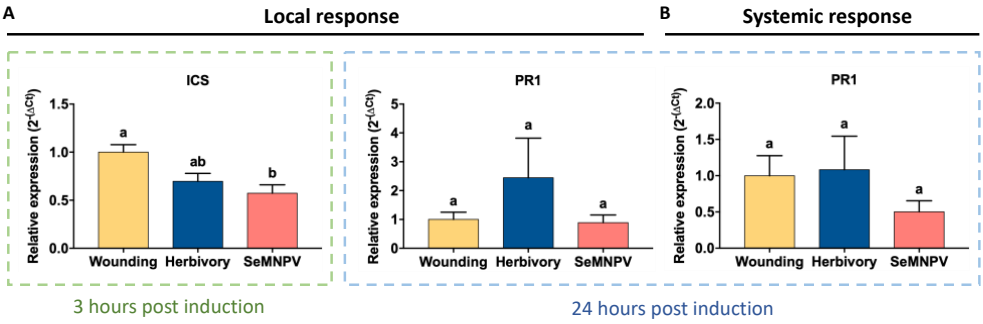

Figure 19. Gene expression of SA-related genes at local and systemic level.
Relative expression of tomato SA genes of leaflets exposed to 3 hours of damage by mechanical wounding or feeding by mock- or SeMNPV-infected larvae. Defense response was recorded at the local site of damage (A) and in a leaflet adjacent to that locally damaged (B). *ICS* expression was recorded after 3 hours of feeding, whereas *PR1* expression was analyzed at 24 hours post induction. Mean values were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Error bars represent standard error of the mean (SEM). Different letters indicate significant differences among the treatments. *ICS*: isochorismate synthase; *PR1*: pathogenesis-related protein 1.

3.3.1.3. Phytohormone levels

Since changes in the expression of some JA marker genes were detected in locally damaged leaflets, the abundance of the defense hormones OPDA, JA, and JA-Ile belonging to jasmonate pathway were also measured in those samples at 3 and 24 hours post induction (Fig. 20). Insect herbivory, regardless the presence of the virus, induced the accumulation of JA (Tukey HSD test, $P < 0.05$; Fig. 20B) and JA-Ile (Tukey HSD test, $P < 0.001$; Fig. 20C) at 3 hours post induction, thus confirming the activation of JA-defense responses.

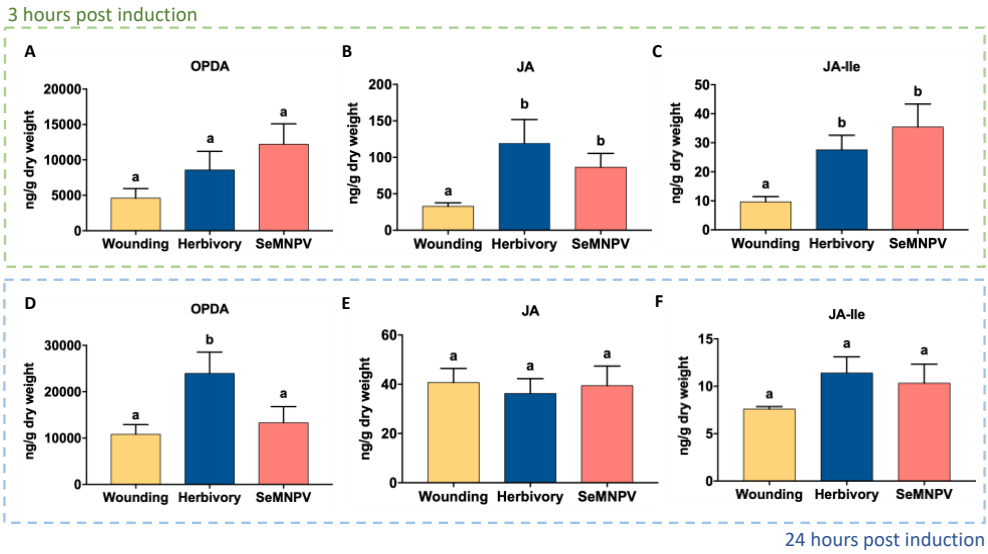

Figure 20. Levels of JA pathway-related phytohormones.
Abundance of phytohormones in leaflets exposed to 3 hours of mechanical wounding or feeding by mock- or SeMNPV-infected larvae. Hormone content was recorded at 3 (A, B, C) and 24 hours post induction (D, E, F). Mean values were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Error bars depict standard error of the mean (SEM). Different letters denote significant differences among the treatments. OPDA: 12-oxo-phytodienoic acid; JA: jasmonic acid; JA-Ile: jasmonic acid-isoleucine conjugate.

At 24 hours post induction, tomato leaflets exposed to herbivory by mock-infected larvae significantly accumulated OPDA (Tukey HSD test, $P = 0.0302$; Fig. 20D), whereas the presence of the virus reduced the amount of this phytohormone (Tukey HSD test, $P = 0.0249$; Fig. 20D), suggesting that SeMNPV infection may influence the accumulation of the JA-precursor OPDA in locally damaged tomato leaflets.

In contrast, insect herbivory did not induce the accumulation of SA-related phytohormones after 3 hours of feeding (Tukey HSD test, $P > 0.05$; Fig. 21), showing that SA signaling pathway is not activated with *S. exigua* herbivory, neither after herbivory in SeMNPV-infected larvae, suggesting that this virus is not involved in the modulation of SA pathway in tomato (Tukey HSD test, $P > 0.05$; Fig. 21).

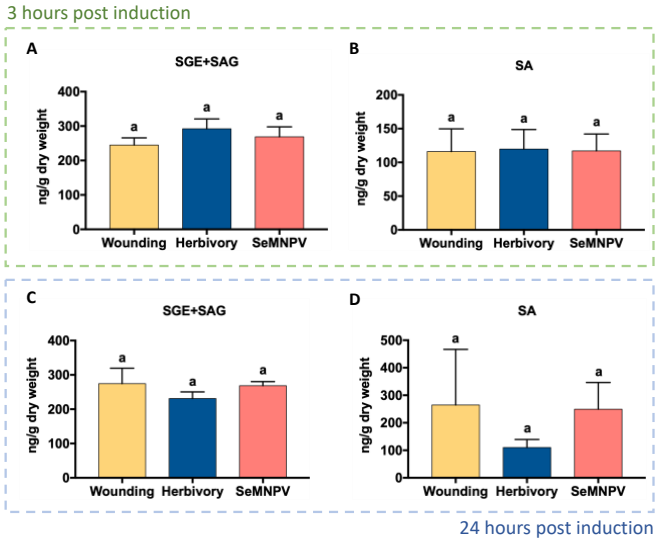

Figure 21. Levels of SA pathway-related phytohormones.
Abundance of phytohormones in leaflets exposed to 3 hours of mechanical wounding or feeding by mock- or SeMNPV-infected larvae. Hormone content was recorded at 3 (A, B) and 24 hours post induction (C, D). Mean values were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Error bars represent standard error of the mean (SEM). Different letters indicate significant differences among the treatments. SA: salicylic acid; SGE: salicylic acid glucose ester; SAG: salicylic acid glucoside.

3.3.2. Impact of infection on *S. exigua* oral secretion composition

After analysing the effect of SeMNPV on tomato herbivory responses, we explored changes in the protein content of *S. exigua* oral secretions after SeMNPV infection, to identify putative effectors that may be associated with the modulation of defense response. Proteomic analysis identified a total of 360 proteins in larval oral secretions, from which 6 were significantly up-regulated and 1 was significantly down-regulated in infected samples compared to non-infected ones ($P < 0.05$; Fig. 22).

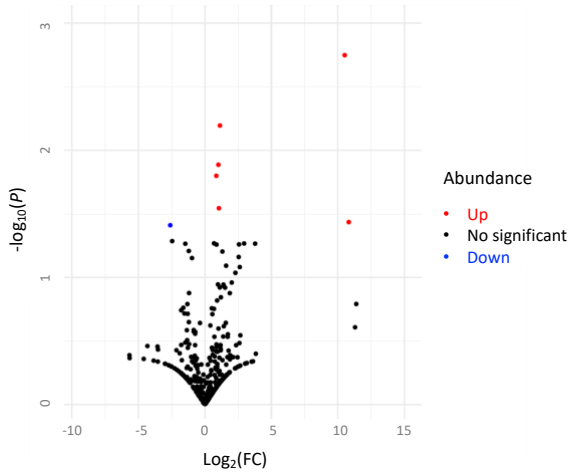

Figure 22. Volcano plot representing the protein abundance changes after baculovirus infection.
The differently up-regulated proteins in *S. exigua* oral secretions are depicted in red, whereas in blue is represented the only down-regulated protein. Two biological replicates were performed at different times. Differences in protein abundance were analyzed using an unpaired t-test analysis.

Specifically, baculovirus infection led to a significant increase in the abundance of the proteins: aldehyde dehydrogenase, dihydrolipoyl dehydrogenase, triosephosphate isomerase, cytochrome b-c1 complex subunit Rieske, peroxisomal multifunctional enzyme type 2, and an uncharacterized protein (Table 3). In contrast, SeMNPV treatment was responsible for a 5-fold reduction in the levels of only one protein: a lipase domain-containing protein of unknown function (Table 3).

Table 3. Proteins differentially expressed in *S. exigua* oral secretion after baculovirus infection.

Accession	Description	Fold change[1]	*P*-value
A0A835GFK7	Uncharacterized protein	1803.5	0.037
A0A922ME02	aldehyde dehydrogenase (NAD(+))	1457.8	0.002
A0A835L504	Dihydrolipoyl dehydrogenase	2.2	0.006
A0A835GTS1	Peroxisomal multifunctional enzyme type 2	2.1	0.029
A0A835LCX1	Triosephosphate isomerase	2.0	0.013
H9BBE7	Cytochrome b-c1 complex subunit Rieske, mitochondrial	1.8	0.016
A0A835GSR7	Lipase domain-containing protein	0.2	0.039

[1]Fold change corresponds to the relative abundance of proteins regulated after SeMNPV treatment in larval oral secretions compared to non-infected ones. Two biological replicates were performed at different times. Differences in protein abundance were analyzed using an unpaired t-test analysis.

3.4. Discussion

Plants have evolved several defensive strategies to prevent or overcome the attack of herbivorous insects. Among them are the production of antiherbivore defenses, such as the synthesis of toxic chemical compounds, which interfere with the growth, physiology, and survival of the insect (Mithöfer & Boland, 2012; Yactayo-Chang et al., 2020). In this context, potential allies of plants may be microbial entomopathogens, which can further impact the health of the attackers (Sabbahi et al., 2022; Elliot et al., 2000). Among them, baculovirus may indirectly contribute to the protection of the plant by infecting and killing the insect (Ruiu, 2018; Clem & Passarelli, 2013). Despite the hypothesis that the interaction with baculovirus may be beneficial for the host plant, the relationship among this entomopathogen, the larval host and the plant remains poorly understood. In this study, we found that tomato defense response to herbivory by larvae of the chewing caterpillar *Spodoptera exigua* infected with a lethal concentration of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) was lower compared to that elicited by mock-infected larvae. Moreover, we also detected changes in the protein composition of the oral secretion (OS) of *S. exigua* after SeMNPV infection compared to mock-infected larvae. These findings suggest that baculovirus infection triggers a lower activation of tomato antiherbivore responses by altering caterpillar's OS protein profile, thus influencing the outcome of plant-insect interactions.

The main plant hormones modulating inducible defenses against insects and pathogens are jasmonic acid (JA) and salicylic acid (SA) (Smith et al., 2009; Reymond & Farmer, 1998; Stroud et al., 2022). In this work, feeding of tomato plants by mock-infected *S. exigua* larvae for 3 hours led to a strong activation of JA pathway in damaged leaflets. This local induction was reflected in the

overexpression of some JA biosynthesis genes, as well as the JA-responsive genes *PIN2* and *PPO*, which drive the production of anti-digestive protease inhibitors and the oxidation of phenols to highly reactive quinones, respectively, in tomato (Kim et al., 2012; Taranto et al., 2017; Constabel & Barbehenn, 2008). Moreover, a systemic induction of the JA pathway was also reported in unwounded leaflets (Tian et al., 2014; Kloth & Dicke, 2022; Sun et al., 2011). In contrast, SA pathway was not activated after *S. exigua* herbivory since no changes in gene expression nor in phytohormone accumulation were detected. These results are in line with previous studies which have reported the activation of JA antiherbivore responses after herbivory by *S. exigua* and other lepidopteran larvae in different plant species, like tomato (Tzin et al., 2017; Al-Zahrani et al., 2020; Guo et al., 2019a; Hu et al., 2021). It is well known that feeding by chewing caterpillars cause massive damage in plant tissues, releasing DAMPs and HAMPs in the injury site (Tanaka & Heil, 2021; Acevedo et al., 2015; Felton & Tumlinson, 2008; Mithöfer & Boland, 2008), whose perception by the plant triggers the activation of the jasmonate pathway and, ultimately, the synthesis of a repertoire of toxic or deterrent compounds (Campos et al. 2014; Wasternack and Strnad 2019; Li et al. 2022; Frattini et al. 2024).

After feeding by baculovirus-infected larvae, the tomato anti-herbivory response showed some changes. In particular, quantitative RT-PCR analysis revealed a significant reduction in the expression levels of the early JA biosynthesis gene *OPR3* at 3 hpi, and the late JA responsive-gene *PPO* at 24 hpi, when compared to feeding by mock-infected larvae. In addition, it was also detected a lower accumulation of the phytohormone OPDA, the substrate of *OPR3* gene, in the damaged leaflets at 24 hpi. No changes in the expression levels of SA-responsive genes nor the accumulation of SA-related

phytohormones were detected. These findings suggest a role of baculoviral infection in the interplay between *S. exigua* and tomato JA signalling pathway.

Previous studies have reported the ability of viruses to alter host plant defenses by different ways. Some insect-borne plant viruses are involved in the suppression of antiherbivore plant responses by directly interfering with JA signalling pathway, thus enhancing the fitness of the insect vector and promoting viral transmission (Zhao & Li, 2021; Wu & Ye, 2020). For example, it has been found that the pathogenicity factor βC1 of the Tomato yellow leaf curl China virus (TYLCCNV) interferes with the dimerization of the transcription factor MYC2, inhibiting the expression of JA defense genes (Li et al., 2014), whereas the C2 protein of the tomato yellow leaf curl virus (TYLCV) hinders plant responses by compromising the degradation of JAZ1 repressor protein (Li et al., 2019c). Suppression of JA pathway by begomoviruses was translated into a lower synthesis of repellent terpenes, thus improving the performance of the whitefly *Bemisia tabaci*, the insect vector of both plant viruses. Viruses can also exert indirect influence on plant defensive mechanisms through modifications in the composition of the insect host's saliva or oral secretions. This modulation has been observed in the case of polydnaviruses (PDVs), obligate mutualistic viruses associated to parasitic wasp of caterpillars. Tan et al. (2018) reported that a PDV of the parasitoid *Microplitis croceipes* suppressed salivary GOX of *Helicoverpa zea* parasitized caterpillars, impairing the elicitation of tomato defenses. This effect increased the growth rate of the caterpillar, improving host suitability for the parasitoid, which may enhance the performance of the virus.

Limited research has focused on the influence of baculovirus infection on plant defense induction. A paper published by Pan et al. (2019) reported that Autographa californica multiple nucleopolyhedrovirus (AcMNPV)-challenged

H. zea larvae at 96 hpi induced higher plant responses (*i.e.* proteinase inhibitors) than mock-infected larvae after feeding on tomato. This led to a lower growth of larvae fed on leaflets previously damaged by infected larvae. From an ecological point of view, the negative impact on larval biomass may not benefit the virus because it may interfere with OBs production and viral dispersal. These results are contrary to our findings, since herbivory exerted by SeMNPV-challenged *S. exigua* larvae caused a reduction in the activation of tomato defenses. In addition, we observed a defense modulation at 72 hpi, unlike Pan et al. (2019) who reported an effect at 96 hpi, but not at 72 hpi. They infected larvae with a sublethal concentration of AcMNPV, so it is possible that the virus required more time to establish a systemic infection and mediate host changes responsible for the higher defense induction. In agreement with our findings, a role of SeMNPV infection in modulating plant defenses was also reported by Wan et al. (2017). They observed an increase in soybean volatile diversity after feeding by SeMNPV-infected *S. exigua* larvae, which enhanced the attraction of *Microplitis pallidipes* female wasps, which are endoparasitoids of *S. exigua.* The higher recruitment of *M. pallidipes* wasps to plants exposed to infected larvae may increase the contact of the parasitoids with the virus, thus facilitating its transmission when seeking new hosts. Overall, all these findings point to a role of baculovirus infections in shaping plant-insect interactions. However, more studies are needed to evaluate the extent of this modulation to different plant-insect-virus systems, since it seems that viral infections may act as both effector or elicitor of plant defenses, and to unravel the ecological consequences of these modulations.

Insect oral secretions (OS), which are composed by a mixture of regurgitant and saliva, have a crucial role in mediating plant defense responses

due to the presence of molecules acting as elicitor or effectors (Acevedo et al., 2015; Bonaventure, 2012). It has been previously reported that the composition of insect's OS can be influenced by some viruses, as in the case of PDVs, and ultimately produce a change in the plant defense response (Tan et al., 2019; Cusumano et al., 2018; Zhu et al., 2018). Based on this, we hypothesized that the observed reduction of tomato induced defenses after herbivory by infected larvae could be an indirect effect of baculovirus infection on caterpillar's OS content. Specifically, we evaluated the protein composition of *S. exigua* OS between mock- and SeMNPV-infected larvae due to the reported role of proteins in eliciting plant defenses (Zhang et al., 2024). Proteomic analysis showed a 5-fold decline in the abundance of a lipase domain-containing protein in OS samples from infected larvae. Insect-derived lipases are known to participate in the activation of jasmonate pathway since they allow the release of polyunsaturated fatty acids from the chloroplast membrane and their availability to JA biosynthetic enzymes (Bonaventure et al., 2011a; Kimberlin et al., 2022). Zhang et al. (2024) showed that the induction of maize defense responses by *Spodoptera frugiperda* herbivory is mainly attributed to lipase activity in OS, which corroborated previous findings by Acevedo et al. (2018) that identified the enzyme phospholipase C (PLC) in the saliva of *S. frugiperda*, as capable of inducing the overexpression of protease inhibitors in maize. Similarly, in the grasshopper *Schistocerca gregaria*, lipases present in the OS are involved in the accumulation of OPDA and JA in *Arabidopsis* plants (Schäfer et al., 2011). Thus, the reduction in lipase content in the OS of SeMNPV-infected *S. exigua* larvae may explain the lower induction of the jasmonate pathway in tomato plants.

We also identified other six proteins which were upregulated in the larval OS after baculovirus infection: aldehyde dehydrogenase (NAD(+)),

dihydrolipoyl dehydrogenase, peroxisomal multifunctional enzyme type 2, triosephosphate isomerase, cytochrome b-c1 complex subunit Rieske, and an uncharacterized protein. Aldehyde dehydrogenases (ALDHs) are enzymes involved in the oxidation of highly reactive aldehyde to non-toxic carboxylic acids (Shortall et al., 2021). Rivera-Pérez et al. (2013) documented the conversion of farnesal into farnesoic acid by ALDHs in the *corpora allata* of mosquitoes. In addition, an upregulation of ALDH levels was also detected after permethrin exposure in *Anopheles gambiae* (Vontas et al., 2005). Besides ALDHs, the cytochrome b-c1 complex subunit Rieske seems to be also associated to detoxification processes since it participates in the oxidation of quinones, toxic compounds for several lepidopteran insects (Ten Brink et al., 2013). Taking this into account, we hypothesize that the overexpression of these enzymes in *S. exigua* OS after viral infection may play a role in the detoxifying plant antiherbivore compounds. An increased detoxification rate, associated with the reduction in the activation of jasmonate defenses, may collectively render plants less toxic to the attacker. Consequently, this would allow for greater herbivore growth and, in turn, a higher production of OBs, which would be beneficial for the baculovirus. Moreover, the suppression of JA responses in tomato plants may not necessarily be detrimental for the plant, as the induction of defenses is energetically costly, and the control of the insect attacker might be efficiently achieved through the increased production of baculovirus. However, further studies are needed to confirm this intriguing hypothesis and to better understand the mechanisms underlying the effects of plant defenses on infected larvae in different plant-insect-virus systems.

Previous studies of our group found that baculovirus infection was involved in the induction of REPAT proteins in the midgut of lepidopteran larvae

(Herrero et al., 2007; Navarro-Cerrillo et al., 2013). The REPAT proteins HARP1 and HAS1 have been detected in the OS of the cotton bollworm *Helicoverpa armigera*, and identified as effectors of plant JA-defenses HARP1 (Chen et al., 2019, 2023). The HAS1 homolog VRLP4, present in the oral secretions of *S. frugiperda* larvae, was found to contribute to the suppression of maize plant defenses (Zhang et al., 2024). Based on the above, we initially hypothesized that the reduction in tomato defenses after baculovirus infection could be caused by a overaccumulation of REPAT proteins in the OS of *S. exigua* infected-caterpillars. However, no differences in REPAT protein content were observed between the OS of mock- and SeMNPV-infected larvae, thus pointing that the modulation of tomato defense response genes observed in our system was not due to a differential accumulation of some REPATs in the OS of SeMNPV-infected larvae.

In conclusion, our research showed that baculovirus infection interferes with the activation of jasmonate signaling pathway after *S. exigua* herbivory in tomato. Changes in larval OS composition induced by SeMNPV may underlie this effect. These results suggest that this viral entomopathogen could have a role in altering plant-insect interplay through the modulation of plant tomato defenses. Overall, these findings could be taken into consideration in integrated pest management programs due to their potential impact on multitrophic interactions.

GENERAL DISCUSSION

Entomopathogens are a diverse group of microorganisms which have evolved specialized mechanisms to infect and kill insects. Due to their high specificity and lack of toxicity to non-target organisms or the environment, they contribute to sustainable agriculture and reduce reliance on chemical pesticides (Smagghe et al., 2023; Sabbahi et al., 2022). Among these natural enemies, bacteria and viruses are commonly used as biological control agents in integrated pest management programs. The most commercialized bacterial and viral entomopathogens are *Bacillus thuringiensis* (Bt) and baculoviruses, respectively. Their mode of action has been studied extensively due to their potential in controlling insect herbivory (Bel et al., 2020; Heckel, 2020; Boogaard et al., 2018; Rohrmann GF, 2019). In both cases, the infection begins when caterpillars consume food contaminated with either Bt spores or virions-containing occlusion bodies (OBs). Bt is characterized by the synthesis of parasporal toxins which, after binding to receptors in the insect's gut lining, create pores that lead to insect's septicemia and death (Pinos & Hernández-Martínez, 2019; Palma et al., 2014). Instead, baculovirus replicate in the midgut after OBs dissolution and virions release, triggering both primary and secondary infections and causing larval liquefaction and death (Clem & Passarelli, 2013; Cory & Myers, 2003).

These natural enemies, together with nematodes and fungal entomopathogens, are recognized as key regulators of insect populations since they deeply influence the interaction between insects and plants. The top-down pressure exerted by entomopathogens is known to enhance plant growth and fitness, thus shaping the structure and dynamics of agroecosystems. However, **the potential impact of plants on entomopathogen effectiveness against insect pests (bottom-up effects) needs further exploration, some of them being part of the scope of this thesis**. Some studies have delved into these interactions, showing that plants can promote or interfere with the persistence

and/or efficacy of entomopathogens (Cory & Hoover, 2006; Shikano, 2017). Moreover, entomopathogenic action can be indirectly influenced by some plant traits through the modulation of insect susceptibility (Biere & Tack, 2013; Janmaat & Myers, 2005; Wang et al., 2022). Despite the progress made, additional research is necessary to fully elucidate this topic.

As entomopathogens can serve as natural allies against herbivory, we hypothesized that plant defenses act in concert with them to potentiate their ability in decimating insect populations. **In the first chapter of this thesis, we further explored the concept investigating whether constitutive and/or inducible tomato defenses contribute to Bt lethality against *Spodoptera exigua*.** Evaluating the effects of constitutive antiherbivore defenses on larval growth and mortality presents some challenges due to the activation of inducible plant responses during insect feeding. To address this, we established a bioassay system using a leaf-based artificial diet prepared with lyophilized leaves mixed with a diluted artificial diet at 5 % to contribute to the preservation and digestibility of the artificial form. This method allowed us to expose the insects to the phytochemicals produced by the plant at the exact moment of sampling, without any confounding effects triggered by subsequent insect feeding. To assess the stability of plant antiherbivore metabolites in the lyophilized leaves, we conducted preliminary experiments in which the leaves were stored at either -80 ºC or 29 ºC for 10 weeks (Suppl. Figure S2). Subsequently, leaf-based artificial diets were prepared to feed *S. exigua* larvae, and the growth parameters of the latter were recorded. The results confirmed the full preservation of the growth-inhibitory effects of lyophilized foliar compounds following exposure to 29 ºC.

After confirming the suitability of the method, we analyzed the effect of constitutive defenses from fifteen wild species and forty cultivated varieties on larval performance. The results showed that the defenses in wild species had a greater impact on larval growth compared to those in cultivated varieties. These differences in constitutive resistance could be due to the domestication of tomato, a process that has prioritized higher yield and fruit quality over defensive traits against insect herbivores (Chaudhary, 2013; Gasparini et al., 2024). As commercial traits have been selected during breeding programs at expense of constitutive resistance, it is likely that these domesticated crops allocate resources to defense only when attacked (Paudel et al., 2019). Domesticated varieties usually show higher rates of inducibility in response to herbivore attack, supporting the idea of a negative correlation between constitutive and inducible defenses (Karban & Baldwin, 1997). Notwithstanding these findings, our study revealed a significant influence of the plant genotype on *S. exigua* larval growth among the fifty-five tomato varieties, indicating that the effects of selective breeding are not as uniform as previously thought (Meyer et al., 2012). Regarding the effect of domestication on Bt-induced mortality, no differences were detected between both wild and cultivated varieties, suggesting that while constitutive defenses against caterpillars may have changed, those potentially amplifying Bt toxicity remained unaffected.

Subsequently, we assessed the influence of both constitutive tomato defenses on the efficacy of Bt against *S. exigua* larvae. We found that the negative effects of non-induced plant diets derived from the fifty tomato varieties positively correlated with the enhanced Bt-triggered mortality. Then, a smaller panel of tomato genotypes was selected and treated with methyljasmonate (MeJA) to activate JA-dependent defenses and prepare plant-based diets to feed *S. exigua* larvae. Since the same pattern was observed in larvae fed on artificial

diets derived from MeJA-treated plants, it seems to indicate that both constitutive and inducible tomato defenses play a role in enhancing the efficacy of Bt against *S. exigua*. The increased lethality of Bt against *S. exigua* that we observed after induction with MeJA is consistent with findings by Shikano et al. (2018), who reported similar effects of MeJA-induced tomato defenses on *Helicoverpa zea* larvae. Given the wide range of bioactive compounds produced by tomatoes, we propose several mechanisms by which plant defenses could enhance Bt lethality. One possible mechanism is the disruption of the peritrophic membrane – a protective layer in the larval gut against pathogens – by plant-produced tannins, chitinases or proteases (Caccia et al., 2012; Napoleão et al., 2019). Another strategy could involve the interference with the insect's digestive process through PPO enzymes, lectins or proteinase inhibitors, which reduce the availability of aminoacids and proteins, leading to nutritional deficiencies that may accelerate Bt-triggered larval mortality (Bhonwong et al., 2009; Fan et al., 2020). Furthermore, certain toxic phytochemicals such as triterpenoids, alkaloids, or flavonoids may directly compromise insect immune status, making it more difficult to clear the bacterial pathogen and thereby worsening the infection (Lampert, 2012). Considering that tomato plants produce a plethora of defenses to overcome insect herbivory, it is likely that the enhanced lethality of Bt results from the combined effects of multiple plant compounds. From an ecological perspective, the positive interaction between plant defenses and Bt is potentially mutually beneficial. By boosting Bt efficacy against *S. exigua*, plant defenses help reduce insect damage and increase plant fitness. At the same time, the effective control of the insect leads to its body disintegration and release of Bt spores, thus supporting the survival and spread of the bacterial entomopathogen.

We also identified potential compounds responsible for both larval damage and increased Bt susceptibility**.** After confirming that the leaf metabolome rearranged following MeJA treatment and comparing the profiles of the six elicited cultivated varieties, we found that the triterpenoid saponin soyasapogenol B base + O-HexA, Hex, dHex, 1 malonyl (SB) was the only compound overaccumulated in all the selected varieties after defense elicitation. It belongs to the family of saponins, a class of plant secondary metabolites involved in triggering cellular toxicity and altering insect digestion and immunity (Geyter et al., 2007; Tian et al., 2021). These detrimental effects are consistent with the observed reduction in larval growth after MeJA treatment and may explain the enhancement in the efficacy of Bt against *S. exigua.* Moreover, metabolomic analysis also provided other five compounds overaccumulated in the majority of selected varieties, such as the flavonoid catechin or the polyphenol syringaldehyde, with reported antiherbivore properties (Ruttanaphan et al., 2023; Regnault-Roger et al., 2004). The differential induction of these metabolites in each variety may be attributed to the fact that the identity and content of plant defensive compounds is greatly influenced by plant species, variety, or cultivar (Francini et al., 2020; Klepacka et al., 2011).

Overall, our results from the first chapter indicate that both constitutive and inducible tomato chemical defenses enhance the lethality of a bacterial entomopathogen against *S. exigua.* The contribution of plant defenses to the improved action of a third trophic level demonstrates their role in modulating insect-entomopathogen interactions and highlights the potential benefits of combining plant defense traits and the use of microbial entomopathogens in sustainable agricultural practices.

The interplay between plant and insects may be further modulated by the action of beneficial soil microorganisms (Pineda et al., 2010). For instance, most

land plants establish mutualistic associations with arbuscular mycorrhizal fungi (AMF). This symbiosis improves the nutritional status and growth of the plant, but also render plants more resistant to abiotic and biotic stress, including pest attack (Bennett & Groten, 2022). Given the previously observed impact of tomato defenses on Bt-induced lethality, we hypothesized that mycorrhization may further influence the outcome of the interaction among tomato, *S. exigua* and its entomopathogens.

Therefore**, in the second chapter of this thesis we investigated whether the mycorrhization of tomato plants with the AMF *Funneliformis mosseae* had an impact on the susceptibility of *S. exigua* larvae to the entomopathogens Bt and Baculovirus**. We prepared leaf-based artificial diets from control and mycorrhizal plants (exposed or not to previous herbivory) to elucidate the impact of plant-derived compounds on larval development. Herbivory-induced defenses were responsible for a significant decrease in caterpillar growth, either with or without AMF symbiosis. This is likely attributed to the activation of the jasmonate pathway which, after being induced upon herbivory, is known to trigger the production of secondary metabolites and defensive proteins with toxic or deterrent properties against insects (Li et al. 2022; Anjali et al. 2023; Frattini et al. 2024). Additionally, when mycorrhization was combined with previous herbivory there was a significant interaction among both factors, which may be explained by the priming of antiherbivore responses. It has been previously reported the ability of mycorrhizal plants to elicit stronger and faster JA defenses after subsequent biotic attack (Jung et al., 2012; Martinez-Medina et al., 2016a). Moreover, the benefits of mycorrhiza-induced resistance (MIR) has been observed in other mycorrhiza-insect-plant systems at both below- and aboveground level (Poveda et al., 2020; Schoenherr et al., 2019; Manresa-Grao et al., 2022; Ramírez-Serrano et al., 2022). Instead, larval growth

was not impaired upon feeding on diets derived from mycorrhized plants but not exposed to herbivory. This is consistent with the fact that mycorrhizal plants undergo only a slight induction of defenses in the absence of stress, unlike the high metabolic reconfiguration which takes place in mycorrhizal plants subjected to herbivory (Rivero et al., 2021).

After confirming the negative impact on larval development, we evaluated the influence of both mycorrhization and herbivory on *S. exigua* susceptibility to sublethal concentrations of Bt and Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV). Feeding infected caterpillars with diets derived from plants exposed to these factors, either individually or in combination, increased larval mortality by 20 %. Unlike many studies which have focused on the effect of individual plant compounds on the lethality of Bt against lepidopteran larvae (Konecka et al., 2020; Wang et al., 2021a; Krischik et al., 1988; Isayama et al., 2021), our work provides a broader perspective by examining the influence of the full spectrum of plant defenses induced after herbivory on the insect pest. This positive contribution to Bt efficacy aligns with the results obtained in the previous chapter of this thesis, where defenses induced by synthetic herbivory also increased larval mortality triggered by Bt. Antiherbivore compounds induced by insect damage, and also primed in mycorrhizal plants, may enhance Bt lethality against *S. exigua* by altering insect digestion, immunity, or peritrophic membrane integrity (Shi et al., 2020; Howe & Herde, 2015; War et al., 2012). These alterations could disturb larval physiology and increase its susceptibility to pathogens. Additionally, mycorrhizal plants, even without herbivory, increased the lethality of Bt, and significantly enhanced the efficacy of baculovirus. Given the subtle changes in defensive metabolites of mycorrhizal plants in the absence of stress (Rivero et al., 2021), we speculate that the enhanced efficacy of both entomopathogens with

this diet may be due to alterations in plant nutrition, which could affect larval physiology and susceptibility to pathogens. These results show that AMF colonization in tomatoes, whether exposed to herbivory or not, increases the susceptibility of *S. exigua* to the bacterial entomopathogen Bt and has a positive or neutral effect on baculovirus efficacy.

Due to the strong correlation between immune status and pathogen susceptibility, we focused on the impact of plant compounds on phenoloxidase (PO) activity, a marker of insect immunity (Lu et al., 2014), to better understand the mechanisms behind the enhanced lethality of entomopathogens**.** Enzymatic analysis showed a reduction in PO activity only after ingestion of diets derived from plants exposed to herbivory. This effect, along with the lack of direct interference of plant compounds with PO activity, suggests that chemical defenses induced in tomato leaves after insect attack interfere with the immune status of *S. exigua*. Since PO plays a key role in insect cellular and humoral responses against pathogens (Wang et al., 2020; Huang et al., 2022), it is likely that the reduction in PO activity may interfere with the insect's ability to remove the pathogen, thereby contributing to higher larval susceptibility to Bt.

Finally, we investigated whether mycorrhization and/or herbivory mediated changes in the gut microbiota of larvae, due to their reported role in modulating insect metabolism, development, and immunity (Zhang et al., 2022; Wu et al., 2016). No changes in bacterial composition, load, or diversity were found among insects fed on the different plant diets, suggesting that changes in leaf nutrition or defense compounds induced by mycorrhization and/or herbivory had a little impact on the caterpillar's gut microbiota, indicating that these factors are unlikely to be the primary drivers of the observed enhanced effectiveness of both entomopathogens. However, Di Lelio *et al.* (2023) reported that the association of tomato plants with the soil beneficial fungus *Trichoderma*

afroharzianum disrupted the gut microbiota of *S. littoralis,* leading to nutritional deficiencies and impairing larval development and survival. Hence, the identity of the fungus colonizing the host plant appears to be crucial in influencing the effect of the interaction on the insect pest.

Overall, these findings demonstrate the compatibility of bacterial and viral entomopathogens with mycorrhiza in the control of *S. exigua* in tomato. While this combination of AM inoculation and entomopathogens offers a potential strategy for biological control, additional research is required to evaluate the applicability of this approach in other crop systems using different insects, host plants, or AMF species.

After exploring the tomato-mediated bottom-up effects on the *S. exigua*-entomopathogens relationship, **the third chapter of this thesis delves into the potential top-down effects exerted by viral entomopathogens on plant-insect interactions through the modulation of plant defense elicitation.** Some viruses have been reported to interfere with the jasmonate defensive pathway of plants. For example, polydnaviruses have been shown to suppress plant defenses by modifying the composition of insect oral secretions (OS) or saliva (Tan et al., 2018; Zhu et al., 2018). Changes in the elicitors or effectors present in these secretions play a crucial role in modulating antiherbivore pathways (Peiffer & Felton, 2009; Wang et al., 2023). Given the recent discoveries about the role of certain REPAT proteins present in insect OS in suppressing plant defenses (Chen et al., 2019, 2023; Wang et al., 2024), and the overexpression of some of them in the midgut of baculovirus-challenged lepidopteran larvae (Navarro-Cerrillo et al., 2013; Herrero et al., 2007), we hypothesized that baculovirus infection could interfere with plant response to herbivory through changes in the REPAT composition of insect OS.

To address this, we focused on the effect of SeMNPV infection of *S. exigua* larvae on the induction of tomato plant defenses**.** Tomato herbivory by non-infected (mock-infected) larvae for 3 hours led to the overexpression of genetic markers associated with the JA signaling pathway, as well as the accumulation of the phytohormones JA and JA-Ile, the bioactive form of JA. This confirmed the local induction of jasmonate pathway in the damaged leaflets, which may be explained by the deposition of DAMPs and HAMPs on the injury site by chewing larvae. The plant perceives these signals and subsequently triggers the production of antiherbivore compounds (Snoeck et al., 2022; Acevedo et al., 2015). Additionally, an increase in the expression levels of the late JA responsive genes, *PIN2* and *PPO*, was also detected in leaflets distal to those locally damaged, indicating systemic induction of the jasmonate pathway (Truman et al., 2007; Sun et al., 2011).

After confirming the activation of JA-dependent defenses following herbivory, we evaluated the influence of baculovirus infection on the elicitation of tomato defenses. Feeding by infected *S. exigua* larvae led to a reduction in the expression levels of the JA-related genes *OPR3* and *PPO*, as well as in the accumulation of the phytohormone OPDA, the substrate of *OPR3*. These results suggest that baculovirus infection may suppress the jasmonate pathway in tomato damaged leaflets, thereby influencing the interaction between *S. exigua* and the plant's defense mechanisms.

To further validate the suppressive effect of baculovirus infection on tomato defenses, additional experiments could involve feeding *S. exigua* larvae on previously damaged leaflets. This approach would help determine if the suppression of defenses makes tomato leaflets less toxic to the insect, potentially enhancing larval growth. Previous findings reported by Pan et al. (2019) and Wan et al. (2017) differ from our study, as they observed an increase in direct

and indirect plant defenses, respectively, upon baculovirus infection in lepidopteran larvae. However, the use of different baculovirus-insect-plant systems in these studies may result in a different modulation of JA-dependent responses, which are highly context-dependant (Xiao et al., 2019; Basu et al., 2023).

To delve deeper into the mechanisms underlying this suppression, and based on the involvement of some viruses in altering insect secretions, we performed proteomic analysis of the OS from mock- and infected-larvae. This study revealed a five-fold reduction in the abundance of a lipase-domain-containing protein in the OS of baculovirus-infected larvae. This finding may help explain the observed decrease in plant defense induction, as insect lipases are widely implicated in the elicitation of defense responses (Zhang et al., 2024; Acevedo et al., 2018; Schäfer et al., 2011). Moreover, lipase activity is known to increase the availability of polyunsaturated fatty acids to JA biosynthetic enzymes, thereby contributing to the activation of the jasmonate pathway (Kimberlin et al., 2022; Schäfer et al., 2011).

Baculovirus infection also led to the upregulation of six proteins in the insect OS, including an aldehyde dehydrogenase - an enzyme that converts highly reactive aldehydes into non-toxic carboxylic acids (Islam & Ghosh, 2022; Stiti et al., 2011). Given the potential role of some of these upregulated proteins in the detoxification of plant compounds, along with the suppression of jasmonate-dependent defenses, these results suggest that baculovirus-mediated changes in both the plant and insect may enhance the suitability of the host plant for the insect. Increased larval growth would promote greater production of OBs and their subsequent dispersal in the environment. Thus, more efficient utilization of the tomato plant by *S. exigua* may ultimately benefit baculovirus production. In addition, the reduced induction of antiherbivore defenses might

be advantageous also for the plant, as it may redirect energy and resources toward plant growth. Since the baculovirus infection effectively eliminates the insect pest, the plant can avoid investing heavily in costly defense mechanisms.

Finally, since no differences in REPAT composition were observed in the OS of mock- and baculovirus-infected *S. exigua* larvae, we cannot attribute the suppression of tomato defenses to changes in the abundance of these proteins following baculovirus infection, although other factors such as post-translational changes could be of relevance for their action. In fact, previous studies revealed the glycosylated nature of REPAT1, modification which is not detectable in proteomic analysis and that may have a role in the protein function (Herrero et al., 2007). It would also be worth examining whether Bt can modulate plant defenses through alterations in the content or composition of these proteins in insect OS, since previous research have also detected the overexpression of some REPAT protein in lepidopteran larvae after Bt infection (Herrero et al., 2007; Hernández-Martínez et al., 2010b).

Overall, the results presented in this thesis demonstrate that tomato antiherbivore defenses - whether constitutive or induced by herbivory or mycorrhizal symbiosis - significantly enhance the insecticidal efficacy of Bt in the tomato-*S. exigua* system. In addition, plant defenses either positively or neutrally impact the effectiveness of baculovirus against *S. exigua* (Fig. 23). These findings suggest that integrating plant varieties with specific defensive traits, AMF inoculation, and the use of bacterial and viral entomopathogens could be a promising strategy to bolster the tomato resistance against biotic stress.

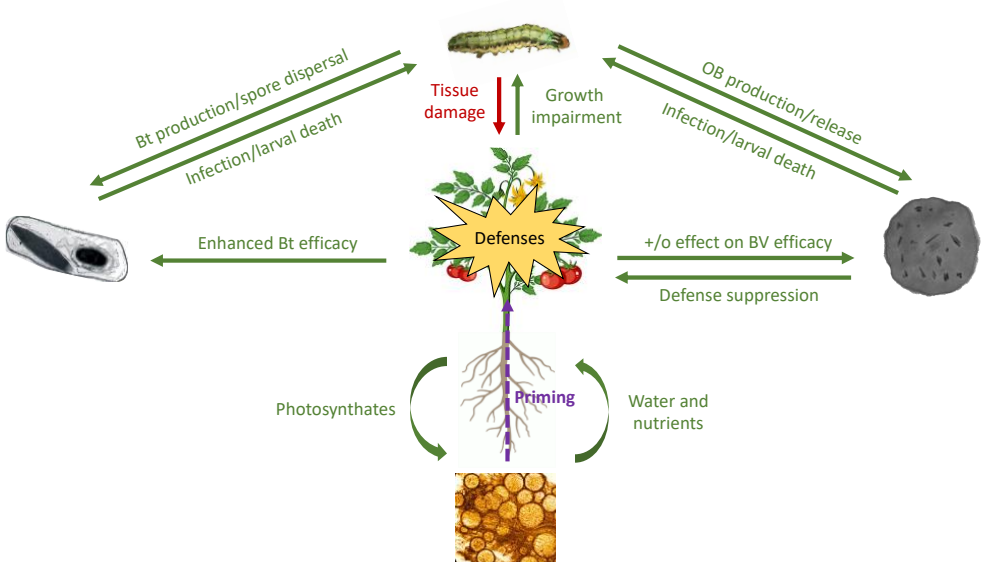

Figure 23. Overview of multitrophic interactions studied in this research.
Interplay among the tomato plant, the arbuscular mycorrhizal fungus *F. mosseae*, the insect pest *S. exigua* and the associated entomopathogens *B. thuringiensis* and baculovirus.

Beyond the plant-mediated bottom-up effects on insect-pathogen interactions, we also observed how baculovirus infection modulates tomato defenses (Fig. 23). This provides new insights into the top-down effects of entomopathogens on insect-plant relationships, which should be taken into account when applying baculoviruses as biological control agents in sustainable pest management programs. Further research is needed to decipher the complex interactions among plant defenses, insect pests, and microbial entomopathogens. Moreover, it would be beneficial to extrapolate these studies to other agroecosystems as well as evaluate these effects on other organisms, like parasitoids or predators.

CONCLUSIONS

1. Tomato constitutive and inducible defenses contribute to the lethality of the bacterial entomopathogen *Bacillus thuringiensis* (Bt) against the lepidopteran pest *Spodoptera exigua.*

2. The overaccumulation of the triterpenoid saponin soyasapogenol B base + O-HexA, Hex, dHex, 1 malonyl in the leaves of tomato plants may be involved in the plant protection against herbivory and enhancement of the efficacy of Bt.

3. Mycorrhizal symbiosis of tomato roots with the arbuscular mycorrhizal fungus *Funneliformis mosseae* coupled with prior herbivory disrupts *S. exigua* growth, increases larval susceptibility to Bt, and has positive or neutral impact on the lethality of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV).

4. Leaf compounds of tomato plants exposed to prior herbivory negatively influence the immune status of *S. exigua* by interfering with the activity of the enzyme marker phenoloxidase.

5. SeMNPV infection impairs the activation of the jasmonic acid (JA) pathway after *S. exigua* herbivory in tomato plants.

6. The reduction in the content of a lipase domain-containing protein in the oral secretion of SeMNPV-infected *S. exigua* larvae may explain the suppression of JA-dependent defense responses in tomato.

7. Combining plant breeding, mycorrhizal inoculation, and entomopathogen application could be an effective strategy to improve tomato resistance to insect pests.

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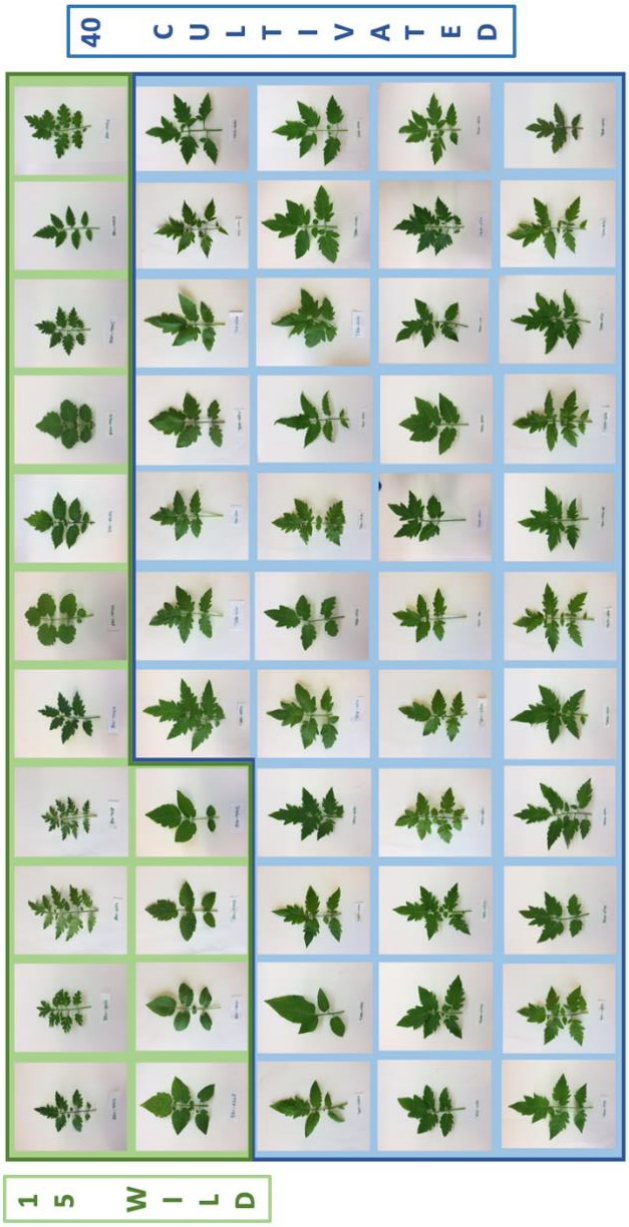
ANNEX

Supplementary Table S1. Impact of fifteen wild species and forty cultivated tomato varieties on *S. exigua* growth and mortality. Highlighted cultivated varieties represent those with contrasting phenotypes (green: low constitutive resistance; red: high constitutive resistance).

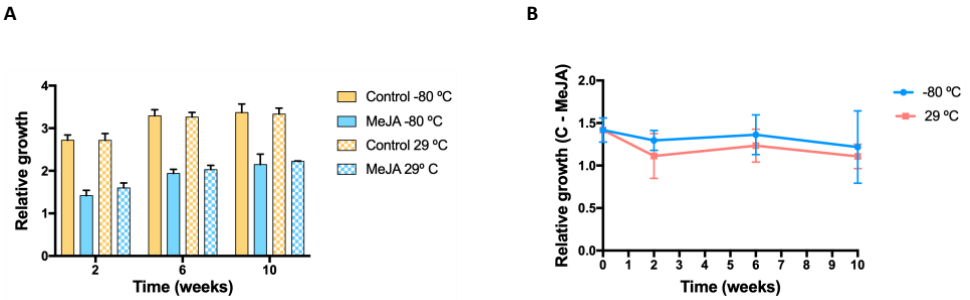
Group	Internal code	Selected variety	Accession number	ID TRADITOM	Species	Growth (L2)	Error (SEM)	Control mortality % (L2)	Error (SEM)	Bt mortality % (L2)	Error (SEM)
Wild species	1S		BGV007977		*Solanum chmielewskii*	18.5	4.3	22.3	9.0	40.6	3.1
	2S		BGV008147		*Solanum chilense*	28.1	3.0	29.6	17.1	66.7	0.0
	3S		BGV008154		*Solanum chilense*	22.8	0.1	17.4	11.2	31.3	18.8
	4S		BGV008078		*Solanum chilense*	23.0	5.4	19.0.	12.3	31.3	18.8
	5S		BGV007957		*Solanum peruvianum*	15.7	4.5	31.3	6.3	40.6	28.1
	6S		BGV007972		*Solanum peruvianum*	44.7	5.7	6.5	0.2	22.7	3.9
	7S		BGV006081		*Solanum habrochaites*	18.6	7.4	28.1	3.1	35.4	2.1
	8S		BGV007965		*Solanum habrochaites*	20.5	1.5	26.0	7.3	40.6	21.9
	9S		BGV007955		*Solanum huaylasense*	24.4	3.2	19.6	7.1	56.3	25.0
	10S		BGV007959		*Solanum huaylasense*	14.8	1.7	22.9	10.4	65.6	21.9
	11S		BGV007956		*Solanum huaylasense*	35.3	18.3	16.3	3.8	28.1	15.6
	12S		BGV006208		*Solanum pimpinellifolium*	39.8	12.4	3.1	3.1	15.6	9.4
	13S		BGV007944		*Solanum pimpinellifolium*	44.3	16.2	15.6	15.6	15.6	9.4
	14S		BGV007993		*Solanum pimpinellifolium*	37.0	23.0	0.0	0.0	28.1	15.6
	15S		BGV007951		*Solanum pimpinellifolium*	37.3	8.0	3.3	3.3	40.6	9.4
Cultivated varieties	1T		BGV016155	TRBA0750	*S. lycopersicum*	33.4	7.2	12.5	6.3	45.2	1.5
	2T		BGV016116	TRBA1020	*S. lycopersicum*	45.3	25.6	0.0	0.0	9.4	9.4
	3T	4	BGV016151	TRBA1500	*S. lycopersicum*	55.1	15.5	0.0	0.0	12.1	0.4
	4T			TRPA0270	*S. lycopersicum*	34.9	21.2	14.3	14.3	33.3	2.0
	5T		BGV014181	TRVA2500	*S. lycopersicum*	58.6	12.5	3.1	3.1	31.9	11.9
	6T			TRPO0170	*S. lycopersicum*	25.6	11.8	15.8	9.2	34.4	15.6
	7T		V711278	TRV0060	*S. lycopersicum*	22.1	9.8	28.1	9.4	46.9	40.6
	8T		V710109	TRV0230	*S. lycopersicum*	24.8	15.3	37.9	24.6	35.3	35.3
	9T		T100819	TRMO0800	*S. lycopersicum*	22.9	11.1	24.6	18.3	37.5	31.2
	10T		GRC488/04	TRTH0710	*S. lycopersicum*	29.0	10.6	15.6	15.6	25.0	12.5
	11T		T101774	TRMO0030	*S. lycopersicum*	49.9	0.2	3.1	3.1	6.3	6.3
	12T		BFV003890	TRVA1040	*S. lycopersicum*	83.8	9.2	3.1	3.1	6.3	6.3
	13T			TRBA1800	*S. lycopersicum*	53.1	18.8	9.4	3.1	22.7	3.9
	14T			TRPO2430	*S. lycopersicum*	76.3	31.5	3.1	3.1	28.1	28.1
	15T		FMA101	TRCA1220	*S. lycopersicum*	62.0	21.5	2.9	2.9	21.9	15.6
	16T	6	BGV013965	TRVA2490	*S. lycopersicum*	109.9	35.0	0.0	0.0	6.3	0.0
	17T		BGV016122	TRBA1480	*S. lycopersicum*	51.4	13.6	3.1	3.1	34.4	9.4
	18T		BGV016157	TRBA1490	*S. lycopersicum*	50.3	17.6	9.4	3.1	15.6	3.1
	19T			TRPA0130	*S. lycopersicum*	31.1	6.9	12.5	6.3	28.1	21.9
	20T	1		TRPA0140	*S. lycopersicum*	27.9	3.2	9.4	9.4	46.9	15.6
	21T			TRPO0310	*S. lycopersicum*	21.4	5.3	25.0	6.3	43.8	6.3
	22T			TRTH0030	*S. lycopersicum*	44.5	14.2	9.4	3.1	46.9	3.1
	23T		V710142	TRV0380	*S. lycopersicum*	20.0	12.1	15.6	15.6	60.6	20.6
	24T		V710363	TRV11600	*S. lycopersicum*	28.5	10.9	15.6	3.1	41.3	28.0
	25T	5	BGV016232	TRVA0020	*S. lycopersicum*	66.7	28.6	3.1	3.1	32.1	10.7
	26T		LC433	TRCA0010	*S. lycopersicum*	38.3	10.6	9.4	9.4	46.9	28.1
	27T		LC427	TRCA1110	*S. lycopersicum*	36.8	10.0	34.4	34.4	53.1	15.6
	28T		BGV005718	TRVA2150	*S. lycopersicum*	49.6	29.9	16.7	16.7	46.9	15.6
	29T		BGV000110	TRVA0150	*S. lycopersicum*	32.1	23.3	9.8	3.5	50.0	31.2
	30T		BGV004123	TRVA1100	*S. lycopersicum*	55.7	9.5	15.6	15.6	53.1	15.6
	31T		V710314	TRV0050	*S. lycopersicum*	30.0	15.1	25.9	14.1	37.5	6.3
	32T		LC167	TRCA0700	*S. lycopersicum*	34.2	8.7	13.4	0.9	27.7	15.2
	33T		T101948	TRMO0040	*S. lycopersicum*	28.9	3.6	12.5	0.0	37.5	6.3
	34T		T100001	TRMO0110	*S. lycopersicum*	25.8	3.9	22.7	4.0	37.5	6.3
	35T	2	GRC1133A/04	TRTH0600	*S. lycopersicum*	13.1	3.9	20.1	1.3	62.5	6.3
	36T		V710411	TRV1940	*S. lycopersicum*	19.8	4.6	6.3	6.3	59.4	3.1
	37T			TRTH0280	*S. lycopersicum*	32.1	2.3	15.6	9.4	40.6	3.2
	38T		T100028	TRMO0130	*S. lycopersicum*	33.4	14.8	22.9	10.4	43.8	6.3
	39T	3	GR0034/05	TRTH2490	*S. lycopersicum*	28.0	13.7	15.6	3.1	60.7	1.9
	40T		BGV001031	TRVA0040	*S. lycopersicum*	11.9	2.7	15.6	3.1	54.8	1.5

Supplementary Table S2. List of the primers used for RT-qPCR.

Primer name	Target gene	Sequence (5' – 3')
SlEF_F	Solanum lycopersicum elongation factor 1-alpha (*SlEF*)	GATTGGTGGTATTGGAACTGTC
SlEF_R		AGCTTCGTGGTGCATCTC
AOS_F	Solanum lycopersicum allene oxide synthase (*AOS*)	AACAGTGTGCCGGAAAAGAC
AOS_R		AATGGAGATGCACCGACTTC
OPR3_F	Solanum lycopersicum 12-oxophytodienoate reductase 3 (*OPR3*)	GCATATGGGCAAACTGAAGC
OPR3_R		ATGAATGTCCCCTGATACGC
PIN2_F	Solanum lycopersicum wound-induced proteinase inhibitor 2 (*PIN2*)	GAAAATCGTTAATTTATCCCAC
PIN2_R		ACATACAAACTTTCCATCTTTA
PPO_F	Solanum lycopersicum polyphenol oxidase (*PPO*)	CACCACCAACTTTTGGAACC
PPO_R		TACGCTGGCGATAACTGAAC
ICS_F	Solanum lycopersicum isochorismate synthase (*ICS*)	CGCCTCAGCAT TTGTAGTGA
ICS_R		CTTGAACACAC AGCCTCCAA
PR1_R	Lycopersicon esculentum PR (pathogenesis related) protein 1 (*PR1*)	GTGGGATCGGATTGATATCCT
PR1_F		CCTAAGCCACGATACCATGAA



Supplementary Figure S1.
Leaflets corresponding to fifteen wild tomato species and forty cultivated varieties used in this research.



Supplementary Figure S2. Preservation of growth-inhibitory effects of MeJA-diets after long-term exposure at 29 ºC.

(A). Relative growth of newly molted fourth instar *S. exigua* larvae after feeding for 48 hours on plant-based artificial diets prepared from leaf powder (control or MeJA-treated) stored at either -80 or 29 ºC after 2, 6, and 10 weeks. (B) Difference in relative growth of larvae reared on control and MeJA-treated diets after 48 hours. Error bars depict standard error of the mean (SEM).

PUBLICATIONS

Biological Control 198 (2024) 105624

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Constitutive and inducible tomato defenses contribute to *Bacillus thuringiensis* lethality against *Spodoptera exigua*

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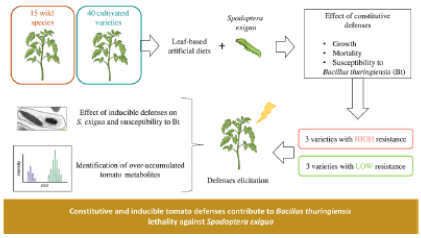
HIGHLIGHTS

- Leaf-based artificial diets allow to study the effect of chemical defenses on insects.
- Tomato defenses enhanced the lethality of a bacterial entomopathogen.
- Over-accumulated plant metabolites were identified after elicitation of defenses.
- Plant defense elicitors contribute to the action of *Bacillus thuringiensis*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Lepidoptera
Bacterial entomopathogen
Tomato screening
Plant defenses
Tritrophic interactions

ABSTRACT

In nature, insect herbivory exerts continuous selective pressure on plants that, in turn, have developed a wide array of constitutive and inducible defenses to fight against attackers. Since plant defenses may affect higher trophic levels, including entomopathogens, further research is required to understand how plant compounds influence insect-pathogens interactions and their implications for integrated pest management programs. Here, we evaluated the impact of tomato defenses on the lethality produced by the bacterial entomopathogen *Bacillus thuringiensis* (Bt) against second instar larvae of *Spodoptera exigua*. We first examined the effect of constitutive defenses from fifteen wild tomato species and forty cultivated varieties (*Solanum lycopersicum*) on *S. exigua* larval growth and susceptibility to Bt. The results showed larvae fed on wild tomato species had a reduced larval growth compared to larvae fed on cultivated varieties, whereas susceptibility to Bt was similar between both groups. We then selected six cultivated varieties, including those with high and low impacts on larval growth and Bt-induced mortality, to further explore the effect of inducible defenses. Elicitation of defenses by methyl jasmonate (MeJA) reduced larval growth and increased basal mortality. Additionally, when larvae were infected with Bt, MeJA treatment further increased their susceptibility to the entomopathogen. Metabolomic analysis confirmed a reprogramming of tomato leaf metabolism following MeJA elicitation, with an induced accumulation of bioactive compounds, such as saponins or flavonoids, known for their anti-herbivory properties in most

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https://doi.org/10.1016/j.biocontrol.2024.105624
Received 1 May 2024; Received in revised form 19 September 2024; Accepted 24 September 2024
Available online 26 September 2024
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Research Article

Received: 12 November 2021 Revised: 22 June 2022 Accepted article published: 29 June 2022 Published online in Wiley Online Library: 20 July 2022

(wileyonlinelibrary.com) DOI 10.1002/ps.7058

Compatibility of mycorrhiza-induced resistance with viral and bacterial entomopathogens in the control of *Spodoptera exigua* in tomato

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Abstract

BACKGROUND: Arbuscular mycorrhizal fungi (AMF) are soil-borne microorganisms that establish mutualistic associations with roots of most terrestrial plants. This symbiosis results in nutritional and defensive benefits to the host plant, usually conferring protection against biotic stresses, but its indirect impact on third trophic levels is still unknown. In the present work, we explore whether the symbiosis of tomato plants with *Funneliformis mosseae* (and/or exposition to herbivory) influences the interaction of the generalist pest *Spodoptera exigua* (Lepidoptera: Noctuidae) with bacterial (*Bacillus thuringiensis*) and viral (baculovirus, SeMNPV) natural entomopathogens.

RESULTS: Symbiosis with AMF and previous herbivory reduces the relative growth of *S. exigua*, increases its susceptibility to a sublethal dose of *B. thuringiensis* and has positive or neutral impact on the lethality of SeMNPV. Reduction of the phenoloxidase activity, a marker of the insect immune response, was associated with the larval feeding on plant material previously exposed to herbivory but not to the AMF. In addition, no changes in the insect gut microbiota could be associated with the observed changes in larval growth and susceptibility to the entomopathogens.

CONCLUSION: Our findings provide the first evidence of compatibility of AMF symbiosis in tomato with the use of bacterial and viral entomopathogens, contributing to the development of novel approaches to combine the beneficial effect of AMF and entomopathogens in biological pest control.
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Keywords: arbuscular mycorrhizal fungi; *Spodoptera exigua*; microbial entomopathogens; *Solanum lycopersicum*; integrated pest management

1 INTRODUCTION

Plants must constantly cope with adverse environmental factors, consequently they have evolved several strategies to face them.[1,2] They display multiple defence mechanisms to deal with diverse stressors.[3] The plant microbiota is known to modulate such defence mechanisms and beneficial soil microorganisms can increase plant stress resistance/tolerance.[4,5] In this context, the association with arbuscular mycorrhizal fungi (AMF) deserve special attention. These obligate biotrophs belong to the phylum *Glomeromycota* and form symbiotic interactions with more than 80% of land plants, including most agricultural crops.[6] In this interaction, AMF colonize the root cortex and develop an extraradical mycelium, increasing the acquisition of water and inorganic nutrients (mainly phosphate and ammonia) of the plant. In return, the fungus receives photosynthates for the maintenance of mycorrhizal structures.[7] Besides improving plant nutritional status and growth, this symbiosis also improves the ability of the plant to overcome abiotic stresses such as salinity, drought or the presence of heavy metals.[8,9]

Mycorrhization is also involved in enhancing plant defense against a broad spectrum of pathogens and pests, a phenomenon known as mycorrhiza-induced resistance (MIR).[10–12] The

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¿Es posible una mejora en el uso de entomopatógenos mediante la selección de variedades vegetales?

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RESUMEN

Las plantas están sometidas al ataque constante de los herbívoros por lo que han desarrollado un arsenal de estrategias para reducir su impacto. Entre ellas, se encuentra la habilidad para reclutar un tercer nivel trófico, los enemigos naturales, tales como insectos predadores y parasitoides. No obstante, cabe resaltar la acción de un grupo adicional, los entomopatógenos, microorganismos presentes de forma natural en el entorno y que son capaces de infectar un amplio rango de insectos. Actualmente, diferentes bacterias, virus, nematodos y hongos son utilizados como biopesticidas en la protección de cultivos agrícolas; sin embargo, no alcanzan los niveles de efectividad proporcionados por los pesticidas químicos. Por ello, en este ensayo planteamos una serie de mecanismos que podrían ser utilizados por la planta para incrementar la eficacia de los entomopatógenos y que podrían incluirse entre los objetivos de los programas de mejora vegetal con el fin de obtener variedades que contribuyan a la acción de estos enemigos naturales.

PALABRAS CLAVE: interacciones tritróficas, herbivoría, control biológico, diversidad vegetal, manejo integrado de plagas.

INTRODUCCIÓN

La relación entre plantas e insectos herbívoros comenzó aproximadamente hace 400 millones de años, constituyendo una de las interacciones más grandes y complejas entre organismos (Labandeira, 2013). A lo largo del tiempo ha tenido lugar un proceso de coevolución como consecuencia de la presión selectiva ejercida entre ambos (Ehrlich y Raven, 1964). Esto ha supuesto el desarrollo de mecanismos de defensa por parte de las plantas para hacer frente a los herbívoros, así como la adaptación de estos últimos para evadir o contrarrestar dichas defensas (Mello y Silva-Filho, 2002; Jander, 2014). Entre las diferentes estrategias adoptadas por la planta, se encuentran la presencia de barreras físicas (tricomas, espinas) y químicas (metabolitos secundarios tóxicos, proteínas de defensa, compuestos volátiles, etc.), cuyo principal objetivo consiste en la alteración de la fisiología y/o comportamiento del fitófago (Schuman y Baldwin, 2016). En respuesta a estas defensas directas, los herbívoros son capaces de llevar a cabo procesos de detoxificación o secuestro de los fitoquímicos así como mecanismos de evitación (Heidel-Fischer y Vogel, 2015; Erb y Robert, 2016; War *et al.*, 2018).

Las plantas también pueden reducir la presión de los herbívoros recurriendo a la ayuda de un tercer nivel trófico: los enemigos naturales de los fitófagos (Price *et al.*, 1980). A partir de la década de los 80, diferentes estudios demostraron que, entre los volátiles inducidos por herbívora (HIPV), había una parte utilizada en la comunicación inter-específica. Estas defensas indirectas actúan como "una llamada de socorro" que atrae a parasitoides y predadores, permitiéndoles detectar la presencia del herbívoro y proteger así a la planta que los emite (Dicke y Baldwin, 2009). Hoy en día, se conoce que la relación entre una planta huésped y su herbívoro no es exclusiva y limitada a dos actores, sino que forma parte de un contexto multitrófico mucho más complejo. Éste abarca también a insectos predadores, parasitoides y polinizadores, plantas limítrofes y, por último, pero no menos importante, a aquellos microorganismos presentes en la planta y en los insectos. Entre estos microorganismos, hay una gran categoría de enemigos naturales representada por los entomopatógenos. Estos organismos se han estudiado detalladamente por su efecto sobre los insectos dianas y su uso aplicado en la agricultura, pero los trabajos disponibles actualmente pasan a menudo por alto el análisis de las interacciones ecológicas de los entomopatógenos con otros niveles tróficos. En este ensayo nos planteamos cómo estos microorganismos capaces de infectar insectos y, en general invertebrados, podrían representar un ulterior reservorio de socorro multitrófico para las plantas y cómo la selección varietal podría mejorar la protección frente a los insectos mediante la inclusión de criterios dirigidos a incrementar la acción de los de entomopatógenos.