Deciphering role of different diet on herbivore oral constituents and their role in modulating plant defense

by

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10BB15J26035

A thesis submitted to the

Academy of Scientific & Innovative Research

for the award of the degree of

DOCTOR OF PHILOSOPHY

in

SCIENCE

Under the supervision of

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April-2022

CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled, **"Deciphering role of different diet on herbivore oral constituents and their role in modulating plant defense"**, submitted by **Mr. Gopal S. Kallure** to the Academy of Scientific and Innovative Research (AcSIR) in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Science, embodies original research work carried-out by the student. We, further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research materials obtained from other sources and used in this research work have been duly acknowledged in the thesis. Images, illustrations, figures, tables etc., used in the thesis from other sources, have also been duly cited and acknowledged.

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Mr. Gopal S. Kallure

Date: 13/04/2022

Dr. Ashok P. Giri

Date: 13/04/2022.

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I Mr. Gopal S. Kallure, a Ph.D. student of the Academy of Scientific and Innovative Research (AcSIR) with Registration No. 10BB15J26035 hereby undertake that the thesis entitled **"Deciphering role of different diet on herbivore oral constituents and their role in modulating plant defense"** has been prepared by me and that the document reports original work carried out by me and is free of any plagiarism in compliance with the UGC Regulations on *"Promotion of Academic Integrity and Prevention of Plagiarism in Higher Educational Institutions (2018)"* and the CSIR Guidelines for *"Ethics in Research and in Governance (2020)"*.

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Signature of the Supervisor

Name: Dr. Ashok P. Giri

Date: 13/04/2022.

Place: Pune

I dedicate this thesis to.....

Parents who were always there for me and encouraged me throughout this whole journey

Acknowledgement

Many people have helped me throughout my long career as a research scholar. My research supervisor, Dr. Ashok P. Giri, is the first to be acknowledged and given credit for this effort. Throughout the years, he has been a continuous source of encouragement and has been especially supportive during my difficult moments. I have to mention, it was his encouragement that helped me stay focused on my studies and finish my Ph.D. He introduced me about plant molecular biology, particularly the fascinating topic of plant-insect interactions. Our group works on a variety of topics, and this exposure allowed me to obtain a broad range of knowledge in one place. He also taught me how to be a researcher, including how to deliver a presentation and how to write a manuscript. I really admire the fact that he allowed me to pursue my interests in science and never said no to any of my suggestions. His consistent encouragement to work in a multidisciplinary environment, study a variety of techniques, and present my work wherever possible have given me immense confidence and helped to shape my career. He used to put pressure on me and set tough deadlines, which I now appreciate because if he hadn't, I would have slid into the "comfort zone," which is perilous for a researcher. He has put lot of effort with me during manuscript preparation and presentations, and I will always thank him for what I have learned from him.

I also thank my DAC members, Dr. Leelavati Narlikar, Dr. Mahesh Kulkarni and Dr. Bhushan Chaudhari for their timely suggestions and evaluation of my research work.

My gratitude towards scientists from NCL, Dr. Mahesh Kulkarni, for helping in Mass spectrometric analysis, Dr. B. Santhakumari, who allowed to work on Triple-TOF. Specially I Would thank Dr. Vitthal Barvkar from SPP university for providing LC-MS facility and also for helping in metabolite data analysis and critical suggestions in manuscript writing.

Also, I would like to express my sincere gratitude to Dr. Ashish Lele, Director, CSIR- NCL, and past directors, Dr. Ashwini Kumar Nangia, Dr. K. Vijayamohanan Pillai, for giving an opportunity to work at this esteemed institute.

I am also grateful to HOD, Biochemical Sciences division for providing the lab infrastructure and facilities. I am thankful to the DST-INSPIRE for providing financial aid. I also acknowledge the funding from DBT, DST and CSIR that helped to do scientific work smoothly.

I express my deepest gratitude to Dr. Balkrishna Shinde, who has been my support system during all these years, not just scientifically, but also as a friend. Being my senior, his constant piece of advice helped me stay on track. He has always been available for discussion, experimental design, manuscript corrections. Also, I am thankful to Dr. Archana Kumari for her help in review and manuscript writing.

I am also grateful to Dr. Rakesh Joshi and Dr. Kritikumar Kondhare for their continual scientific suggestions. I also thank Dr. Narendra Kadoo, who has always been available for discussion, and was continuously supportive of my research work.

I would like to specifically thank, Dr. Anand Sukeerthi, Dr. Sneha Singh, Bhakti Dayama, Dr. Nalini, Debopriya Roy, Ashwini Bohite who has been best friends at NCL, and have been companions with me in all my good and bad times. They have aided me in troubleshooting numerous scientific challenges by sharing their experiences and knowledge. They were the one who were always there for me, sharing the little nuances of our lives, and I will treasure the memories we shared at NCL.

I was fortunate to work closely with wonderful people in my lab, Dr. Tejas Chirmade, Dr. Nidhi Saikhedkar, Shounak Jagdale, Ranjit Barbole, Santosh Lavhale, Veena Dewangan, Vaishnavi Salunkhe, Monika Mathur, Meenakshi Kapase, Ashwini Rane, Aruna Patil, Tejas Vare, Rutuja Kale, Sonali Gaikwad, Pranjali Oak, Gayatri Salunke, Amrita Ghosh, Tanuja Gurav, Ravindra Vidhate, Pooja Deshmukh, and Sonal Madliya who were partners in everything and for their direct or indirect help in conducting my research work. I am especially thankful to my seniors, Dr. Vishal Dawkar, Dr. Purushottam Lomate, Dr. Raviraj Kalunke, Dr. Amey Bhide, Dr. Sheon Mary, Dr. Rahul Tanpure, and Dr. Neha Mahajan for their guidance in handling the projects. It was due of these people that I learned appropriate scientific experiments and teamwork culture.

I have been blessed enough to be surrounded by a wonderful group of people Dr. Amar Yeware, Dr. Sonia Agrawal, Shrikant Nikam, Dr. Govinda Navale, Dr. Rahul Jagtap, Jagjivan Sabane. They have encouraged my progress as I started on this wonderful path, and they have always been by my side and supported me.

I am also thankful to Dr. Yogesh Mahajan, Mr. Jagtap and his team along with the people from horticulture department for their help in all greenhouse experiments starting from pot filling to maintains of plants in good condition.

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Abbreviations:

CFMID	Competitive Fragmentation Modeling for Metabolite Identification
CGA	Chlorogenic acid
Ca2+	Calcium
Ct	Cycle threshold
Da	Dalton
ET	Ethylene
FACs	Fatty acid amino acid conjugates
GOX	Glucose oxidase
Gln	Glutamine
Glu	Glutamic acid
H_2O_2	Hydrogen peroxide
HAPMs	Herbivore-Associated Molecular Patterns
HCL	Hydrochloric acid
IDA	Information Dependent Acquisition
JA	Jasmonic acid
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
MEGA	Molecular Evolutionary Genetics Analysis version
Mg	Milligram
Min	Minutes
Ml	Milliliter
NaCl	Sodium chloride
OS	Oral secretion
OSAD	Oral secretion collected from *H. armigera* fed on artificial diet
OSH	Oral secretion collected from *H. armigera* fed on tomato leaves
OSNH	Oral secretion collected from *H. armigera* fed on Capsicum leaves
PCA	Principal component analysis

PPM	Parts per million
ROS	Reactive oxygen species
RPM	Revolutions Per Minute
RT	Room temperature
SA	Salicylic acid
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SWATH	Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra
Sl	*Solanum lycopersicum*
TMHMM	Transmembrane Helices; Hidden Markov Model
W	Wound
h	Hours
kDa	Kilodalton
qRT PCR	Real-Time Quantitative Reverse Transcription PCR
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
%	Percentage

Synopsis

Chapter 1: Introduction

For more than 350 million years, there have been ongoing dynamic interactions between plants and insects. In several cases, insects cause-specific feeding damage with ensuing herbivore-associated molecular patterns that invoke characteristic defense responses. During feeding on plant tissue, insects release oral secretions (OSs) containing a repertoire of molecules affecting plant defense. Some of these OS components might elicit a defense response to combat insect attacks (elicitors), while some might suppress the plant defenses (suppressors/effectors). Insect OSs includes regurgitant and saliva with distinct origins and compositions. Regurgitant's arise from the foregut and midgut while saliva is released from the labial gland through the spinneret (Eichenseer et al., 1999; Peiffer and Felton, 2005a). *Helicoverpa armigera* (Hübner) is a polyphagous insect, feeding on a wide range of host plants. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant range but also specific tissues of the host plant. Few reports suggest that the synthesis and function of OS components might depend on the host plant and associated microorganisms. Diet is crucial factor for the development and reproduction of herbivorous insects. However, despite decades of research on plant-insect interaction, a focused study on diet formulation influencing the interaction between plant and insect is mostly elusive. Further, identification and functional characterization of herbivorous salivary proteins as effectors/elicitors will enrich our knowledge about plant-insect interaction.

Chapter 2: Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense

Little is known about how insect herbivores' oral secretion (OS) modulates the plant defense responses in tomato. We have collected the OS of *H. armigera* fed on tomato (host), Capsicum (Non-host) leaves and artificial diet (AD). The treatment of *H. armigera* OS_{AD}, OS_{H} and OS_{NH} on wounded tomato leaves showed differential expression of (i) genes involved in JA and SA biosynthesis and their responsive genes and (ii) biosynthetic pathway genes of chlorogenic acid (CGA) and trehalose, which exhibited increased accumulation along with several other plant defensive metabolites. Principal component analysis of identified mass peaks from tomato leaves after wound and different *H. armigera* OS treatments shows different cluster of

metabolites across the treatment. Specifically, high levels of CGA were detected after OS_H and OS_{NH} treatments in tomato leaves. There was higher expression of the genes involved in phenylpropanoid biosynthesis, which may lead to the increased accumulation of CGA and related metabolites. Along with phenylpropanoid pathway, the OS treatments lead to a higher accumulation of signaling sugars trehalose, which was evident with the induced expression of trehalose biosynthetic genes (SlTPS1 and SlTPP). Consistent with a higher accumulation of CGA, we also observed increased expression of CGA biosynthetic pathway genes in OS_H and OS_{NH} treated tomato leaves. Thus, CGA can be considered as a strong anti-nutritive, altering the herbivore feeding behavior, growth, and survival. In the insect bioassay, CGA significantly inhibited *H. armigera* larval growth. Our results underline the differential accumulation of plant and insect OS metabolites and identified potential plant metabolite(s) affecting insect growth and development.

Chapter 3: Integrated omics approach for analysis of oral secretion constituents from *Helicoverpa armigera*

Metabolites from insect OS have been reported. However, equivalent data comparing the impact of different plant-based diet on insect OS composition is limited. *H. armigera* larvae prefer tomato plants (*Solanum lycopersicum* L.; host) as their diet compared to the Capsicum plants (*Capsicum annum* L.; non-host). This was also evident when *H. armigera* larvae fed on Capsicum plants, they showed delayed growth and development. To understand the *H. armigera* diet preferences and impact of different diets on the OS composition, oral secretion of larvae fed either on tomato (OS_H) or capsicum (OS_{NH}) leaves, or an artificial diet (OS_{AD}) were analyzed using Liquid Chromatography - Quadrupole Time of Flight- Mass Spectrometry (LC-QTOF-MS). Non-targeted metabolic analysis identified total 1296 mass peaks in OS_{AD}, OS_H, and OS_{NH}. Principal component analysis showed clear separation of all identified mass peaks from OS_{AD}, OS_H, and OS_{NH} contributed by PC1 (48.6 %) and PC2 (41.4 %). Among identified mass peaks, 599, 634 and 505 were from OS_{AD}, OS_H, and OS_{NH}, respectively. From all three OS metabolites, 75 were common, 165 (75 + 90) were shared by OS_{AD} and OS_H, 112 (75 + 37) were common between OS_{AD} and OS_{NH}, and 240 (75 + 165) were shared between OS_H, and OS_{NH}. Interestingly, 397, 304, 228 distinct mass peaks were identified from OS_{AD}, OS_H, and OS_{NH}, respectively. Moreover, radar plot analysis of classes of identified mass peaks showed that phospholipids, alkaloids and terpenoids were higher in OS_{AD}, OS_H, and OS_{NH}, respectively. From identified mass peaks on the basis of peak area, 43 significantly ($P < 0.05$)

identified metabolites were confirmed by using standard parameters and MS/MS fragmentation patterns. The analysis of these metabolites showed differential occurrence across the OS_{AD}, OS_H, and OS_{NH}. Further, the untargeted proteomic analysis of OS_{AD}, OS_H, and OS_{NH} generated spectral library of 221 proteins through IDA. Out of a total of 221 proteins, OS_{NH} and OS_H have 118 and 103 proteins, respectively. Among these 27 and 56 proteins were common and showed differential accumulation in OS_H and OS_{NH} as compared to OS_{AD}. In addition, as compared to OS_{AD} 16 and 4 proteins were found in OS_H with high and low abundance, respectively. Similarly, 19 and 16 proteins were identified as high and low abundant in OS_{NH},respectively. The highly accumulated proteins from OS_H and OS_{NH} were belongs to digestive enzymes and some of them are uncharacterized.

Chapter 4: Identification and functional characterization of salivary secretory proteins from *Helicoverpa armigera*

During feeding on plant tissue, insects release oral secretions (OSs) containing a repertoire of molecules affecting plant defense (effectors). To unravel the function of other uncharacterized secretory proteins we retrieved the transcriptome data of fourth-instar *H. armigera* larvae fed on artificial diet, tomato and *Capsicum annuum* leaves from CSIRO database. Genes coding for salivary secreted proteins like proteins have been identified from the transcriptome and proteomic data of *H. armigera*. For functional characterization, we have cloned and recombinantly expressed the uncharacterized secretory proteins (HARPs) of *H. armigera*. We have purified the recombinant proteins by affinity chromatography and confirmed by western blot using anti-His antibody. To understand the impact of uncharacterized HARPs, we applied the recombinantly purified HARPs on mechanically wounded tomato plants and analyzed the plant defenses. The application of recombinantly purified HARPs on wounded tomato leaves shows differential expression of jasmonic acid biosynthetic and responsive genes. However, no effect on the expression of salicylic acid biosynthesis was observed in response to HARPs treatments. We also observed the higher transcript abundance of HARP1 and HARP5 in foregut, midgut and hindgut of *H. armigera* fed on Capsicum (non-host) as compared to tomato (host) leaves and artificial diet.

Chapter 5: Summary and future prospects

Overall, our study reveals that the treatment of *H. armigera* OS affects the expression of phytohormones biosynthesis and accumulation of specialized metabolites in tomato plants.

Moreover, we discovered that diet has a significant impact on *H. armigera* OS composition, as we observed differences in metabolite and protein accumulation in OS of such larvae. Furthermore, research on *H. armigera* secretory proteins reveals that these proteins have a high degree of similarity. The *H. armigera* secretory proteins may be present in the combination to influence the JA response in tomato plants. However, further experimental validation is necessary for in detail understanding of plant-insect coevolution at molecular level. Long term plans include to understand the overall mechanisms of OS components perception by plants and underlying such molecular interactions.

Organization of thesis

The thesis is organized into five chapters, the contents of which are as described here:

Chapter 1: Introduction

This chapter focuses the subjects that lead to the creation of the thesis. The interplay between plant-insect is explained by giving special emphasis on herbivore oral constituents and plant defense. This chapter deals with the details of characterized components of insect Oral Secretions (Oss), which regulates the plant defenses that either benefit host plants or feeding insects. Further, factors that potentially influence the OS compositions are highlighted viz., host plant (diet), and associated microorganisms of the insect and plant. The possible chemical, and biochemical markers of plant defense that are common between natural insect feeding and OS treatments are presented. Overall, this chapters summarizes the current updates on chemical cues that unravel the molecular dynamics of the plant-insect interactions and provide future perspectives in the area

Chapter 2: Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense

Helicoverpa armigera (Hübner) is a polyphagous insect, feeds on wide range of host plants. In this chapter we have collected the OS from *H. armigera* fed on different artificial Diet (OS_{AD}), tomato-host (OS_H) and Capsicum-non-host (OS_{NH}) leaves. We quantified the expression of several defense marker genes on mechanically wounded tomato leaves treated with OS_H, OS_{NH}, and OS_{AD}, which are known to mimic insect infestation. Further we performed both non-targeted and targeted metabolite analyses of OS-treated tomato leaves to correlate the gene

expression with defense metabolites and, subsequently, the effect of the selected metabolite on larval growth is studied.

Chapter 3: Integrated omics approach for analysis of oral secretion constituents from *Helicoverpa armigera*

Plant specialized metabolites often modulate interactions between plants and insects, which can substantially affect herbivores and their fitness. Solanaceae family plants like tomato, eggplant, potato, Capsicum etc. are rich in phenolic compounds, glycoalkaloids, and defensive proteins such as proteinase inhibitors (Bhat et al., 2005; Felton, 2005; Kennedy, 2003). Hence, based on this we hypothesized that *H. armigera* may secretes different enzymes, proteins or metabolites on plant feeding sites through oral secretion. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant range but also specific tissues of the host plant. To comprehend the impact of a plant-based diet on *H. armigera*, we analyzed the metabolites, FACs and proteins from OS of *H. armigera* fed on tomato as host (OS_H) and Capsicum as non-host (OS_{NH}) plants along with artificial diet (OS_{AD}).

Chapter 4: Identification and functional characterization of salivary secretory proteins from *Helicoverpa armigera*

Our proteomic analysis of OS from *H. armigera* fed on artificial diet, tomato and Capsicum plants suggest differential accumulation of secretory proteins (Chapter 3). In this study we have selected six secretory proteins (HARP1 to 6) identified from OS of *H. armigera* for functional characterization. Further *In silico* analysis of selected HARPs are presented. For functional characterization selected HARPs are cloned, recombinantly expressed and purified from bacterial systems. Further, the transcript level of Jasmonic acid and Salicylic acid biosynthesis and responsive genes are shown in response to mechanical wounding and recombinantly purified HARPs treatments.

Chapter 5: Summary and future prospects

This chapter highlights the important findings of this work and the possible futureavenues generated by this research.

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Chapter 2: Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense

Chapter 3: Integrated omics approach for analysis of oral secretion constituents from *Helicoverpa armigera*

Chapter 4: Identification and functional characterization of salivary secretory proteins from *Helicoverpa armigera*

Chapter 5: Summary and future prospects

Abstract

Name of the Student: Gopal S. Kallure **Registration No.:** 10BB15J26035

Faculty of Study: Biological Science **Year of Submission:** 2022

AcSIR academic center/CSIR Lab: CSIR-National Chemical Laboratory, Pune

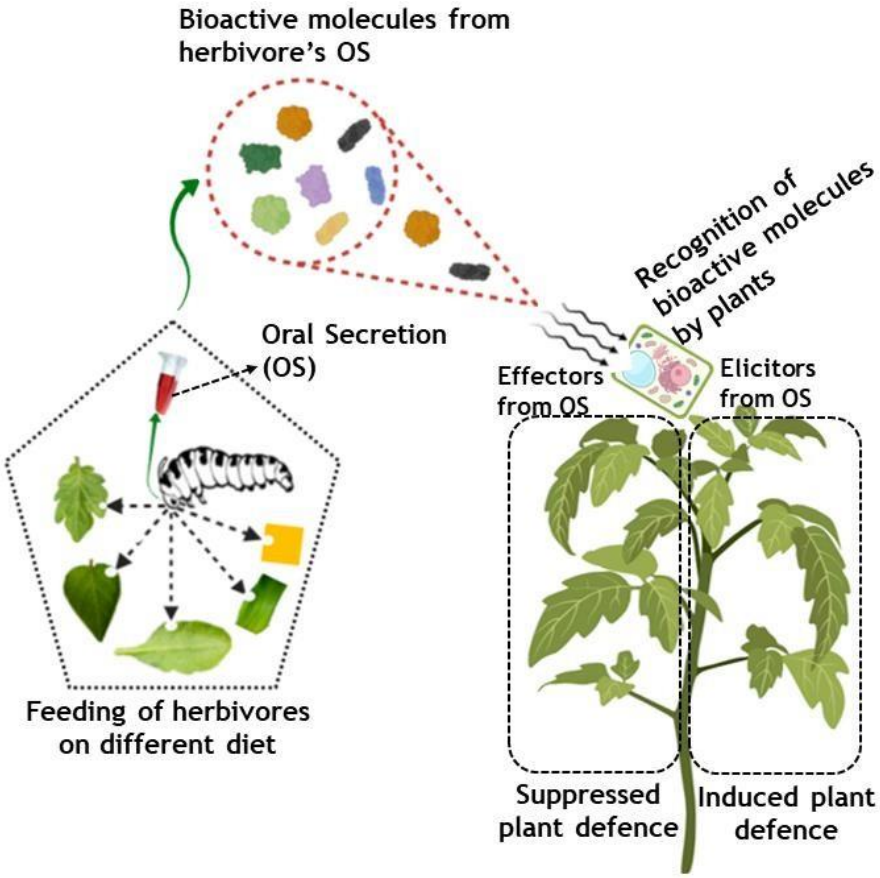
Name of the Supervisor: Dr. Ashok P. Giri

Title of the thesis: Deciphering role of different diets on herbivore oral constituents and their role in modulating plant defense

Little is known about how different plant-based diets influence the insect herbivores' oral secretion (OS) composition and eventually the plant defense responses. We collected the OS from *Helicoverpa armigera* fed on the host tomato (OS_H), non-host *Capsicum* (OS_{NH}) plants, and artificial diet (OS_{AD}). Interestingly, the treatment of *H. armigera* OS_{AD}, OS_H and OS_{NH} on wounded tomato leaves showed a differential expression of genes involved in biosynthesis and/or signaling pathways of jasmonic acid (JA), salicylic acid (SA) chlorogenic acid (CGA), and trehalose. There was higher expression of genes involved in the phenylpropanoid biosynthesis pathway, which may lead to the increased accumulation of CGA and related metabolites. Specifically, high levels of CGA were detected after OS_H and OS_{NH} treatments in tomato leaves. The insect bioassays demonstrated that CGA significantly inhibits *H. armigera* larval growth. Further, we analyzed the metabolite accumulations in OS_{AD}, OS_H and OS_{NH} using Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry. We observed that OS_H and OS_{NH} samples were enriched with alkaloids and terpenoids, respectively; while OS_{AD} sample was abundant in phospholipids. Also, we found differential accumulation of fatty acid amino acid conjugates in *H. armigera* OS. Further, in proteomic analysis of *H. armigera* OS, we found the differential and diet specific accumulation of proteins in OS. The *in-silico* analysis of proteomics data gave insights on the accumulation of salivary secretory proteins, which possess high similarity with *Helicoverpa armigera* R-like protein 1 (HARP1 a known effector protein of *H. armigera)*. Also, we observed the presence of single amino acid substituted uncharacterized HARP1 like secretory proteins in OS. For functional characterization, we have recombinantly expressed and purified the HARP1-like secretory proteins (HARP1 to 6) of *H. armigera*. The application of recombinantly purified HARPs individually as well as in combination on wounded tomato plants shows the differential expression of JA biosynthesis ad responsive genes expression without affecting SA biosynthetic and responsive genes. We also observed the higher transcript abundance of HARP1 and HARP5 in foregut, midgut and hindgut of *H. armigera* fed on Capsicum (non-host) as compared to tomato (host) leaves and artificial diet. Overall, our work highlights the role of diet on differential accumulation of bioactive molecules in insect OS and the OS components differentially modulate the tomato plant defense.

Chapter 1

Introduction and review of the literature



Contents of Chapter 1 have been published as review article…

G.S. Kallure, A. Kumari, B.A. Shinde, A.P. Giri, Characterized constituents of insect herbivore oral secretions and their influence on the regulation of plant defenses, **Phytochemistry**, 193 (2022) 113008.

1.1 Plant-insect interaction interplay

For more than 350 million years, there have been ongoing dynamic interactions between plants and insects (Ehrlich and Raven, 1964; Mishra et al., 2015). Some of the insects shows beneficial impact towards plant while many of them have adverse effect on plant growth (**Fig. 1.1**). Based on the host range of the insects, they have been classified as generalists or specialists (Ehrlich and Raven, 1964). Generalists feed on several plant species from different families, whereas specialists feed on one or more plant species of the same family.

Figure 1.1: Impact of insect infestation on plants A. Monarch butterfly feeding on a flower helps in pollination (©Amy Lynn Grover) **B.** A honeybee collecting nectar helps in pollination (©Peter Del). **C.** Ants help in seed dispersal (©Ozark Bill Duncan) **D.** Tobacco hornworm feeding on plant leaves causing severe plant damage (©Texas A&M University). **E.** Aphids sucking plant sap result in plant damage (©lnzyx) **F.** Insects transmit viruses to plants resulting in plant death (©EPPO global database).

Another classification is based on their feeding strategies inflicting mechanical damage of different intensities on plants (**Fig. 1.2**). Large herbivore insects are chewing insects that cause damage, with sharp and powerful mandibles evolved for munching, snipping, or tearing. Sucking insects have needle-like mouthparts (stylets) used to suck content from specific cells, such as phloem and xylem feeders, causing overall less physical damage. In all cases, oral secretions (OSs) of insects are bound to be encountered by the plants and potentially play an important role in insect-plant interactions. (Acevedo et al., 2015; Alborn et al., 1997; Basu et al., 2018; Chung et al., 2013; Felton and Tumlinson, 2008; Lou and Baldwin, 2003; Ray et al., 2015; Reymond, 2013).

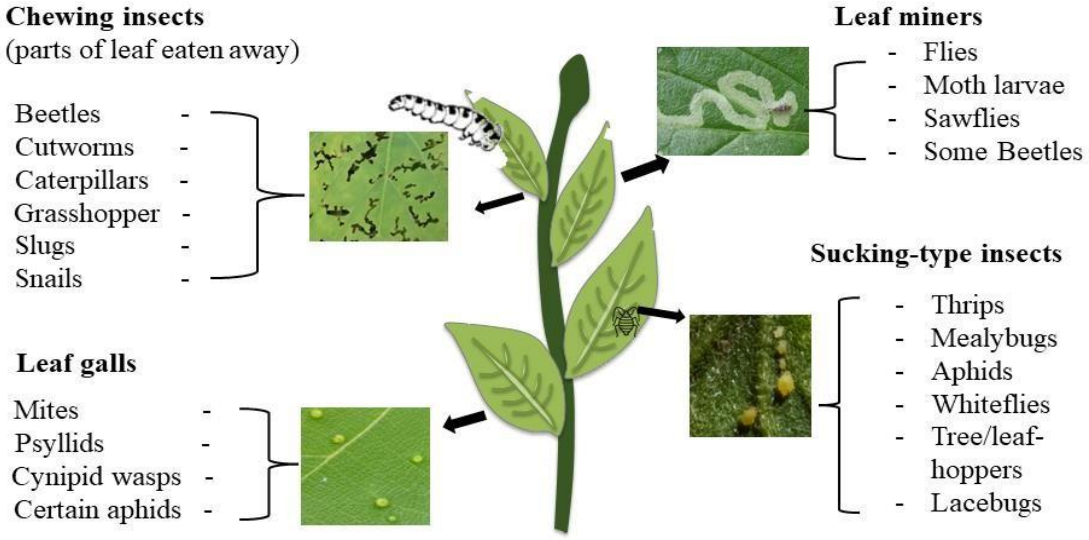


Figure 1.2: Overview of different herbivores based on their feeding pattern.

Many plants respond to feeding damage by a rapid release of lipoxygenase pathway produced green leafy volatiles and by upregulating a more complex defensive response to OS-related herbivore-associated molecular patterns (**Fig. 1.3**) (Acevedo et al., 2015; C. Chen and Mao, 2020; Felton et al., 2014; Paré and Tumlinson, 1999; Qi et al., 2018; Schmelz, 2015; Yoshinaga, 2016).

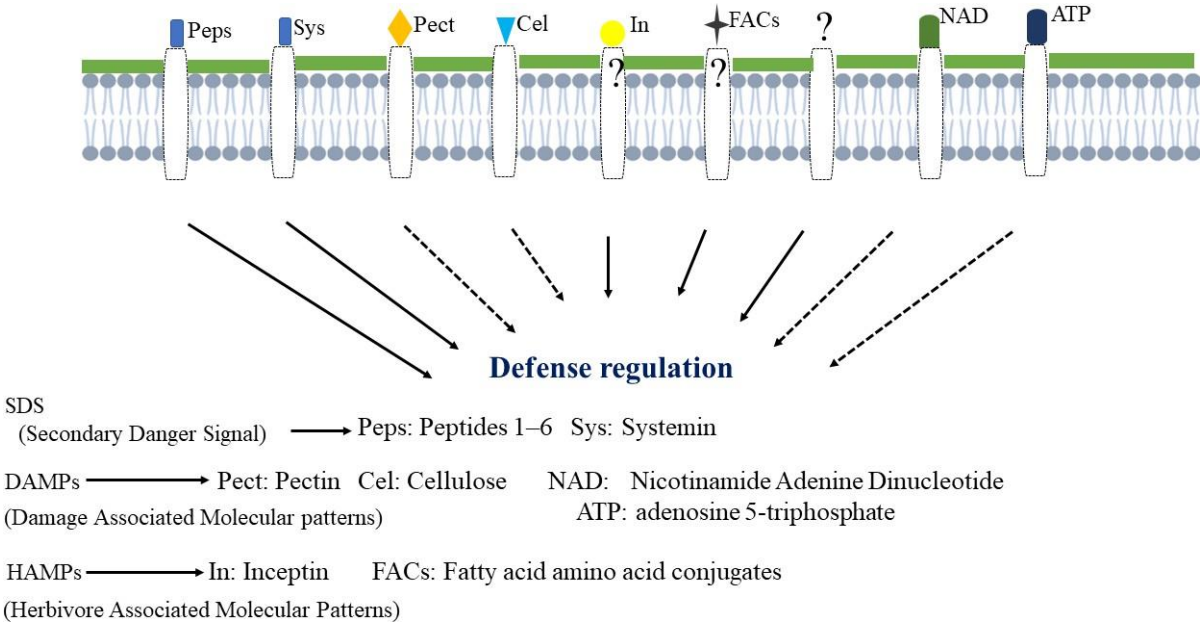


Figure 1.3: Recognition of known or putative ligands associated with insect attack. Question mark indicates unidentified bioactive molecules from insects and receptors from plants (Erb and Reymond 2019)**.**

Early signaling responses to insect damage might start with calcium flux, variation of plasma membrane potential, reactive oxygen species production, and phosphorylation cascades (Farmer et al., 2020; Zebelo and Maffei, 2015), which can further lead to systemic signaling affecting parts of the plant distant from the damaged tissue. (Schilmiller and Howe, 2005; Turlings and Tumlinson, 1992; Zebelo and Maffei, 2015). Thus, specific defense preparation proceeds by signaling networks through modulating the levels of numerous kinases, transcription factors, phytohormones, specialized metabolites, and defensive proteins that might compromise plant growth (**Fig. 1.4**) (Erb and Reymond, 2019). Insect OSs includes regurgitant and saliva with distinct origins and compositions. Regurgitant's arise from the foregut and midgut while saliva is released from the labial gland through the spinneret (Eichenseer et al., 1999; Peiffer and Felton, 2005). The components of OS trigger or suppress plant defense and are known as elicitors or suppressors, respectively (Alborn and Schmelz, 2008; Louis et al., 2013; Musser et al., 2002). Few reports suggest that the synthesis and function of OS components might depend on the host plant or the feeding part of the plant or the associated microbes (**Fig. 1.5**). Different plant-based diets are known to influence the herbivore OSs constituents and, eventually, the plant defense responses.

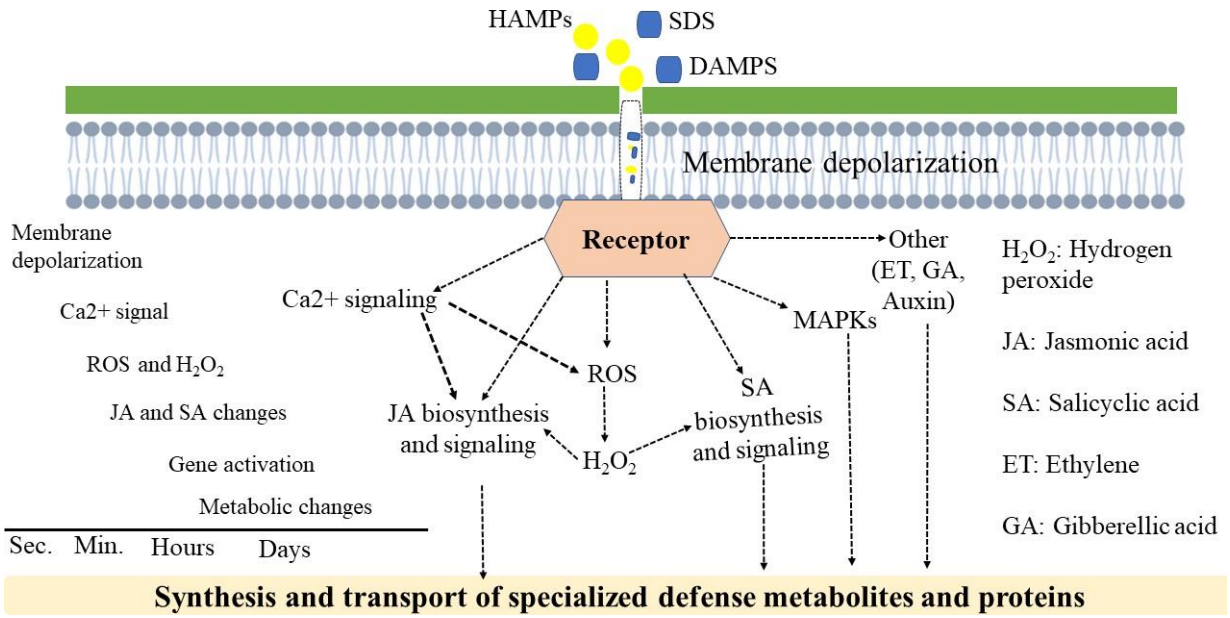


Figure 1.4: Activation of plant defense in response to herbivore attack.

The fatty acid components of fatty acid amides are directly diet-related and have been suggested to affect insect feeding choice.-Thus, as part of the diet of an herbivore, plants defense proteins and metabolites of host plants may influence the OS composition (Acevedo et al., 2015).

Over 300 comprehensive reviews covering the nature and general features of plant-insect interactions have been published since 2010. As per our survey, only twelve reviews used the term OS in the title, abstract, or keywords. Among these (Schmelz, 2015) comprehensively reviewed the magnitude and direction of plant responses orchestrated through OS components. In particular, constituents of OSs of insects and their role in plant defense has been recently reviewed (Erb and Reymond, 2019; Stahl et al., 2018). The role of insect OS constituents in suppressing herbivore- specific induced defenses and activating inaccurate plant defense has also been highlighted (Felton et al., 2014; Hogenhout and Bos, 2011).

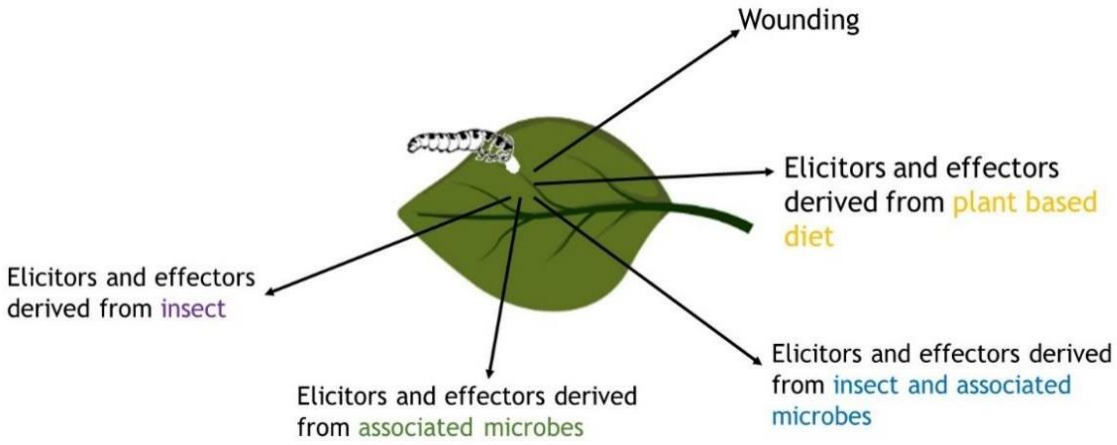


Figure 1.5: Release of bioactive molecules from herbivore oral secretions.

From this information, we gain some insights that the most characterized OS components are proteinaceous molecules such as glucose oxidase (GOX), several aphid proteins (ApC002, MpC002, Bsp9, Me10, Me23, Bt56), proteases, lipases, and *Helicoverpa armigera* R-like proteins (HARPs), while small molecules include fatty acid amino acid conjugates (FACs), peptides, oligosaccharides, amino acids, fatty acids, sugars, etc. (**Fig. 1.6**). Recently (Chen and Mao, 2020) summarized newly identified elicitors and effectors from insects and their target proteins in the plants. Active molecules were characterized as inducer or suppressor of plant defense. FACs elicit volatile emission and isoflavonid synthesis. Proteinaceous elicitors induces jasmonicacid (JA) and Salicylic acid (SA) biosynthesis and signaling leading to induce plant defense by protease inhibitors synthesis and release of volatiles. GOX induces reactive oxygen species (ROS) through hydrogen peroxide H_2O_2. Proteinaceous effectors either suppress the hydrogen peroxide H_2O_2, JA/SA biosynthesis and signaling or the interaction of transcription factors (WRKY) with kinases (**Fig. 1.6**).

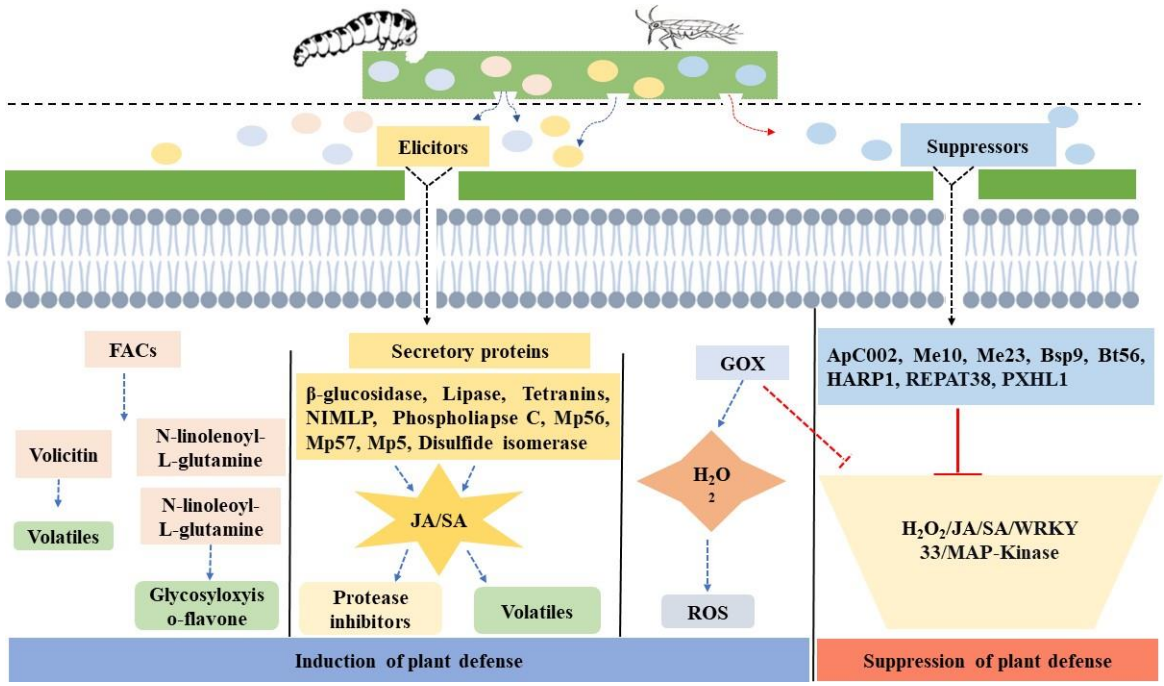


Figure 1.6: Schematic presentation of identified bioactive molecules from the oral secretion/mouthpart of insects.

1.2 Characterized constituents of the OSs of insect herbivore

1.2.1 GOX – an enzyme from insect OS that regulates plant defense response

GOX was reported as one of the abundant proteins in the saliva of *Helicoverpa zea* Hübner (Noctuidae) during their active feeding stage and has been secreted into the wounded plant part (Eichenseer et al., 1999; Musser et al., 2006; Peiffer and Felton, 2005). In the presence of D-glucose, GOX catalyzes the production of D-gluconic acid and H_2O_2 (Eichenseer et al., 1999). A higher level of H_2O_2 is believed to be the main factor responsible for the altered plant defenseby eliciting a salicylic acid (SA) burst and decreasing the jasmonic acid (JA) and (ET) levels (Diezel et al., 2009; Mittler et al., 2004). GOX has been recognized as a plant defense modulatorin several plant species, including *Nicotiana attenuata* Torrey (Solanaceae), *Medicago truncatula* Gaertn. (Fabaceae), *Solanum lycopersicum* Linnaeus (Solanaceae), and *Arabidopsis thaliana* Linnaeus (Brassicaceae) (Bede et al., 2006; Diezel et al., 2009; Lè Ne Weech et al., 2008) (**Table 1.1**). Furthermore, GOX was first identified as a suppressor molecule from the saliva of *H. zea*, suppressing nicotine production and defense responses in the *N. tabacum* (Musser et al., 2002). The feeding and survival of larvae was superior when fed on GOX-treatedtobacco leaves (Musser et al., 2005, 2002; Zong and Wang, 2004). The generalist behavior of insects might also be linked to the higher synthesis, activity, and stability of GOX (Eichenseer et al., 2010; Yang et al., 2017), consequently acting as a suppressor of plant defense so these insects can feed more on plants.

Interestingly, GOX inhibits the release of herbivore-induced plant volatiles by stomatal closure in tomato and soybean plants (Lin et al., 2021), these responses are plant species-dependent. Possibly, volatiles are synthesized and build up in the intercellular spaces of the leaf, and diffuse to the atmosphere through guard cells.

GOX may also act as a primary elicitor by activating the defense responses, which was first reported in tomatoes (Tian et al., 2012). Variable defense responses have been reported in other Solanaceae plants, including bell pepper (*Capsicum annuum* Linnaeus; Solanaceae), habanero pepper (*C. chinense*), and tomatillo (*Physalis philadelphica* Lamarck; Solanaceae) (Lin et al., 2020). These defense responses were possibly impacted by the availability of GOX substrates, D-glucose, in the host plants, emphasizing the importance of plant species and the quality of the host (**Table 1.1**)

However, it is possible that in addition to the response to an oxidative H_2O_2 burst, some plants might have GOX-specific receptors that trigger an additional defensive response. Yet, no such receptors have thus far been identified. The maximum quantity of GOX was reported from aggressively feeding larvae (Zong and Wang, 2004), but insect diets also seem imperative for the synthesis and secretion of GOX. For example, when *H. zea* larvae were reared on different host plants, varied amounts of GOX were detected in their salivary secretion (Peiffer and Felton, 2005). Overall, the quantity and quality of saliva constituents are inversely related to the quality of the host (Merkx-Jacques and Bede, 2004, 2005; Peiffer and Felton, 2005; Rivera-Vega et al., 2017). We can conclude that GOX synthesis and secretion are host-specific (Afshar et al., 2010; Merkx-Jacques and Bede, 2005, 2004; Peiffer and Felton, 2005), while they also vary among different caterpillar species depending on their feeding behavior (Eichenseer et al., 2010). Furthermore, to determine the dietary components essential for the GOX activity, lepidopteran insects were fed on their host plant and chemically defined artificial diets supplemented with sugars and other specialized metabolites (Hu et al., 2008). These comparisons have established that in general, proteins and carbohydrates could modulate GOX activity, insect growth, and development; however, phenolic components might have no impact on GOX activity (Babic et al., 2008; Hu et al., 2008; Simpson and Raubenheimer, 1993).

Table 1.1: Glucose oxidase (GOX) identified from insects oral secretions/mouth parts and its potential/proposed functions in respective insects and host plants.

Insect sp.	Feeding on	Insect response	Plant response	References
Helicoverpa armigera *Menduca sexta*	Artificial Diet Tobacco Tomato Cotton Soybean Geranium	Host plant dependent Glucose oxidase activity	Suppression of plant defense	(Eichenseer et al., 2010)
H. zea	Tomato Cotton Tobacco	Differential Glucose oxidase activity	Host plant affects herbivores oral secretion constitutes	(Peiffer and Felton, 2005)
H. armigera *H. assulta*	Hot pepper Tobacco Cotton	Higher glucose oxidase activity in generalist insect compared to specialized insect	Plant dietary component affect herbivory glucose oxidase activity	(Yang et al., 2017)
H. armigera *H. zea* *H. assulta*	Artificial Diet	Higher glucose oxidase activity in generalist insect than specialist insect	Suppression of nicotine induction in plants	(Zong and Wang, 2004)
M. sexta *Spodoptera exigua*	Tobacco	Glucose oxidase activity is lower in *M. sexta* oral secretion than *S. exigua*	Modulation of cross-talk between Salicylic acid (SA), Ethylene (ET) and Jasmonic acid (JA) pathways	(Diezel et al., 2009)
H. zea	Artificial Diet	Increased survival rate of insects	Suppression of induced resistance in *N. tabacum*.	(Bede et al., 2006)
S. exigua	Artificial diet (differ carbohydrate/protein)	Insect salivary GOX activity is diet-dependent	Not known	(Babic et al., 2008)
H. zea	Wheat germ and casein-based artificial diet	Glucose oxidase is the main protein identified in insect saliva	Burst of Jasmonic acid and induction of late defense gene expression	(Tian et al., 2012)
H. zea	Tomato, Soybean	Host plant dependent GOX activity for stomatal closure	Inhibits the release of herbivore-induced plant volatiles by stomatal closure	(Lin et al., 2021)

1.2.2 FACs – components of lepidopteran OS as elicitors of plant defense responses

Fatty acid amino acid conjugates (FACs) are among the most abundant OS bioactive molecules present in lepidopteran insects (Alborn et al., 1997; Yoshinaga et al., 2010), and are synthesized by the conjugation of fatty acid(s) and amino acid(s). A common feature among the discovered FACs is the conjugation of either L-glutamine or L-glutamic acid of insect origin with the linolenic acid or other free fatty acids derived from plant lipids. This chemical modification allows plants to distinguish herbivore attacks and to have specific defense responses (Paré et al., 1998). FACs are familiar in the Noctuidae family, but with variable quantities in different species (Mori et al., 2003). Several FACs, such as N-linoleoyl-L-glutamine, N-linoleoyl-L-glutamic acid, volicitin (N-17-hydroxylinolenoyl-L-glutamine), and N-hydroxylinolenoyl-L-glutamic acid were discovered from the OS of herbivore insects (Alborn et al., 2000; Halitschke et al., 2001; Mori et al., 2003; Paré et al., 1998; Pohnert et al., 1999; Tumlinson and Lait, 2005; Yoshinaga et al., 2010) (**Table 1.2**). Caeliferins, a family of sulfoxy fatty acids, have been identified from the OS of grasshopper *Schistocerca americana* Drury (Acrididae). Caeliferins trigger the release of terpenoid-like volatiles from maize seedlings (Alborn et al., 2007).

The most potent FAC, volicitin, was identified from *Spodoptera exigua* Hübner (Noctuidae) OS (Alborn et al., 1997), which contains two asymmetric carbons. Interestingly, synthetic volicitin with a D-glutamine conjugate did not show any activity (Alborn et al., 2000, 1997), suggesting that a structural configuration of amino acids has a significant role in the bioactivity of FACs. Similarly, hydroxylation on carbon 17 of linolenic acid is also important for bioactivity (Alborn et al., 2000). Most volicitin- and fatty acid amide-induced plant volatiles are terpenoids released in response to inducible upregulation of terpene synthases. In addition, FAC induction also often results in the release of indole which was found to be due to induced indole-3-glycerol phosphate lyase activity (Frey et al., 2000). Following this discovery, several other volatile components were also characterized after volicitin treatment on damaged plants (Gaquerel et al., 2009). FACs of *S. litura* Fabricius (Noctuidae) N-linolenoyl-L-glutamine and N-linoleoyl-L-glutamine have been found to induce the accumulation of isoflavone 7-O-glucosides and isoflavone 7-O-(6″-O-malonyl-β-glucosides) in soybean (Nakata et al., 2016). From the perspective of the insects, activating the plant defense or attracting their enemies is an intriguing behavior. One plausible explanation is that FACs might function in nitrogen assimilation by regulating the supply of amino acids in the insects midgut (Yoshinaga et al., 2008). Thus, we can presume that insects have developed a mechanism to modify plant-based linolenic acid into the FACs, which plants perceive as messengers to activate defense mechanisms.

As mentioned earlier, in several insect species, FACs are synthesized with two different conjugates, glutamine (Gln) and glutamic acid (Glu). Bioassays with synthetic conjugates confirm that glutamine conjugates are more active in inducing plant volatiles than glutamic acid conjugates (Alborn et al., 2003). Furthermore, the diet of insects significantly influences the composition of these conjugates with OS of insects (Alborn et al., 2003, 2000, 1997; Halitschke et al., 2001). Importantly, plant-based linolenic acid has a significant impact on the synthesis of volicitin and is considered the backbone of FACs. For instance, insect larvae were devoid of volicitin if they fed on the fruits of *Physalis angulate*, which lack linolenic acid (De Moraes and Mescher, 2004). Furthermore, the impact of the closely related host on the synthesis and activity of FACs was analyzed. A single plant species may elicit discrete responses to different FACs, and a single FAC may trigger diverse responses to the related plant species (Xu et al., 2015). Many plants, e.g., tomato, Arabidopsis, and cowpea, have been observed not to respond to FAC treatment. In contrast, FAC treatment had a strong effect on tobacco, eggplant, and corn plants, with upregulation of phytohormones and release of a suite of volatiles (Schmelz et al., 2009). A recent study, including wild species of tomato and other genera of Solanaceae, had demonstrated that the plant response to FACs does not follow phylogenetic relationships. Instead, responses to FACs are ancestral traits that may have been lost during the evolution or domestication of Solanaceae species (Grissett et al., 2020).

Moreover, another prospect is the presence of the variable side chains, carboxylic acid and amine of N-linolenoyl-L-glutamic acid (18:3-GLU) and N-linolenoyl-L-glutamine (18:3-GLN), which mediate different defense responses in host plants (Alborn et al., 2003). It is possible that not yet known receptors in the host plants could relate the differential activity with FACs.

Table 1.2: Different Fatty acid amino acid conjugates (FACs) identified from insects oral secretions/mouth parts and potential/proposed functions.

Insect sp.	Feeding on	Molecule	Insect response	Plant response	Reference
M. sexta	Tobacco	FACs	FACs along with other molecules act as insect elicitors	Transcriptional and proteomic changes	(Giri et al., 2006)
M. sexta	Tobacco	*N*-linolenoyl-L-Glu *N*-linoleoyl-L-Glu *N*-palmitoyl-L-Glu *N*-linolenoyl-L-Gln *N*-linoleoyl-L-Gln *N*-palmitoyl-L-Gln	Glu-Conjugate fatty acid is more abundant than Gln-conjugate fatty acid	Induced JA accumulation, and volatile release	(Halitschke et al., 2001)
M. sexta	*Nicotiana attenuata*	*N*-linolenoyl-L-Gln *N*-linolenoyl-L-Glu Hydroxyoctadecatrienoic acid	Not known	Induced and suppressed suites of volatiles	(Gaquerel et al., 2009)
M. sexta	Tobacco	FACs	18:3-Glu major elicitor in insect oral secretion	Induced JA biosynthesis and differential monoterpene emission	(VanDoorn et al., 2010)

S. litura	Artificial Diet enriched with amino acid	Glutamine type FACs	Role in nitrogen assimilation and function as storage of glutamine in gut lumen	Not known	(Yoshinaga et al., 2008)
Heliothis virescens, *S. exigua*, *S. frugiperda*, *S. littoralis*, *Epirrita autumnata*, *Operophtera*	Artificial Diet, *Betula pendula* leaves	Fatty Acid Amides	Amount of fatty acid conjugates is species specific	Not known	(Pohnert et al., 1999)
S. exigua	Corn seedlings	Volicitin	L-glutamine conjugated volicitin identified from oral secretion	Emission of volatile compounds	(Alborn et al., 1997)
H. subflexa	*Physalis angulata* fruit	Volicitin	Adaptive to dietary deficiency Reduced susceptibility to natural enemies	Differential accumulation of volatiles against fruit feeding caterpillar compared to leaf feeding and linolenic acid-treated leaves	(De Moraes and Mescher, 2004)
Not known	Not known	Synthetic volicitin	Not known	Increased emission of indole and sesquiterpene volatiles	(Lawrence and Novak, 2004)

S. exigua	Isotopically labelled corn seedlings	Volicitin	Chemical modification of ingested linolenic acid by insect	Triggered the release of plant volatile	(Paré et al., 1998)
H. armigera *S. litura* *Mythimna separata* *Agrius convolvuli*	Cabbage Rice leaves Sweet potato	Volicitin related compounds	Species specific synthesis of volicitin related compounds in insect oral secretion	Not known	(Mori et al., 2003)
Shistocerca americana	Maize	Caeliferins	Not known	Triggers release of terpenoid-like volatiles	(Alborn et al., 2007)

1.2.3 Secretory proteins in insect OS as elicitors and suppressor of plant defense

The proteins from herbivore OS have been characterized as elicitors and suppressors of plant defenses (**Table 1.3**). The proteinaceous elicitor β- glucosidase from *Pieris brassicae* Linnaeus (Pieridae) OS activates volatile emission from cabbage leaves (Mattiacci et al., 1995). Lipase and phospholipase C from the OS of *Schistocercagregaria* Forsskål (Acrididae) and *S. frugiperda* Smith (Noctuidae) have been found to elicit theaccumulation of oxylipin and protease inhibitors in *Arabidopsis* and corn, respectively (Acevedoet al., 2018; Schäfer et al., 2011). Another group of elicitor proteins called tetranins from *Tetranychus urticae* Koch (Tetranychidae) activates SA and JA biosynthesis in kidney beans andeggplants (Iida et al., 2019). The secretory protein NlMLP of the sucking insect *N. lugens* Stål (Delphacidae) induces Ca^{2+} mobilization and JA signaling in *Oryza sativa* Linnaeus (Poaceae) (Shangguan et al., 2018). Induced plant defenses have been observed in *N. tabacum* against individual secretory proteins Mp56, Mp57, and Mp58 from *Myzus persicae* Sulzer (Aphididae) aphids. However, the synthesis and secretion of these proteins was mostly dependent on the hostplants (Elzinga et al., 2014). The transient expression of the secretory enzyme disulfide isomerase of the brown planthopper *Laodelphax striatellus* Fallén (Delphacidae) induces JA signaling and callose deposition in *N. benthamiana* (Fu et al., 2021).

In addition to elicitors from OS, the suppressor-like proteins have also been identified (**Table 1.3**). The protein C002 has been identified from the mouthpart of *Acyrthosiphon pisum* Harris (Aphididae) while feeding on phloem sap of host plant Fava beans (*Vicia fava* Linnaeus; Fabaceae). Knockdown of the *ApC002* gene in *A. pisum* resulted in altered feeding behavior; specifically, the aphids were unable to assess the phloem cells. (Elzinga et al., 2014; Mutti et al., 2008). The functional characterization of *Macrosiphum euphorbiae* Thomas (Aphididae) saliva resulted in the identification of Me10 and Me23 as effector proteins that suppress the plant defense of *N. benthamiana*, allowing aphids to increase their population (Atamian et al., 2013). Aphid effectors were demonstrated to be fast evolving and provided aphid-host specificity by promoting colonization on specific plant species (Pitino and Hogenhout, 2013).

Other salivary proteins, Armet, ACE1 and ACE2, from *A. pisum* were reported as suppressor proteins. The induced transcriptional response due to Armet proteins in tobacco plants benefits insects. Knockdown of these suppressors altered the feeding performance of aphids on plants. These proteins are crucial for the adaptation of *A. pisum* to different plants, as they modulate the required transcriptional responses in both host and nonhost plants.

Higher transcript levels of Armet, ACE1 and ACE2 were observed in *A. pisum* when fed on a plant-based diet as compared to an artificial diet (Wang et al., 2015a, 2015b). Thus, these findings signify the importance of the plant diet on the alteration of the synthesis and secretion of effector-like proteins by the aphids. The migration inhibitory factor protein from *M. persicae*saliva was identified as another critical component for the improved aphid survival, fecundity, and feeding on different host plants. The transient expression of migration inhibitory factors in *N. benthamiana* suppressed the transcript levels of defense genes and reduced callose deposition (Naessens et al., 2015). Functional characterization of two salivary secretory proteins, Bsp9 and Bt56, from *Bemisia tabaci* Gennadius (Aleyrodidae), have revealed that both the proteins modulate the defense signaling in tomato and tobacco, respectively. Bsp9 improves the feeding of *B. tabaci* on tomato plants by suppressing the activation of WRKY33 and MAP-kinase interactions, while Bt56 modulates SA signaling in tobacco through a KNOTTED 1-like transcription factor (Wang et al., 2019; Xu et al., 2019a). Stink bugs also activates MPKs pathway by releasing their saliva to the developing soybean seeds that modifies the seed cell wall and may activate defense metabolic pathways (Giacometti et al., 2020, 2016). The overexpression of effector protein RpC002 in barley resulted in enhanced susceptibility towards *Rhopalosiphum padi* Linnaeus (Aphididae) and not against *M. persicae*. Reduced transcript levels were observed for defense signaling genes in RpC002 transgenic barley lines (Escudero-Martinez et al., 2020). *In silico* analysis of spider mites (*Tetranychidae sp.*) has identified Tu28 and Tu84 and its homolog Te84, which can account for suppression of SA defense. Transient expression of three of these proteins in tobacco plants promoted the reproductive performance of *T. urticae* (Villarroel et al., 2016). In the brown planthopper (*N. lugens*), secretory calcium-binding protein-1 (NlSEF1) functions as a suppressor. The recombinant NlSEF1 protein was reported to reduce H_2O_2 production in rice. Furthermore, knockdown of NISEF1 in *N. lugens* resulted in reduced feeding performance, leading to higher mortality (Ye et al., 2017). Recently, two other effector proteins were reported that target the ROS pathway to promote their performance on host plants. Salivary ferritin, BtFer1 from the whitefly *B. tabaci*, suppress the ROS burst during feeding on tomato plants (Su et al., 2019). The mirid bug (*Apolygus lucorum* Meyer-Dür; Miridae) releases salivary gland effector A16 in the host cells to interfere with plant susceptibility (Dong et al., 2021). Thus, synthesis and induced levels of novel suppressors may be beneficial for the insects feeding on different host and nonhost plants. Few effector-like proteins have been identified from chewing insects. Recently, the effector *Helicoverpa armigera* R-like protein 1 (HARP1) was identified in OS (Chen et al., 2019). HARP1 protein has stabilized

JAZ proteins involved in JA-mediated defenseresponses in Arabidopsis. The overexpression of HARP1 in the nonhost plant *N. benthamiana* helped *Plutella xylostella* Linnaeus (Plutellidae) to improve their feeding performance. The induced accumulation of HARP1 protein was found in *H. armigera* OS collected from insects feeding on Arabidopsis plants compared to artificial diet (Chen et al., 2019), suggesting that dietcould play an important role in the biosynthesis and secretion of suppressor-like proteins in insects. Similar to HARP1, REPAT38 from *S. exigua* interacts with JAZ proteins of host plantsto impede JA signaling. Hence, the secretion of effector-like proteins through OS could help insects to feed on multiple plants by modulating the defenses. Many HARP1-like proteins are conserved in lepidopteran insects; however, their functional role has yet to be elucidated. To minimize the feeding performance of insects on multiple plants, the role of dietary components and their significance on the accumulation of effector-like proteins in OS needs to be explored. The use of knockdown and RNAi to silence the expression of suppressor transcripts in insects can help to reduce the insect infestation. Similarly, understanding the digestive physiology of insects will also provide new strategies for the management of insect pests (Lomate and Bonning,2016). Few proteinaceous elicitors have also been identified from the OS of herbivores; hence studying the differentially accumulated elicitor-like protein from insects will help to engineer plants with enhanced defense against herbivores.

Table 1.3: Salivary proteins characterized as elicitor and suppressor from insects oral secretions/mouth parts and potential/proposed function in respective insects and host plants.

Elicitors					
Insect sp.	**Feeding on**	**Molecules**	**Insect response**	**Plant response**	**Reference**
Pieris brassicae	Brussels sprouts	β-glucosidase	Attractive parasitic wasp	Activates volatile emission	(Mattiacci et al., 1995)
Myzus persicae	Tobacco	Mp56 Mp57 Mp58	Decreased aphid reproduction	Activated plant defense responses	(Elzinga et al., 2014)
M. persicae	Not known	Mp10 Mp42	Reduced aphid fecundity	Triggered defense response	(Bos et al., 2010)
A. pisum	Fava beans	ACE1 ACE2	ACE1 and ACE2 work together to modulate *A. pisum* feeding and survival on plant	ACEs can hydrolyze systemin or other signal molecules that induce plant immune reactions	(Wang et al., 2015b)
Schistocerca gregaria	*Arabidopsis*	Lipase	Not known	Elicit accumulation of oxylipin	(Schäfer et al., 2011)
S. frugiperda	Rice and Maize	Phospho-Lipase C	Larval growth negatively regulated	Accumulation of protease inhibitors	(Acevedo et al., 2018)
Tetranychus urticae	Kidney bean plants	Tetranins	Increased mortality of insects	Activates expression of SA and JA biosynthesis	(Iida et al., 2019)

Nilaparvata lugens	Susceptible rice cultivar	NlMLP	Inhibition of *NlMLP decrease feeding performance*	Induces Ca^{2+} mobilization and JA signaling	(Shangguan et al., 2018)
Laodelphax striatellus	Susceptible rice cultivar	Disulfide isomerase	Reduced feeding of insects	Induces JA signaling and callose deposition	(Fu et al., 2021)
Plant-derived					
Not known	Tomato	Systemin	Not known	Induces oxidative bursts and accumulation of proteinase inhibitor	(Pearce et al., 1991; Wang et al., 2018)
Not known	*Arabidopsis*	PEPs	Enhanced resistance toward the pathogen *Pythium irregulare* and *Pseudomonas syringae*	Activates H_2O_2 synthesis and defensive gene	(Huffaker et al., 2013; Yamaguchi et al., 2006)
S. frugiperda	Cowpea or Maize	Inceptin	Found only in insects fed on leaf	Promoted JA signaling, ethylene production,	(Schmelz et al., 2006)
M. sexta *Trichoplusia ni*	Tomato, *N. atteunata*	Threonine deaminase	Reduced the level of free threonine	Provides isoleucine (Ile) for biosynthesis of JA-Ile conjugates and activates specific defense responses	(Chen et al., 2007, 2005; Gonzales-Vigil et al., 2011)
Insect-associated microbes					

Macrosiphum euphorbiae-associated Buchnera aphidicola	*Arabidopsis*, Tomato	GroEL	Reduced aphid fecundity	ROS accumulation and Induced expression of pattern-triggered immunity early marker genes	(Chaudhary et al., 2014)
Spodoptera littoralis associated bacteria probably	*Arabidopsis*	Porin-like protein	Act as an insect elicitor	Induces membrane potential changes and cytosolic Ca^{2+} elevations in Arabidopsis and *Vicia faba*	(Guo et al., 2013)
Suppressors					
A. pisum	Fava beans	Armet	Promoted feeding on host plant	Suppression of host plant defense, induction of non-host plant defense,	(Wang et al., 2015a)
Acyrthosiphon pisum	Fava beans	C002	Crucial for feeding of the pea aphid on host plant	Not known	(Mutti et al., 2008)
Bemisia tabaci	Tobacco	Bt56	Promoted whitefly phloem-feeding on host plants	Induction of SA-signaling pathway	(Xu et al., 2019a)
M. persicae	Artificial Diet	MIF	Crucial for aphid survival, fecundity and feeding on host plant	Inhibition of expression of defense related genes and callose deposition	(Naessens et al., 2015)
H. armigera	*Arabidopsis*	HARP1	Improved feeding of oligophagous insect on non-host plant	Inhibition of JA signaling	(Chen et al., 2019)

M. persicae	Tobacco	MpC002 Mp55	Increased aphid fecundity	Suppression of plant defense	(Elzinga et al., 2014)
B. tabaci	Tomato	Bsp9	Promoted performance and preference to host plant	Suppression of plant immune signaling	(Wang et al., 2019)
N. lugens	Rice	NlSEF1		Reduce H_2O_2 production	(Ye et al., 2017)
B. tabaci	Tomato	BtFer1	Promote the performance on host plant	Suppresses the ROS burst during feeding	(Su et al., 2019)
M. euphorbiae	Tomato	Me10 Me23	Increased aphid fecundity	Ability to suppress *N. benthamiana* defense	(Atamian et al., 2013)
M. euphorbiae	Tomato	Me47	Enhanced aphid colonization	Suppression of plant immunity	(Kettles and Kaloshian, 2016)
M. persicae	Tobacco	MpC002	Promotes *M. persicae* colonization on Arabidopsis	Modulation of defense	(Pitino and Hogenhout, 2013)
Tetranychidae sp	Tobacco	Tu28, Tu84, Te84	Promote the reproductive performance of *T. urticae*	Suppression of SA defense	(Villarroel et al., 2016)
Apolygus lucorum	Tobacco	A16	Promote the performance on host plant	degrade toxic oxidation products produced during feeding	(Dong et al., 2020)
Plant-derived					

H. zea	Tomato	Apyrase	Secrete ATP hydrolyzing enzymes that suppress plant defense	Suppresses the defensive genes regulated by the jasmonic acid and ethylene	(Wu et al., 2012)
Insect-associated microbes					
Aster yellows phytoplasma in Macrosteles quadrilineatus	Arabidopsis	SAP11	Increased susceptibility to phytoplasma insect vectors	Changes leaf morphogenesis and reduces the plant defense responses	(Sugio et al., 2011)
Aster yellows phytoplasma in M. quadrilineatus	Arabidopsis	SAP54	Promotes Insect Colonization	Degrade MADS-domain transcription factors, suppresses the flower development	(MacLean et al., 2011)
Aster yellows phytoplasma in *M. quadrilineatus*	Arabidopsis	SAP05	Promotes insect colonization	Control several plant developmental pathway	(Huang et al., 2021)
Tomato yellow leaf curl virus in *B. tabaci*	Tobacco	C2	Promotes survival and reproduction of *B. tabaci*	Suppression of plant defenses by interacting with plant ubiquitin and blocks JA signaling	(P. Li et al., 2019)
Tomato yellow leaf curl China virus in *B. tabaci*	Arabidopsis	βC1	Enhanced performance of the vector whiteflies	Suppresses terpene synthesis by interacting with MYC2 transcription factor	(Li et al., 2014)
Cucumber mosaic virus (CMV) in *M. persicae*	Arabidopsis	2b	Manipulate host's appeal to insect vectors	Blocks JA signaling	(Tungadi et al., 2017)

1.3 Host plants and insect-associated microbes alter the OS composition of insects that differentially tweaks the plant defense responses

1.3.1 Plant-derived peptides and metabolites in the OS of insects responsible for regulating plant defense

The OS typically contains proteins, peptides, oligosaccharides, fatty acids, and a combination of primary and specialized metabolites. Several of these compounds have plant origin and are known to be involved in the regulation of plant defense responses (**Table 1.3**). For example, sheath saliva of brown marmorated stink bug (*Halyomorpha halys* Stål; Pentatomidae) elicited the JA-inducible defense gene proteinase inhibitor 2 (*Pin2*), but this induction was observed only when sheaths had been collected from tomato plants, indicating their plant origin (Peiffer and Felton, 2014). Systemin and HypSys (18 to 20 amino acid) peptides induce defense responses in Solanaceous plants through the JA signaling pathway (Pearce et al., 1991; Wang et al., 2018). Twenty-three-amino acid plant elicitor peptides (Peps), plant ATPase-derived inceptin (11-amino acid) peptide, and protein apyrase (446-amino acid) are found in the OS of chewing insects. These peptides/proteins are known to function as effectors. Peps and inceptins lead to activation of the defense response in the Fabaceae and Cruciferae families via the wound-inducible JA signaling pathway, while apyrase suppresses the JA-dependent signaling in tomato (Schmelz et al., 2007, 2006; Wu et al., 2012). In *N. attenuata,* threonine deaminase (TD) has been demonstrated to provide isoleucine (Ile) for the biosynthesis of JA-Ile conjugates that activate specific defense cascades against insect infestation (Kang et al., 2006). Moreover, feeding of *Manduca sexta* Johannsen (Sphingidae) and *Trichoplusia ni* Hübner (Noctuidae) on tomato plants resulted in the accumulation of active TD in the insect gut without a regulatory domain that continuously catalyzes the cleavage of threonine (Chen et al., 2005). TD inhibits the growth of *M. sexta* not only by reducing the level of free threonine but also by producing the toxic metabolite α-ketobutyrate (Chen et al., 2007, 2005; Kang and Baldwin, 2006). Furthermore, it was found that the chymotrypsin-like proteases of lepidopteran insect origin are responsible for proteolytic cleavage of the regulatory domain of the duplicated TD paralog (TD2), leading to an active form of the enzyme that depletes threonine levels (Gonzales-Vigil et al., 2011). Studies have also reported that *Spodoptera littoralis* Boisduval (Noctuidae) OS contains β-galactofuranose polysaccharides of unknown origin (either from the plant, insect, or associated microorganism), which act as a prominent elicitors of defense responses in Arabidopsis and soybean. These OS polysaccharides are responsible for early events viz. membrane depolarization, elevation in cytosolic Ca^{2+} ions, and generation of reactive oxygen

species in plants (Arimura, 2021; Uemura et al., 2020).

1.3.2 Influence of microbial associates on insect OS composition and response to plant defense

Symbiotic microbes are important for herbivores because they deliver amino acids (Douglas, Angela, 2015), help in digestion (Visôtto et al., 2009), and detoxify specialized metabolites (Hammer and Bowers, 2015; Mason et al., 2015). The ability of insects to exploit some host plants depends, at least in part, on their association with a specific microorganism (Hosokawa et al., 2007; Tsuchida et al., 2004). However, plant metabolites also influence the microbiota of the digestive systems of insects and hence the OS of the insects (Grunseich et al., 2019; Shikano et al., 2017). Evolutionary forces that shape plant-insect interactions may possibly have also impacted the insect microbial interactions (Noman et al., 2020).

There is an increasing amount of evidence wherein insect-associated microbes have influenced plant defense in numerous ways. Recently, Yamasaki et al., (2021) showed that JA biosynthesisand signaling are induced by *S. litura* OS devoid of bacterial isolates; however, their presence activates SA biosynthesis and signaling. Microbes present in the insect OS directly come in contact with plant wounds during insect feeding (Chung et al., 2013). Bacterial symbionts in theOS of Colorado potato beetles (*Leptinotarsa decemlineata* Say; Chrysomelidae) elicit SA- regulated defense. Colonization of these bacteria seems beneficial for insects, as SA upregulationleads to JA downregulation and increases the insect performance (Chung et al., 2013). Depending on the host plants, variable plant defense responses were observed with similar bacterial isolates(Acevedo et al., 2017). Furthermore, some microbe-derived molecules can also modulate plant defenses (**Table 1.3**). Similar to GroEL and porin-like proteins, elicitors from insect-associated microbes induce early defense responses in host plants (Chaudhary et al., 2014; Guo et al., 2013).Herbivore-associated fungi and viruses can also directly induce a higher level of defense in several plants (C. Chen and Mao, 2020; Tan et al., 2018). *Wolbachia* sp. via their host (western corn rootworm) downregulated numerous defense-related genes (Barr et al., 2010). However, some suppressor molecules have been reported from vector-borne insect pathogens, such as SAP11, SAP54, and SAP05 (aster yellows phytoplasma), C2 and βC1 protein (tomato yellow leaf curl China), and 2b protein (cucumber mosaic virus) (W. Huang et al., 2021; P. Li et al., 2019; Li et al., 2014; MacLean et al., 2011; Sugio et al., 2011; Tungadi et al., 2017). SAP11, βC1, C2 and 2b proteins target the synthesis and signaling of the JA pathway and increase the performance of host plants. The phytoplasmic effector SAP54 promotes insect colonization by controlling plant reproduction through degradation of MADS-box proteins**.** However, SAP05

controls several plant developmental pathways for its benefit by degrading of developmental regulators (W. Huang et al., 2021). These molecules suppress plant defense, which is beneficial to the host insects feeding on those plants. In addition to releasing effectors, insect-associated microbes can also indirectly trigger plant defense by influencing insect synthesis of biologically active molecules. For example, the *H. zea* gut-associated bacterium *Enterobacter ludwigii* Hoffmann (Enterobacteriaceae) induces salivary GOX and triggers tomato plant defense (Wang et al., 2017). Similarly, bacteria present in the gut of herbivores has been estimated to play a role in the synthesis of FACs (Spiteller et al., 2000). However, the biosynthesis rate was very slow. Overall, the knowledge emerging from recent studies, including insect-microbe-plant interactions has emphasized the importance of herbivore-associated microbes in modulating plant defense responses (Noman et al., 2020).

1.4 Plant transcriptional reprogramming leading to the induction of common protein and metabolite defensive markers upon insect feeding and OS application

Plant defense is continuously modulated by transcriptional reprogramming by altering the biosynthesis and signaling of phytohormones such as JA, SA and ET in plants (Bodenhausen and Reymond, 2007; Erb and Reymond, 2019; Heidel-Fischer et al., 2014; War et al., 2018). Inducible defense is fascinating, as it provides plants with a flexible and less costly affair. These inducible defenses consist of defense proteins and specialized metabolites, which plants prioritize over growth to defend against herbivores. Inhibitors of protease and amylase are essential plant defensive proteins against various insect pests, including lepidopteran, hemipteran, and coleopteran (Ahn et al., 2007; Jadhav et al., 2016; Parde et al., 2012; Tamhane et al., 2005). Additionally, various studies have reported ribosome-inactivating proteins, *vegetative insecticidal proteins*, pathogenesis-related proteins of various plant origins viz. maize, apple, and Sambucus nigra Linnaeus (Adoxaceae) have insecticidal activity against different insect pests (Gatehouse et al., 1990; Shahidi-Noghabi et al., 2008; Stirpe, 2013; Zhu et al., 2018). *F*urthermore, lectins have a protective function against several insect pests (Vandenborre et al., 2011). Induced accumulation of lectins and hevein-like protein was observed not only upon different insect feeding but also in response to OS in *N. tabacum* and Arabidopsis, respectively. However, mechanical wounding did not affect lectin accumulation (Reymond et al., 2000; Vandenborre et al., 2009a, 2009b). Moreover, identical but lower levels of proteins involved in defense, primary metabolism and transcriptional regulation were observed in native tobacco (*N. attenuata*) against *M. sexta* feeding compared to OS treatment (Giri et al., 2006). Altogether, these reports suggested that the above mentioned proteins (Particularly protease inhibitors,

pathogenesis-related 10, and lectins) are commonly induced in plants by various insect pests and thus can be considered plant defensive protein markers.

Apart from proteins, plants also produce several specialized metabolites (**Table 1.4**) targeting herbivore biological systems, such as nervous, digestive, and endocrine organs (Mishra et al., 2015; War et al., 2018). The response of plants to insect herbivory can be general or insect species-specific. For example, the increase and consistent release of volatiles such as linalool, (E)-ßocimene, (E)-2,4-hexadiene, β-caryophyllene, (E,E)-α-farnesene, α-humulene, 7-epizingiberene, and R-curcumene were reported in Solanaceae plants in response to *Heliothis virescens* Fabricius (Noctuidae) feeding, or OS, and does not differ from the response to feeding by the spotted spider mite (Zhang et al., 2020). Similarly, cucurbitacin is a commonly occurring triterpenoid in cucurbits that affects sap-sucking spider mite's growth (Kaushik et al., 2015). Thus, plant volatiles viz. terpenoids are common and vital cues in plant-insect interactions; however, these molecules could be plant-family-specific. Alkaloids are widely distributed specialized nonvolatile molecules found in more than 20% of vascular plants (Bhambhani et al., 2021). Various reports have shown that alkaloids such as nicotine, α-tomatine, dehydrotomatine, α-solanine, and α-chaconine are induced in Solanaceous plants in response to different chewing insect pests (Steppuhn et al., 2004; Steppuhn and Baldwin, 2007).

Furthermore, phenolic compounds, including phenolic acids, chlorogenic acid, anthocyanins, polyphenols, lignins, coumarins, tannins, stilbens, and flavonoids, accumulate in plants against herbivore attack (Bernards and Båstrup-Spohr, 2008; Vogt, 2010). These metabolites have toxic effects on chewing and sucking herbivores (Bernards and Båstrup-Spohr, 2008). The induced levels of two phenolamides, *p*-coumaroylputrescine and feruloylputrescine, have been reported in *O. sativa* leaves treated with *Mythimna loreyi* Duponchel (Noctuidae) OS (Shinya et al., 2016). Moreover, winter cress (*Barbarea sp.*) plants produce a varied amount of saponin-aglycones viz. oleanolic-acid cellobioside, and hederagenin cellobioside, important in providing resistance to flea beetles (*Phyllotreta vittula* Redtenbacher; Chrysomelidae) (Kuzina et al., 2009). Brassicaceae plants accumulate 3-O- [O- β-D-glucopyranosyl-(1-4)-β-D-glucopyranosyl-hederagenin, a special saponin that is detrimental to *P. xylostella* (Agerbirk et al., 2003). In response to tissue damage, Brassicaceae family members release volatile isothiocyanates as a result of enzymatic degradation of glucosinolates. Species-specific glucosinolate composition will give different volatile profiles, but as with green leafy volatiles, this is the result of mixing a substrate with an enzyme rather than an active induced release (Singh, 2017; Sun et al., 2020). In-line with this approach benzoxazinoids are indole alkaloids found in most of Poaceae family members that are toxic to many chewing herbivore insects

(Dafoe et al., 2011; Maag et al., 2016; Niculaes et al., 2018; Tzin et al., 2017; Wouters et al., 2016). The specialized metabolites appear to be specific to feeding guilds (**Table 1.4**). These specialized metabolites and their biosynthetic pathway enzymes/genes can be considered potential markers viz. alkaloids and phenolics from Solanaceae, cyanogenic glucosides and glucosinolates from *Bracecaceae,* and benzoxazinoids from *Poaceae* during plant-insect interactions. Overall, the studies suggest that early reflection in transcriptional reprogramming of specialized metabolite biosynthesis is necessary for the plant to defend against herbivores. Hence, monitoring the phytohormone and specialized metabolite biosynthesis and signaling can be used as markers (at the level of gene expression, proteins/activities, and metabolites) for obtaining insights into plant-insect interaction studies by designing various mimicry experiments to unravel the complexities.

Table 1.4: Specialized metabolites from different plants identified against herbivore insect pests

Host Plant	Insect Pest	Secondary Metabolite	References
	Sucking insects	**Only against sucking insects**	
Wild tomato (*Solanum habrochaite*)	Silver leaf whitefly (*Bemicia tabaci*), Spider mites (*Tetranychus urticae*)	7-epizingiberene, R-curcumene	(Bleeker et al., 2012, 2011)
Arabidopsis (*Arabidopsis thaliana*)	Asian citrus psyllid (*Diaphorina citri*)	(E)-β-caryophylene	(Alquézar et al., 2017)
Rice (*Oryza sativa*)	Brown plant hopper (*Nilaparvata lugens*)	2-heptanone, 2-heptanol, (+)-limonene, (*E*)-linalool oxide, Linalool, α- curcumene	(Ye et al., 2020))
Cucumber (*Cucumis sativus*)	Spider mites (*T. urticae*)	Cucurbitacin-C	(Balkema-Boomstra et al., 2003)
Arabidopsis (*A. thaliana*)	Green peach aphid (*Myzus persicae*)	Indole- Glucosinolates	(Barth and Jander, 2006; Kim and Jander, 2007)
Pepper (*Capsicum annuum*)	Two-spotted spider mite (*T. urticae*)	Flavonoid-*O*-glucoside, Linalool, (*E*)-ß-ocimene	(Zhang et al., 2020)
	Chewing insects	**Only against chewing insects**	
Maize (*Zea mays*)	Fall armyworm (*Spodoptera frugiperda*), African cotton leafworm (*S. littoralis*)	2-ß-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one	(Glauser et al., 2011)

Cassava (*Manihot esculenta*)	Cassava burrower bug (*Cyrtomenus bergi*)	Cyanogenic glucosides	(Bellotti and Arias V, 1993)
Bitter almond (*Prunus dulcis*)	Flat headed woodborer (*Capnodis tenebronis*)	Amygdalin, Prunasin	(Garrido Vivas and Malagón, 1990)
Arabidopsis (*A. thaliana*)	Flea beetle (*Phyllotreta nemorum*)	Dhurrin	(Tattersall et al., 2001)
Poplar (*Populus tremula*)	Gypsy moth (*Lymantria dispar*)	2-methylbutyronitrile, 3-methylbutyronitrile	(Irmisch et al., 2014)
Neem (*Azadirachta indica*)	Cotton bollworm (*Helicoverpa armigera*)	Azadirachtin-A	(Dawkar et al., 2019)
Norway spruce (*Picea abies*)	European spruce bark beetle (Ips typographus)	D-lemonene, eucalyptol	(Schiebe et al., 2012)
Norway spruce (*P. sitechensis*)	White-pine weevil (*Pissodes strobe*)	Dehydroabictic acid	(Robert et al., 2010)
Wild tobacco (*Nicotiana attenuata*)	Tobacco hornworm (*Manduca sexta*)	17-hydroxygeranyllinalool glucoside	(Heiling et al., 2010)
White mustard (*Sinapis alba*)	Flea beetle (*P. cruciferae*)	Sinalbin	(Bodnaryk, 1991)
Arabidopsis (*A. thaliana*)	(S) Cabbage large butterfly (*P. brassicae*)	Kaempferol-3, 7-dirhamnoside	(Onkokesung et al., 2014)
Citrus (*Citrus maxima*)	Leafcutter ant (*Atta cephalotes*)	Limonene	(Cherrett, 1972)
Winter cress (*Barbarea vulgaris*)	Flea beetle (*P. nemorum*)	Hederagenin cellobioside, Oleanolic acid cellobioside	(Kuzina et al., 2009)
Winter cress (*B. vulgaris*)	Diamond black moth (*Plutella xylostella*)	3-O-[O- β-D-glucopyranosyl-(1-4)-β-Dglucopyranosyl]-hederagenin	(Agerbirk et al., 2003; Shinoda et al., 2002)

Wild tobacco (*N. attenuata*)	Spotted cucumber beetle (*Diabrotica undecimpunctata*), Beet armyworm (*S. exigua*), Pallid-winged grasshopper (*Trimerotropis* spp.)	Nicotine	(Roda et al., 2004; Steppuhn and Baldwin, 2007)
Potato (*S. tuberosum*), Wild potato (*S. chacoense*)	Guatemalan potato moth (*Tecia solanivora*), Colorado potato beetle (*Leptinotarsa decemlineata*)	α-solanine, α-chaconine	(Karlsson et al., 2013) (Sinden et al., 1986)
Pepper (*C. annuum*)	Oriental leafworm (*S. litura*)	Rutin, Vanillic acid Sinapic acid, Syringic acid	(Movva and Pathipati, 2017)
Tomato (*S. lycopersicum*)	Tobacco hornworm (*M. sexta*), Stinkbugs (*Podisusm aculiventris*), Soybean looper moth (*Pseudoplusia includens*), Fall armyworm (*S. frugiperda*), Cabbage looper (*Trichoplusia ni*), Corn earworm (*Heliothis zea*), Beet armyworm (*S. exigua*), Tobacco budworm (*H. virescens*)	Chlorogenic acid, Rutin, Tomatine	(Bloem et al., 1989; Stamp and Osier, 1998; Traugott and Stamp, 1997)
	Sucking and chewing insects	**Against both sucking and chewing insects**	

Rice (*O. sativa*)	(C) Lawn armyworm (*S. mauritia*), (C) Rice skipper (*Parnara guttata*), (S) Brown plant hopper (*N. lugens*)	p-coumaroylputrescine, Feruloylputrescine	(Alamgir et al., 2016)
Nightshade potato (*S. demissum*)	(C) Colorado beetle (*L. decemlineata*), (S) Potato leafhopper (*Empoasca fabae*)	Demissine	(Harborne JB, 1988)
Maize (*Z. mays*)	(C) First-brood european corn borer (*Ostrinia nubilalis*), (S) Maize plant louse (*Rhophalosiphum maydis*)	Dihydroxy-7-methoxy1,4-benzoxazin-3-one-glucoside	(Niemeyer, 1988)
Oilseed rape (*Brassica napus*)	(C) Field slug (*Deroceras reticulatum*)	Glucosinolates	(Glen et al., 1990)
Cabbage (*B. oleracea*)	(S) Cabbage medium butterfly (*Pieris rapae*)	Glucosinolates	(Agrawal and Kurashige, 2003)
Watercress (*Nasturtium officinale*)	(C) Amphipod (*Gammarus pseudolimnaeus*), (C) Physid snail (*Physella* sp.), (S) Limnephilid caddisflies (*Hesperphylax designates*) (S) *Limnephilus* sp.	Glucosinolates	(Newman et al., 1992)
Wild potato (*S. berthaultii*)	(S) Green Peach aphid (*M. persicae*)	O-acylsugars	(Neal et al., 1990)
Wild tobacco (*N. attenuata*)	(C) Tobacco hornworm (*M. sexta*)	O-acylsugars	(Luu et al., 2017)

Wild tomato (*S. galapagense, S. cheesmaniae, S. pimpinellifolium and S. pennellii*)	(S) Silver leaf whitefly (*B. tabaci*), (C) Tomato leaf miner (*Tuta absoluta, T. urticae*) (S) Thrips species	O-acylsugars	(Alba et al., 2009; Leckie et al., 2012; Lucini et al., 2015; Rakha et al., 2017; Vilela De Resende et al., 2006)

Statement of the problem:

Insect OSs includes regurgitant and saliva with distinct origins and compositions. Regurgitant's arise from the foregut and midgut while saliva is released from the labial gland through the spinneret (Eichenseer et al., 1999; Peiffer and Felton, 2005a). *Helicoverpa armigera* (Hübner) is a polyphagous insect, feeding on a wide range of host plants. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant range but also specific tissues of the host plant. Few reports suggest that the synthesis and function of OS components might depend on the host plant and associated microorganisms. Diet is crucial factor for the development and reproduction of herbivorous insects. However, despite decades of research on plant-insect interaction, a focused study on diet influencing the interaction between plant and insect is mostly elusive. Further, identification and functional characterization of herbivorous salivary proteins as effectors/elicitors will enrich our knowledge about plant-insect interaction.

Objectives:

1. To understand the influence of *H. armigera* oral secretion on modulation of tomato plant defense
    - Feeding of *H. armigera* on artificial diet, tomato (Host) and Capsicum (non-host) leaves for obtaining oral secretion (OS)
    - Effect of *H. armigera* OS on jasmonic acid and salicylic acid biosynthesis in tomato plants
    - Metabolic profiling of tomato plants in response to OS from *H. armigera* fed on different diet
    - Effect of individual metabolite on *H. armigera* growth

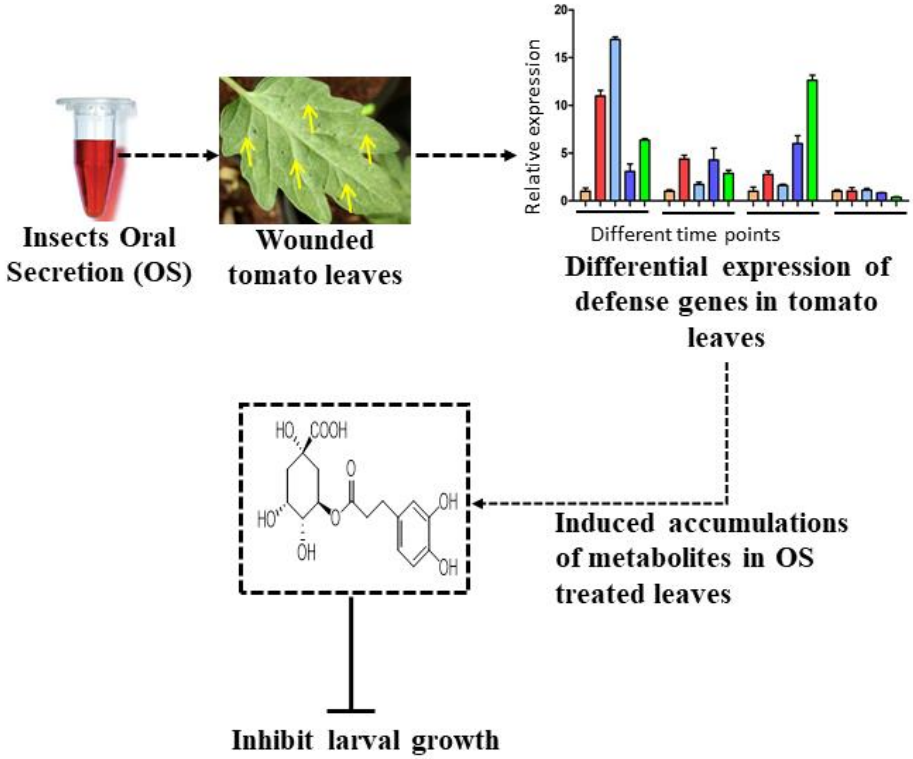
2. To understand impact of diet on the synthesis and secretion of bioactive molecule in *H. armigera* oral secretion
    - Metabolic profiling of OS from *H. armigera* fed on different diet
    - Identification of FACs and phytohormone in *H. armigera* OS
    - Proteomic analysis of *H. armigera* OS

3. Identification and functional characterization of effector like proteins from *H. armigera*
    - Identification of salivary secretory proteins from transcriptome and proteomic data of *H. armigera*

- *In-silico* analysis of *H. armigera* secretory proteins
- Cloning, recombinant expression and purification of *H. armigera* secretory proteins
- Understanding the tomato plant defense response to recombinantly purified secretory proteins of *H. armigera*.

Chapter 2

Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense



Contents of Chapter 2 have been published as research article…

G.S. Kallure, A. Kumari, B.A. Shinde, V. T. Barvkar, A.P. Giri, Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense, **Plant Science**, 314 (2022) 111120.

2.1 Introduction:

Herbivorous insects interact with every plant distinctly and have been categorized as generalists or specialists based on their degree of dietary specialization (Ehrlich and Raven, 1964). Whereas, generalist feeds on several plant species from different families, and specialist feeds on one or related plant species of the same family. Chemical cues from insect oral secretions (OS), which include regurgitant and digestive fluids were perceived by plants to induce defense mechanisms (Acevedo et al., 2015). Further, OS constituents might interact with biomolecules of the plant (Peiffer and Felton, 2005).

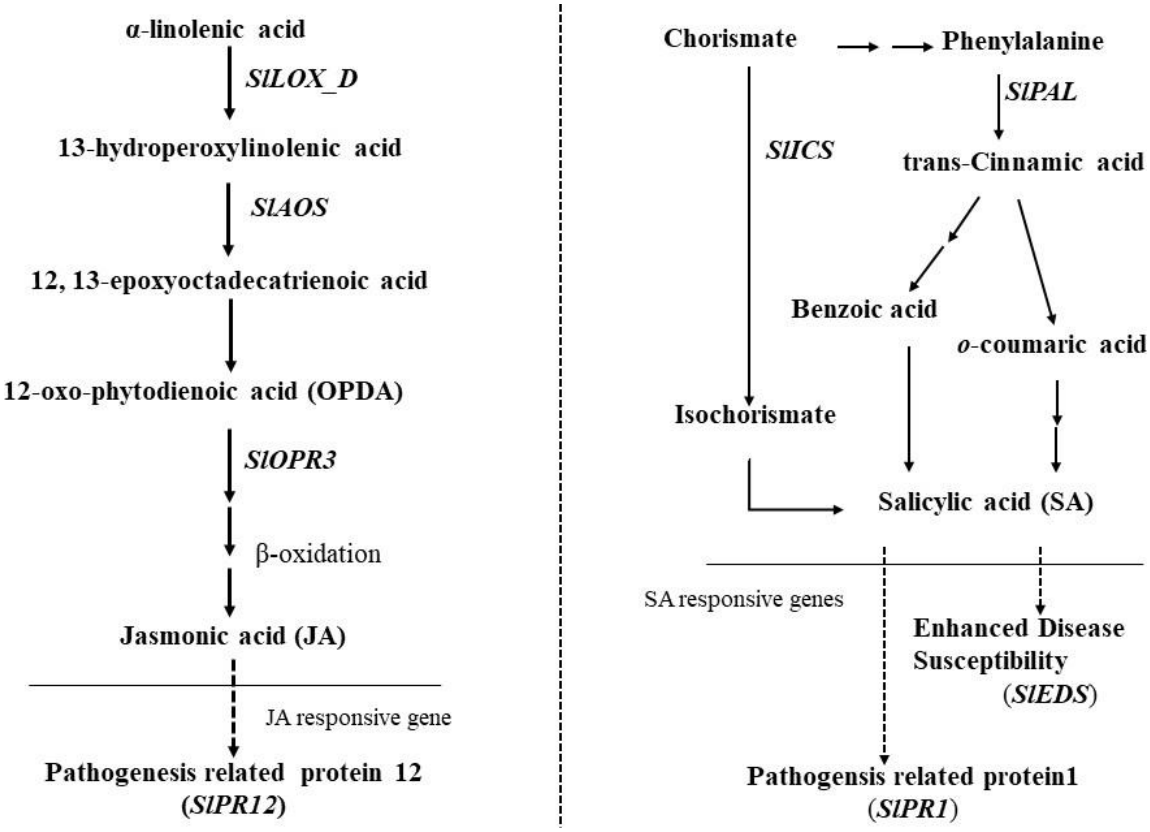


Figure 2.1: Schematic of Jasmonic acid (JA) and Salicylic acid (SA) biosynthetic pathway in plants

Several insects contain highly specialized active molecules in their OS. For example, glucose oxidase (GOX), fatty acid amino acid conjugates (FACs), *Helicoverpa armigera* R-like protein 1 (HARP1), and inceptins are major constituents of insect OS that are well characterized (Chen et al., 2019; Musser et al., 2002; Paré et al., 1998; Schmelz et al., 2006). These active molecules are recognized by plants and trigger (as an elicitor) or suppress (as an effector) plant defense (Louis et al., 2013; Musser et al., 2002). The biosynthesis and signaling of phytohormones is

the first step in the activation of plant defense against herbivore attack and mechanical damage. The series of reactions are involved in the biosynthesis of two major plant phytohormones Jasmonic acid (JA) and Salicylic acid (SA) (**Fig. 2.1**). Wounding alone upregulates jasmonic acid pathway significantly (Acosta and Farmer, 2010; Koo et al., 2009; G. Li et al., 2019; Reymond et al., 2000), whereas the application of insect OS fine-tunes the defense responses with a high degree of specificity to the individual insect attack (Diezel et al.,2009; Giri et al., 2006; Mishra et al., 2015; Schmelz et al., 2006). Apart from plant defensive proteins, plants produce numerous specialized metabolites upon insect attack as chemical defense mechanism *viz,* alkaloids, terpenoids and phenolic compounds (Bernards andBåstrup-Spohr, 2008; Gajger and Dar, 2021; Handrick et al., 2016; Vogt, 2010). Further, infestation by *Manduca sexta* and *H. zea* on tomato plant exhibited differential metabolic profiling. For example, amino acids and phenolics are induced mainly by *H. zea,* whereas the level of nitrogen and carbon transporters are altered by *M. sexta* (Steinbrenner et al., 2011). These plant specialized metabolites often modulate interactions between plants and insects, which can substantially affect herbivores and their fitness. Further, these metabolites are signature cues of any host plant providing evidence about the identity, nutritional value, and physiological status to the feeding insect. Moreover, the altered defense in rice against OS of *Mythimna loreyi* and *Parnara guttata* suggested that the presence of various elicitors in insectOS that are involved in the regulation of plant defense signaling (Shinya et al., 2016). However,studies addressing how different plant-based diets influence herbivores OS and their impact onthe plant defense responses are still limited (Acevedo et al., 2015; Chen and Mao, 2020;Felton et al., 2014; Schmelz, 2015). It is known that after herbivore attacks, plants accumulate chlorogenic acid (CGA) through the phenylpropanoid pathway (Kundu et al., 2018). Phenylalanine is the starting point for the biosynthesis of chlorogenic acid, which is followed by a number of enzyme processes (**Fig. 2.2**).

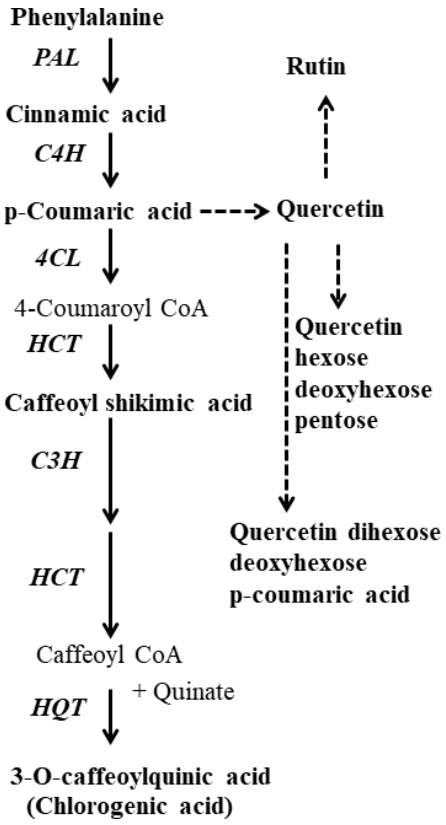


Figure 2.2: Schematic chlorogenic acid biosynthetic pathway in tomato plants

Helicoverpa armigera (Hübner) is a polyphagous insect, feeds on a wide range of host plants. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant range but also specific tissues of the host plant. We quantified the expression of several defense marker genes on mechanically wounded tomato leaves treated with OS_H (*H. armigera* fed on host-tomato), OS_{NH} (*H. armigera* fed on non-host-*Capsicum annuum*), leaves and OS_{AD} (*H. armigera* fed on artificial diet), which are known to mimic insect infestation (Giri et al., 2006; Shinya et al., 2016). Further, we performed both non-targeted and targeted metabolite analyses of OS-treated tomato leaves to correlate the phytohormone (JA) and salicylic acid (SA) responses with defense metabolites and, subsequently, the effect of the selected metabolite(s) on larval growth was studied. Analysis of differentially accumulated plant metabolites was found to be correlatedwith known plant defense response. One of the candidate metabolites identified in this study, chlorogenic acid (CGA), reduced larval growth of *H. armigera*.

2.2 Materials and methods

2.2.1 Insect rearing, collection and preparation of OS

Eggs of *H. armigera* (Cotton bollworm) were obtained from the National Bureau of Agricultural Insect Resources (Bengaluru, India). The hatched 1st instar larvae (n=30) were reared on detached leaves of tomato (Host), *Capsicum annuum* (non-host) (Hybrid hot Pepper-Sitara Gold) plants, and on an artificial diet. The artificial diet consists of chickpea (common host of *H. armigera*) seed powder and other components such as yeast extract, casein hydrolysate, sorbic acid, choline chloride, and vitamins. The detailed composition of the artificial diet is as described earlier (Nagarkatti and Prakash, 1974). Insects were reared under the controlled conditions at 25 °C, 70% relative humidity, and a 14 h light/10 h dark cycle.

The fourth instar larvae were taken for OS collection. The mouthpart of larvae was gently squeezed by hand, and the OS was collected using pipette in a 2 ml Eppendorftube (Chen et al., 2019). Collected OS was centrifuged at 15000 rpm for 10 min at 4 °C. The supernatant was collected and stored at -80 °C for further use.

2.2.2 Treatment of H. armigera OS on tomato leaves and tissue collection

OS was collected from the host, non-host plant, and artificial diet fed *H. armigera* larvae, and diluted as 1:2 with phosphate buffer. One-month-old tomato plants were used for the study. The tomato leaves (fully expanded 3rd and 4th leaf from the main shoot-apex) were mechanically wounded with a pattern wheel and immediately 10 µl of diluted OS was applied. Two leaves each plant and such two plants were considered as one biological replicate and such three replicates were analyzed independently in the present study. Control (unwounded), wounded and OS treated leaves were collected at various time points and snap frozen in liquid nitrogen and kept at -80 °C for further experiments. The experiment was repeated two times. For gene expression analysis, samples were collected at different time point 2, 4, and 24 h after the wounding and OS treatment. For metabolite analysis, samples were collected at 4, 24, 48, and 72 h after OS treatment.

2.2.3 Gene expression analysis by qRT-PCR

Total RNA was isolated from tomato leaves by Spectrum Plant Total RNA kit (Sigma-Aldrich, USA). RNA obtained was treated with DNase I (Thermo Fisher Scientific, USA) and its quantity and quality was determined using NanoDrop 2000 (Thermo Fisher Scientific, USA). DNA-free RNA (1 µg) was used for cDNA synthesis using RevertAid First Strand cDNA Synthesis kit (Thermo scientific, USA).

Gene-specific primers **(Table 2.1)** were designed using GeneRunner software (http://www.generunner.net/) and sequences were obtained from the Sol genomic database. Quantitative real-time PCR (qRT-PCR) was carried out according to (Shinde et al., 2017) and *SlActin* (Solyc11g005330) was used as housekeeping gene. PCR product specificity was confirmed by melting curve analysis. The generated threshold cycle (Ct) was used to calculate the gene expression of treated samples against control samples in terms of fold change (Schmittgen and Livak, 2008).

2.2.4 Liquid Chromatography- Quadrupole Time of Flight- Mass Spectrometry (LC - QTOF-MS) based targeted metabolite profiling

Collected snap frozen leaf samples were grounded to fine powder and weighed prior to extraction. Metabolites from 100 mg of fine-grounded leaf powder were resuspended with 200 µL of extraction solvent (1:2 w/v, tissue; 80% methanol + 0.1% formic acid). The extraction was carried out as per Itkin et al., 2011 (Itkin et al., 2011). The LC-QTOF-MS analysis was performed on Agilent 6530 Q-TOF (Agilent, USA) mass spectrometer connected to HPLC Prime Infinity II1260 system (800 bar). The MS/MS fragmentation data was acquired at 10, 20 and 40 eV collision energy. In the case of targeted metabolite analysis, the peak area of metabolites was determined using Agilent Mass Hunter Qualitative Navigator B.08.00, and Qualitative Workflow B.08.00 using the customized database created using Agilent personal compound database and library. The fold change of each metabolite was calculated by dividing mass feature peak area of sample values against control values. Individual metabolites were confirmed by comparing fragments generated by MS/MS data with standard compound or *in silico* fragments generated by Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) software (http://cfmid.wishartlab.com/) (Allen et al., 2015) and also according to reported experimental fragmentation pattern (Li et al., 2020).

2.2.5 Insect feeding assay with chlorogenic acid

Chlorogenic acid (CGA) (Sigma-Aldrich, USA) was mixed with the artificial diet at 50, 250, and 500-ppm concentrations and used to feed 1st instar *H. armigera* larvae. Each larva was pre-weighed, and those with equal mass were selected for the feeding (n=30). The larvae were kept at 28 °C, and the diet was changed once after two days. Larvae mass was taken at two days of interval, and final observation was done on the 6th day of feeding.

Based on gene function and their involvement in pathway fifteen genes were selected for qRT-PCR analysis

Sr. No.	Gene Name	Function
1	Actin	Actin as internal control (housekeeping gene)
2	Lipoxygenase	The first step in jasmonate biosynthesis is catalyzed by 13-lipoxygenases (LOXs)
3	Allene Oxide Synthase	The resulting 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOTE) is converted by allene oxide synthase (AOS)
4	12-oxophytodienoate reductase 3	OPDA reductase3 (OPR3) is the first enzyme of this pathway catalyzing the reduction of OPDA
5	Pathogenesis related protein 12	Jasmonic acid responsive gene
6	Phenylalanine ammonia-lyase	Phenylalanine ammonia-lyase (PAL) pathway to synthesize Salicylic acid (SA), First enzyme in phenylpropanoid pathway
7	Isochorismate synthase	Isochorismate synthase (ICS) pathway to synthesize SA
8	Enhanced disease susceptibility	SA signaling pathway genes
9	Pathogenesis related protein 1	SA signaling pathway genes
10	p-Coumarate 3-hydroxylase	Chlorogenic acid biosynthetic pathway gene
11	Cinnamic acid 4-hydroxylase	Chlorogenic acid biosynthetic pathway gene
12	Hydroxycinnamoyl Co A shikimate hydroxycinnamoyl transferase	Chlorogenic acid biosynthetic pathway gene

13	Hydroxycinnamoyl Co A quinate hydroxycinnamoyl transferase	Chlorogenic acid biosynthetic pathway gene
14	Trehalose-6-P Synthase	Trehalose biosynthetic pathway gene
15	Trehalose-6-phosphate phosphatase	Trehalose biosynthetic pathway gene

Table 2.1: Detail of primers used for tomato qRT PCR

Sr. No.	Gene Name	Sol ID	Gene Name	Primer Sequence (5'-3')
1	Actin	Solyc11g005330	*Sl*Actin_F	ATGACATGGAGAAGATCTGGCATCA
			*Sl*Actin_R	AGCCTGGATGGCAACATACATAGC
2	Lipoxygenase	Solyc01g099190	*Sl*LOX_D_F	ACTCATCAGCACCGACATCG
			*Sl*LOX_D_R	ACTCTCCAGAAAGAACTCCTGC
3	Allene Oxide Synthase	Solyc11g069800	*Sl*AOS_F	CCGGGACCATTCATCACTTC
			*Sl*AOS_R	GTGAGTTCAGTCGACGGCACG
4	12-oxophytodienoate reductase 3	Solyc07g007870	*Sl*OPR3_F	CAATAGATCATCTTGATGCCATG
			*Sl*OPR3_R	CATATGCTACGTATCGTGGCTG
5	Pathogenesis related protein 12	Solyc04g009590	*Sl*PR12_F	CACTTCACAAATGTCGATCCG
			*Sl*PR12_R	AGCCAAATCCAATGCAGTCTC
6	Phenylalanine ammonia-lyase	Solyco9g007900	*Sl*PAL_F	GGGAAATGGCTGCTGAATC
			*Sl*PAL_R	CACTTTGACCCCATTACTTTTG
7	Isochorismate synthase	Solyco6g071030	*Sl*ICS1_F	CAACATCTTTACGCTCGATTGAG
			*Sl*ICS1_R	CCTCGGTCAAACATTTCAGTTTC
8	Enhanced disease susceptibility	Solyc06g071280	*Sl*EDS1_F	TCGTCGGGTGGCGCTATAGC
			*Sl*EDS1_R	CAAAATGTAGGAAGTAACGAGCCC

9	Pathogenesis related protein 1	Soly01g106620	SlPR1_F	GGGATAGTGGAAGGAAGTG
			SlPR1_R	GATTTCGTAAGTGCCTCCT
10	p-Coumarate 3-hydroxylase	Solyc10g078240	*Sl*C3H_F	TTGGTGGCTACGACATTCCT AAGG
			*Sl*C3H_R	GGTCTGAACTCCAATGGGTT ATTCC
11	Cinnamic acid 4-hydroxylase	Solyc05g047530	*Sl*C4H_F	CCCAGTTTTTGGAAATTGGC TTCA
			*Sl*C4H_R	GCCCCATTCTAAGCAAGAGA ACAT C
12	Hydroxycinnamoyl Co A shikimate hydroxycinnamoyl transferase	Solyc06g074710	*Sl*HCT_F	TCTCCAACCCCTTTTAACGA ACC
			*Sl*HCT_R	CAACTTGTCCTTCTACCACA GGGAA
13	Hydroxycinnamoyl Co A quinate hydroxycinnamoyl transferase	Solycg07g005760	*Sl*HQT_F	CCCAATGGCTGGAAGATTAG CTA
			*Sl*HQT_R	CATGAATCACTTTCAGCCTC AACAA
14	Trehalose-6-P Synthase	Solyco3g007290	*Sl*TPS _F	GTGGAGGAGGAGGAGAAGA AGAGAAG
			*Sl*TPS_R	ATCTAAAGCATATCTGTCCC ATTC
15	Trehalose-6-phosphate phosphatase	Solyc07g006500	*Sl*TPP_F	TCGAGGAGATGATGGATGTA GCA
			*Sl*TPP_R	GAGCTTTGCAACATCGCGTA CAGC

2.3 Results

2.3.1 Induced defense responses are differentially regulated in tomato leaves treated with OS_{AD}, OS_{H} and OS_{NH}

In the plant-insect interactions, a rapid hormonal perturbation forms a signaling network that controls the induced defense responses. A significant transcriptome reprogramming has been observed in plants upon insect attack. The chewing herbivore insects induce plant defense mainly by physical damage followed by molecular signals from the insect OS. Here, we mimicked herbivory feeding by wounding leaves of tomato plant with a pattern wheel and applied *H. armigera* larval OS (OS_H, OS_{NH}, or OS_{AD}, separately). To examine the modulation in phytohormonal signaling and their involvement in induced defense response, expression pattern of SA and JA biosynthetic and responsive genes were assessed. The expression of the *PHENYLALANINE AMMONIA-LYASE* (*SlPAL*), a SA biosynthesis gene and also key enzyme of phenylpropanoid pathway, was significantly upregulated (>2-fold) after 4 h upon W+OS_H and W+OS_{NH} treatments compared to wounded leaves of tomato plants. Further, the expression of *SlPAL* was continued to be significantly higher (>6-fold) till 24 h in response to the W+OS_H treatment (**Fig. 2.3**). In contrast, the expression of another initial gene involved in SA synthesis through iso-chorismate pathway, the *ISO-CHORISMATE SYNTHASE* (*ICS*) was upregulated after 4 h after wounding, however its expression was significantly reduced upon W+OS_H and W+OS_{NH} treatments (**Fig. 2.3**). Additionally, the significant upregulation of SA responsive marker gene *PATHOGENESIS-RELATED PROTEIN 1 (SlPR1)* (>6-fold) after 4 h of treatment with W+OS_H and W+OS_{NH} compared to wounded leaves of tomato plants (**Fig. 2.3**). Although after 24 h the *SlPR1* expression was reduced in all treatments compared to the 4 h treatment, *SlPR1* expression have remained higher (>5-fold) after W+OS_{NH} treatment. The upregulation of another SA responsive gene, *ENHANCED DISEASE SUSCEPTIBILITY (SlEDS)*, was also higher (~10-fold) after 24 h of W+OS_{NH} treatment (**Fig. 2.3**) compared to other treatments (W, W+OS_{AD}, and W+OS_H). Thus, it is evident that SA biosynthetic and signaling pathway more active in W+OS_H and W+OS_{NH} treatments compared to wounded tomato plants through PAL pathway. Besides, we have also compared the impact of insect OS on the jasmonic acid (JA) pathway, one of the phytohormones involved in induction of plant defense against herbivores. JA biosynthetic genes, *LIPOXYGENASE* (*SlLOX_D*) and *ALLENE OXIDE SYNTHASE* (*SlAOS*) were upregulated after 4 h of the wounding (**Fig. 2.4**).

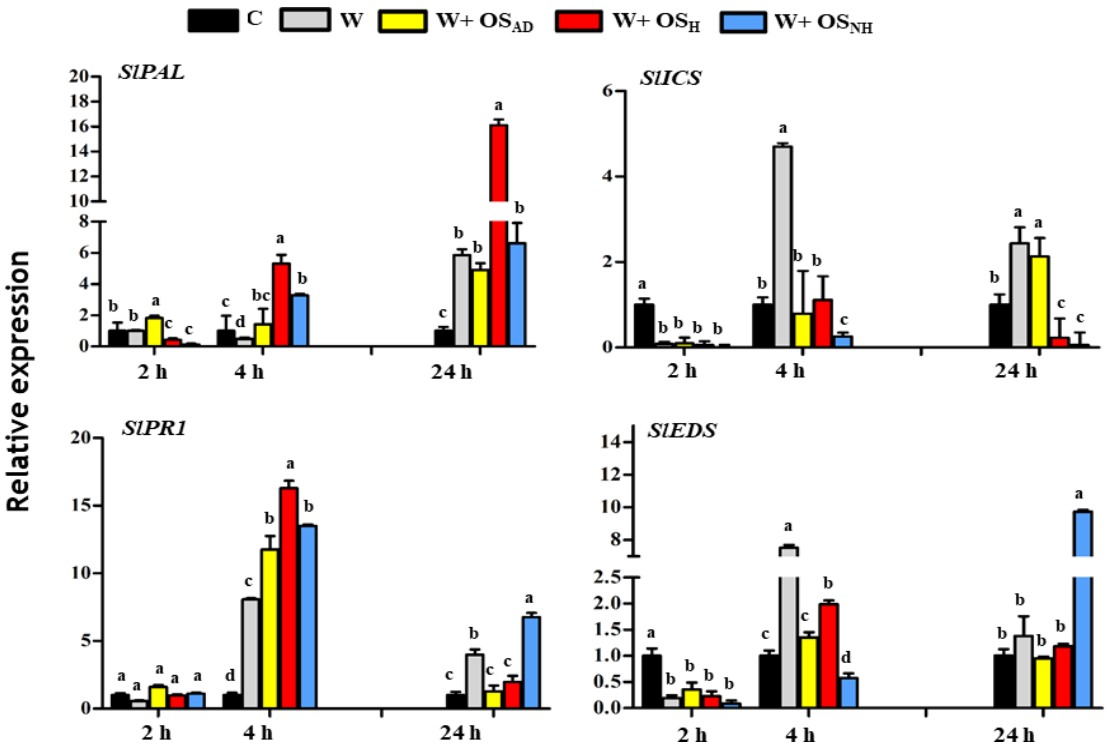


Figure 2.3: Expression profiles of salicylic acid biosynthetic pathway and their responsive genes in tomato. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). *SlPAL* (*PHENYLALANINE AMMONIA-LYASE*), *SlICS* (*ISOCHORISMATE SYNTHASE*), *SlPR1* (*PATHOGENESIS-RELATED PROTEIN 1) SlEDS* (*ENHANCED DISEASE SUSCEPTIBILITY)*, C- unwounded, W- wounded, $W+OS_{AD}$ - wounded and OS of *H. armigera* fed on artificial diet, $W+OS_H$ - wounded and OS of *H. armigera* fed on host (tomato), W+OSNH - wounded and OS of H. armigera fed on non-host (Capsicum) plant leaves. However, expressions of 12-oxophytodienoate reductase 3 (SlOPR3) gene, key enzyme of JA biosynthetic pathway exhibited lower expression upon W+OSH and W+OSNH treatment compared to only wounded leaves (Fig. 2.4). Also, decreased expression of JA responsive gene, PATHOGENESIS-RELATED PROTEIN 12 (SlPR12) with all treatments and time points except at 24 h in wounded plants (Fig. 2.4).

Overall, transcripts of JA biosynthetic and signaling pathway genes were higher in wounded leaves than the W+OSNH treated tomato plants. These findings highlight the critical role of the insect OS components in the interface of plant-insect interactions. Further, the altered transcriptional response in salicylic and jasmonic acid biosynthetic pathway and responsive genes in response to OS treatment might stimulate differential accumulation of the specialized metabolites and defense mechanism.

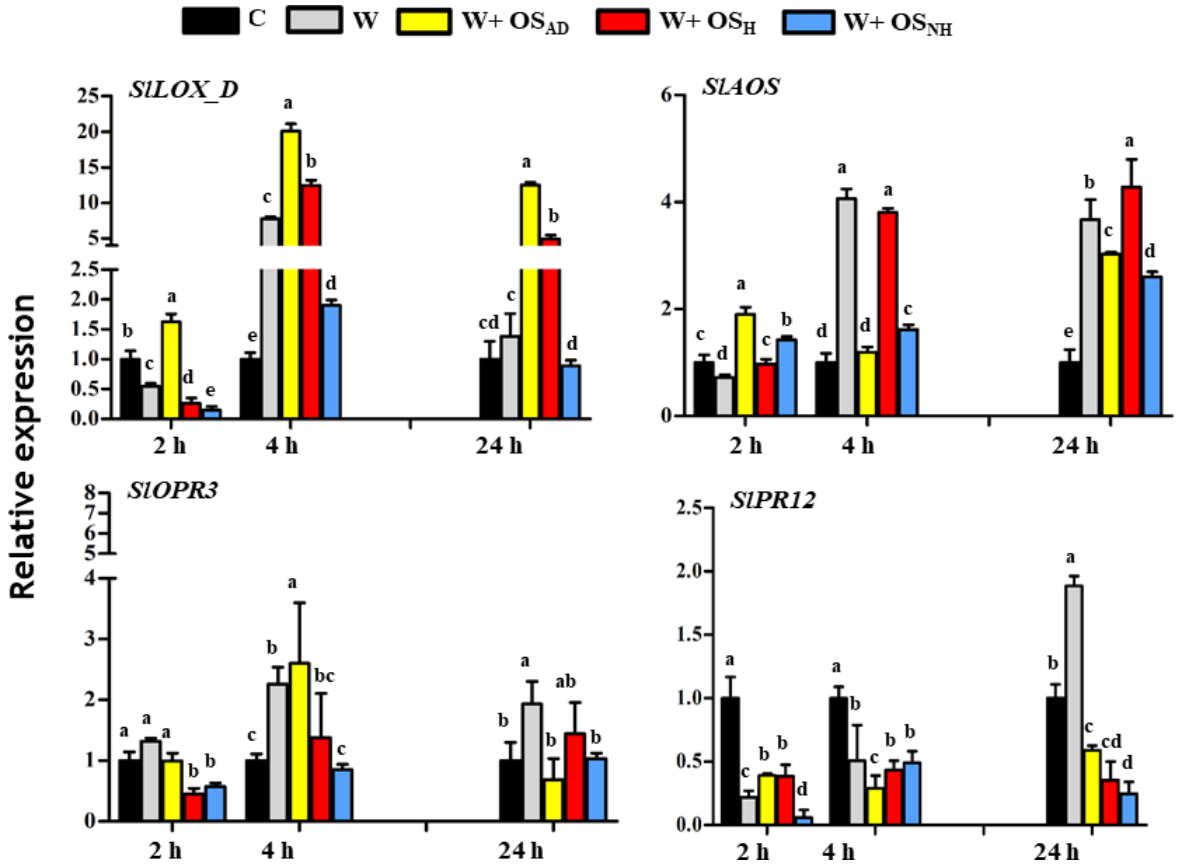


Figure 2.4: Expression profiles of jasmonic acid biosynthetic pathway and their responsive genes in tomato. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). *SlLOX_D*(*LIPOXYGENASE*), *SlAOS* (*ALLENE OXIDE SYNTHASE*), *SlOPR3* (*12-OXOPHYTODIENOATE REDUCTASE 3*), *SlPR12* (*PATHOGENESIS-RELATED PROTEIN 12)*, C- unwounded, W- wounded, W+OSAD - wounded and OS of H. armigera fed on artificial diet, W+OSH - wounded and OS of *H. armigera* fed on host (tomato), W+ OSNH - wounded and OS of *H. armigera* fed on non-host *(Capsicum)* plant leaves.

2.3.2 H. armigera OS modulate specialized metabolites accumulation in tomato leaves

We used targeted and non-targeted LC-QTOF-MS to analyse differentially accumulated metabolites in tomato leaves. Different OS of *H. armigera* larvae (OS_H, OS_{NH}, and OS_{AD}) were applied to the wounded leaves to examine a direct contribution of different OS in plant metabolites accumulation. Metabolic profiling of the treated tomato leaves identified 657 mass peaks, out of which 63 were highly abundant based on peak area. PCA of all identified mass peaks showed clear and separate clusters contributed by PC1 (41.7%) and PC2 (25.4%) at different time points and metabolites from OS_H and OS_{NH} treated plants were distinct and appeared together (**Fig. 2.5**).

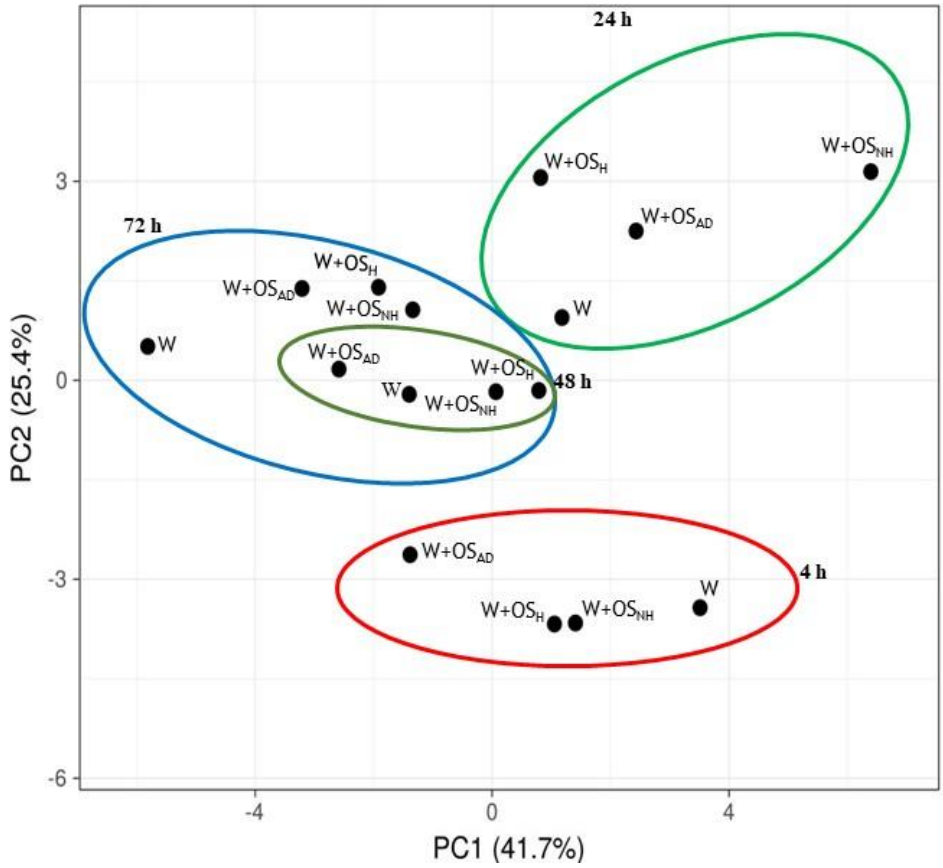


Figure 2.5: Principal component analysis of identified mass peaks from tomato leaves after wound and different *H. armigera* OS treatments with ESI (+) mode. PCA was generated with help of Clustvis web server.

Among them 20 metabolites were confirmed by LC-MS/MS analysis and those metabolites were selected if their induction by wounding and OS treatment (either OS_H, OS_{NH}, or OS_{AD}) was significantly changed (≥1.5-fold change; P <0.05) compared to unwounded tomato leaves (**Table 2.2**). Selected metabolites were differentially accumulated throughout all treated tomato leaves and belong to major groups like amino acids, alkaloids, and phenolics. Further analysis showed the accumulation of most of metabolite after 4 and 24 h of wounding as well as different OS treatments ($OS_{AD,}$ OS_H, and OS_{NH}) compared to unwounded leaves. After 4 h, hydroxyl-tomatine isomer 1 (1.7-fold; P<0.05), *p*-coumaric acid (1.6-fold; P<0.001), rutin (1.7- fold; P<0.05), quercetin-dihexose-deoxyhexose-p-coumaric acid (1.6-fold; P<0.001), quercetin-dihexose-deoxyhexose-pentose (1.9-fold; P<0.001), tyrosine (1.5-fold; P<0.001), and tyramine (2.2-fold; P<0.001) showed induced accumulation in wounded plants, while p- coumaric acid (1.7-fold; P<0.001), caffeoyl shikimic acid (1.5-fold; P<0.001), quercetin- dihexose-deoxyhexose-pentose (1.6-fold; P<0.001), tyrosine (1.6-fold; P<0.001) were higher in $W+OS_{NH}$ (**Fig. 2.6; Table 2.3**). Moreover, cholesterol (>1.5-fold; P<0.001) and hydroxyl-tomatine (>1.5-fold; P<0.05) were significantly upregulated after 24 h of wounding and different OS treatments. However, these metabolites decreased at subsequent time points. Further, hydroxyl-tomatine isomer 1 (2.2-fold; P<0.001) and acetoxy-tomatine isomer (1.7-fold; P<0.01) showed significantly higher accumulation in different OS treatments after 72 h (**Fig. 2.6; Table 2.3**). Among the amino acids; L-phenylalanine (2.2-fold; P<0.05), tryptophan (4.8-fold; P<0.001), tyrosine (1.8-fold; P<0.01) and tyramine (2.4-fold; P<0.01) were significantly higher upon $W+OS_{AD}$, $W+OS_H$, and $W+OS_{NH}$ treated tomato leaves at 24 h. Significantly higher accumulation of a sugar, trehalose was observed in wounded (4.4-fold; P<0.01) as well as upon OS treated leaves ($W+OS_H$: 4.7-fold; P<0.01, and $W+OS_{NH}$: 7.2-fold; P<0.001) (**Fig. 2.6; Table 2.3**). Furthermore, several metabolites of phenylpropanoid pathway were significantly upregulated after 24 h upon $W+OS_{NH}$ treatment like cinnamic acid (2.4-fold; P<0.05), *p*-coumaric acid (1.8-fold; P<0.01), caffeoyl shikimic acid (1.6-fold; P<0.001), 3-O-caffeoylquinic acid (chlorogenic acid (CGA); 1.6-fold; P<0.05), quercetin (1.5-fold; P<0.01), and quercetin-dihexose-deoxyhexose-*p*-coumaric acid (2.2-fold; P<0.001) (**Fig. 2.6; Table 2.3**). Furthermore, quercetin-dihexose-deoxyhexose-pentose (2.5-fold; P<0.001) at 24 h was significantly elevated in the $W+OS_{NH}$ treatment, while rutin (1.5-fold; P<0.05) at 72 h was highly accumulated by the $W+OS_H$ and $W+OS_{NH}$ treatment. This overall modulation in metabolites accumulation by different OS treatments could be the reason for induction of plant defense against herbivore.

Table 2.2: Details of MS/MS fragments of identified metabolites from tomato leaf tissue after different *H. armigera* OS treatments

Metabolite	Chemical Formula	m/z	Mass	Selected ion	Mass error (ppm)	RT (Min)	MS/MS Fragments
Cholesterol[R]	$C_{27}H_{46}O$	404.39	386.35	[M+NH4]+	4.33	15.89	57.06, 246.22, 360.36
Tomatine[S]	$C_{50}H_{83}NO_{21}$	1034.55	1033.54	[M+H]+	3.07	5.79	115.03, 145.04, 273.22, 416.35, 578.40, 1016.54
Dehydrotomatine[R]	$C_{50}H_{81}NO_{21}$	1032.54	1031.54	[M+H]+	1.38	5.59	145.05, 414.33, 576.39
Hydroxy-dehydrotomatine	$C_{50}H_{81}NO_{22}$	1065.55	1047.53	[M+NH4]+	4.77	5.67	-
Tomatidine[S]	$C_{27}H_{45}NO_2$	416.35	415.34	[M+H]+	3.09	6.06	126.12, 147.12, 161.13,173.13, 199.14 255.21, 398.34
Hydroxytomatine isomer 1	$C_{50}H_{83}NO_{22}$	1050.55	1049.54	[M+H]+	1.97	4.90	-
Acetoxy-tomatine isomer	$C_{52}H_{85}NO_{23}$	1092.56	1091.55	[M+H]+	4.01	5.62	-
L-Phenylalanine[R]	$C_9H_{11}NO_2$	166.08	165.07	[M+H]+	4.52	2.94	91.05, 103.05, 120.08
Cinnamic acid[R]	$C_9H_8O_2$	166.08	148.05	[M+NH4]+	5.0	3.42	103.05, 131.05, 149.06
p-Coumaric acid	$C_9H_8O_3$	182.08	164.04	[M+NH4]+	4.89	1.70	-
Caffeoyl shikimic acid	$C_{16}H_{16}O_8$	337.09	336.08	[M+H]+	1.63	4.46	-
3-O-caffeoylquinic acid[S]	$C_{16}H_{18}O_9$	355.10	354.10	[M+H]+	4.75	4.29	55.05, 63.02, 79.05, 117.03, 135.04, 145.02, 163.04
Quercetin	$C_{15}H_{10}O_7$	303.05	302.05	[M+H]+	3.66	4.76	-
Rutin[R]	$C_{27}H_{30}O_{16}$	611.16	610.15	[M+Na]+	4.79	4.76	85.02, 129.05, 147.06, 303.05, 465.10
Quercetin-hexose-deoxyhexose-pentose	$C_{32}H_{38}O_{20}$	743.20	742.19	[M+H]+	4.49	4.58	-

Quercetin-dihexose-deoxyhexose-p-coumaric acid	$C_{42}H_{46}O_{23}$	919.25	918.25	[M+H]+	1.99	5.17	-
Tryptophan[R]	$C_{11}H_{12}N_2O_2$	205.09	204.08	[M+H]+	-1.23	4.16	74.02, 105.07, 132.08, 170.06, 188.07
Tyrosine	$C_9H_{11}NO_3$	182.08	181.07	[M+H]+	-0.84	1.92	-
Tyramine	$C_8H_{11}NO$	138.09	137.08	[M+H]+	3.39	1.72	-
Trehalose[S]	$C_{12}H_{22}O_{11}$	365.11	342.11	[M+Na]+	3.07	1.62	279.15, 335.18

RT: Retention time
Fragmentation pattern matched to Stanadrad (S) or Reference (R) from *insilico* fragments generated by CFM ID software (http:/ /cfmid.wishartlab.com/) (Allen et al., 2014).

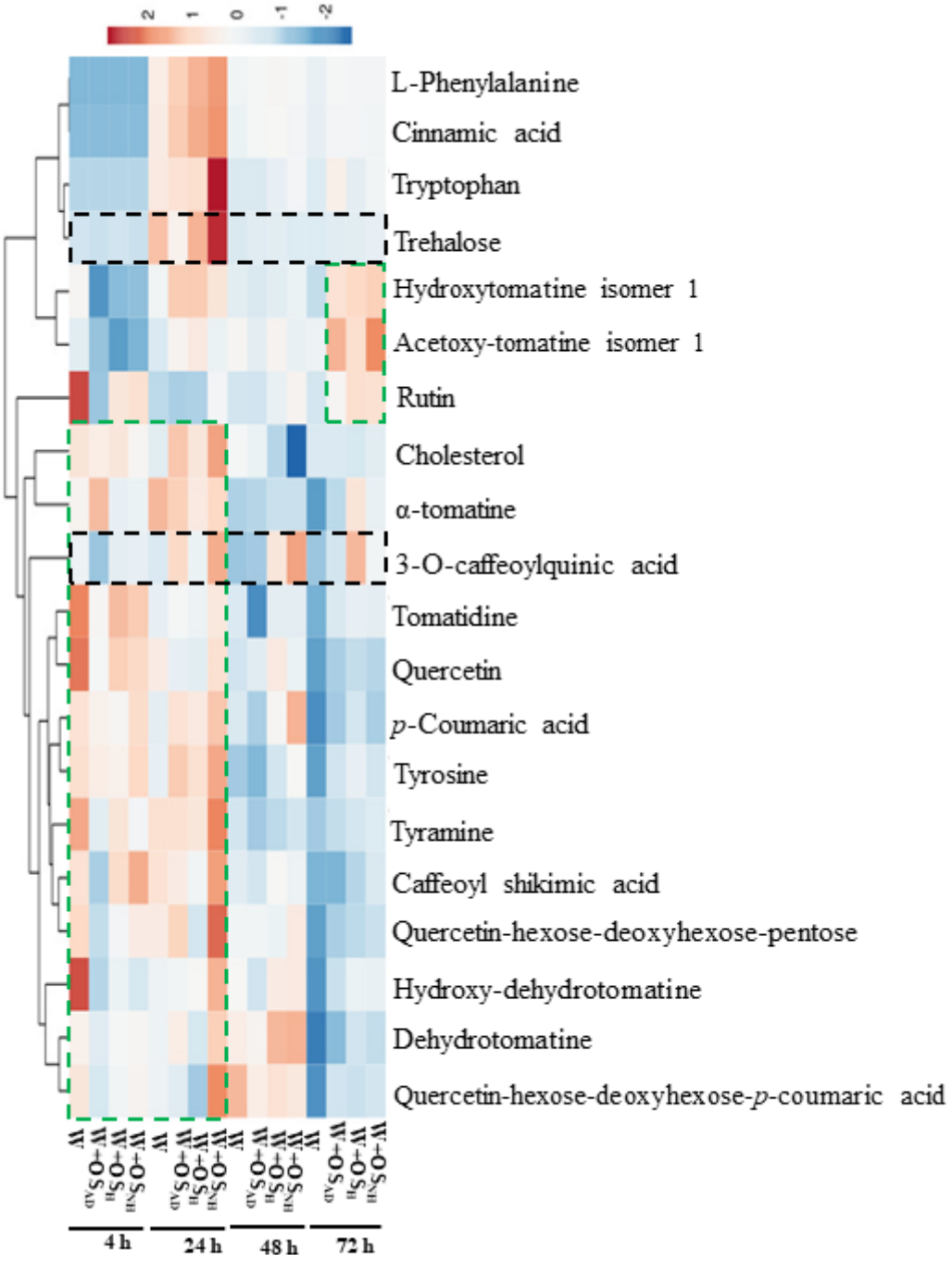


Figure 2.6: Identified metabolites from tomato upon application of oral secretions on wounded leaves. Heatmap of identified metabolites in tomato leaves after wound and different *H. armigera* OS treatments. Fold change was calculated by dividing mass peak area of treated samples against unwounded samples**.**

Table 2.3: **Details of differentially accumulated metabolites (Fold Change) from tomato leaf tissue at 4, 24, 48, and 72 h after insect different OS treatments**

Metabolites	4 h				24 h				48 h				72 h			
	W	W+ OS_{AD}	W+ OS_{H}	W+ OS_{NH}	W	W+ OS_{AD}	W+ OS_{H}	W+ OS_{NH}	W	W+ OS_{AD}	W+ OS_{H}	W+ OS_{NH}	W	W+ OS_{AD}	W+ OS_{H}	W+ OS_{NH}
Cholesterol	1.32	1.22	1.31	1.16	0.95	1.52*	1.30	1.67*	1.11	1.03	0.71	0.14	0.89	0.89	0.85	0.95
Dehydrotomatine	1.08	0.90	1.02	1.05	0.98	1.11	0.83	1.29	1.11	1.06	1.36	1.38	0.41	0.64	0.80	0.77
Hydroxy-dehydrotomatine	1.39	0.86	1.00	0.92	1.01	1.02	1.04	1.25	1.04	0.90	1.09	1.10	0.72	0.89	1.00	0.99
Tomatidine	1.47	1.12	1.35	1.32	1.03	1.09	1.06	1.19	0.97	0.66	1.02	1.02	0.76	0.96	1.03	1.01
α-Tomatine	1.08	1.26	1.00	1.02	1.27	1.22	1.12	1.20	0.86	0.87	0.90	0.90	0.74	0.88	1.15	1.01
Hydroxytomatine isomer 1	1.71*	0.64	0.91	0.92	1.60*	2.19*	2.19*	1.96*	1.44	1.32	1.43	1.55*	1.16	1.97*	2.11*	2.18*
Acetoxy-tomatine isomer	1.10	0.84	0.69	0.79	1.05	1.27	1.35	1.16	1.25	1.11	1.27	1.15	1.18	1.60*	1.44	1.73*
L-Phenylalanine	Nd	Nd	nd	nd	1.30	1.81*	2.05*	2.24*	1.00	1.09	1.14	1.08	0.89	1.07	1.06	1.04
Cinnamic acid	Nd	Nd	nd	nd	1.38	1.92*	2.18*	2.37*	0.93	1.10	1.14	1.08	0.88	1.07	1.06	1.04
***p*-Coumaric acid**	1.64*	1.45	1.41	1.71*	1.22	1.63*	1.54*	1.83*	1.09	0.88	1.41	1.93*	0.48	0.85	1.06	0.88
Caffeoyl shikimic acid	1.33	0.87	1.38	1.52*	1.34	1.26	1.14	1.56*	1.05	0.96	1.18	1.13	0.78	0.78	0.90	1.01
3-O-caffeoylquinic acid	1.09	0.75	1.03	1.05	0.96	1.39	1.08	1.55*	0.76	0.77	1.30	1.58*	0.75	0.90	1.52*	1.11
Quercetin	1.46	0.94	1.22	1.18	1.05	0.86	0.83	1.13	0.71	0.81	1.06	0.89	0.45	0.63	0.69	0.65

Rutin	1.72*	0.73	1.24	1.28	0.81	0.77	0.78	1.07	0.87	0.85	1.02	1.13	0.88	1.11	1.29	1.28
Quercetin-hexose-deoxyhexose-pentose	1.86*	0.75	1.28	1.58*	1.54*	1.89*	0.90	2.50*	1.27	1.26	1.21	1.56*	0.26	0.64	0.74	0.79
Quercetin-dihexose-deoxyhexose-p-coumaric acid	1.62*	0.98	1.30	1.43	1.27	1.10	0.73	2.23*	1.98*	1.51*	1.66*	1.60*	0.31	0.91	0.88	0.95
Tryptophan	nd	Nd	nd	nd	1.91*	2.22*	2.35*	4.86*	0.54	0.51	0.96	1.32	0.69	1.72	0.91	1.26
Tyrosine	1.51*	1.33	1.29	1.57*	1.07	1.64*	1.48	1.78*	0.69	0.60	0.88	1.23	0.47	0.86	1.06	0.87
Tyramine	2.19*	1.27	1.76*	1.43	1.81*	1.82*	1.72*	2.36*	1.07	0.87	0.96	1.07	0.86	0.99	1.09	1.20
Trehalose	nd	Nd	nd	nd	4.44*	2.46*	4.71*	7.22*	0.65	0.97	1.11	0.85	0.86	0.91	1.03	1.34

RT- Retention time; * Asterisk indicates ≥1.5 significant fold change; W- Wound only; W+OS_{AD} - Wound with oral secretion of artificial diet fed *H. armigera*;W+OS_{H} - Wound with oral secretion of host plant (tomato) leaf fed *H. armigera*; W+OS_{NH} - Wound with oral secretion of non-host plant (Capsicum) leaf fed *H. armigera*; nd-not detected

2.3.3 Induced expression of trehalose biosynthetic pathway genes in tomato upon application of H. armigera OS

Significantly higher accumulation of trehalose upon OS treated tomato leaves was noted compared to unwounded plants after 24 h (**Fig. 2.6**). This observation indicated that trehalose might have crucial role in wounding and OS based plant defense induction. The significant increase in the expression of two important trehalose biosynthetic pathway genes (**Fig. 2.7**), *TREHALOSE-6-PHOSPHATE SYNTHASE* 1 (*SlTPS1*) and *TREHALOSE 6-PHOSPHATE PHOSPHATASE* (*SlTPP*), in tomato leaves across wounding and different OS treatments was noted. Consistent with metabolite data, there was upregulation of *SlTPS1* and *SlTPP* (**Fig. 2.7**) transcripts level in all the treatments compared to the wounded tomato leaves. Interestingly, increased expression of *SlTPS1* (>30-fold) and *SlTPP* (>10-fold) after 24 h of W+OS_H and W+OS_{NH} treatment than wounded plants was apparent (**Fig. 2.7**). Elevated upregulation of trehalose biosynthetic genes and induced accumulation of trehalose after OS_H and OS_{NH} treatments underlines the significance of its role in plant defense responses.

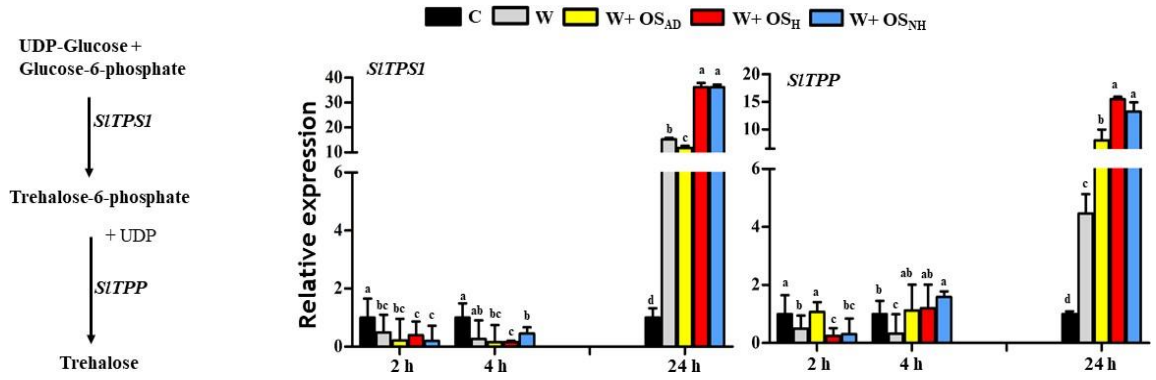


Figure 2.7: Schematic trehalose biosynthetic pathway and Expression profiles of trehalose biosynthetic pathway genes in tomato in response to OS treatments. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). *SlTPS1* (*TREHALOSE-6-PHOSPHATE SYNTHASE*), *SlTPP* (*TREHALOSE 6-PHOSPHATE PHOSPHATASE*); C- unwounded, W- wounded, W+OS_{AD} – wounded and OS of *H. armigera* fed on artificial diet, W+OS_H - wounded and OS of *H. armigera* fed on host(tomato) plant leaves, W+OS_{NH} - wounded and OS of *H. armigera* fed on non-host (Capsicum) plant leaves.

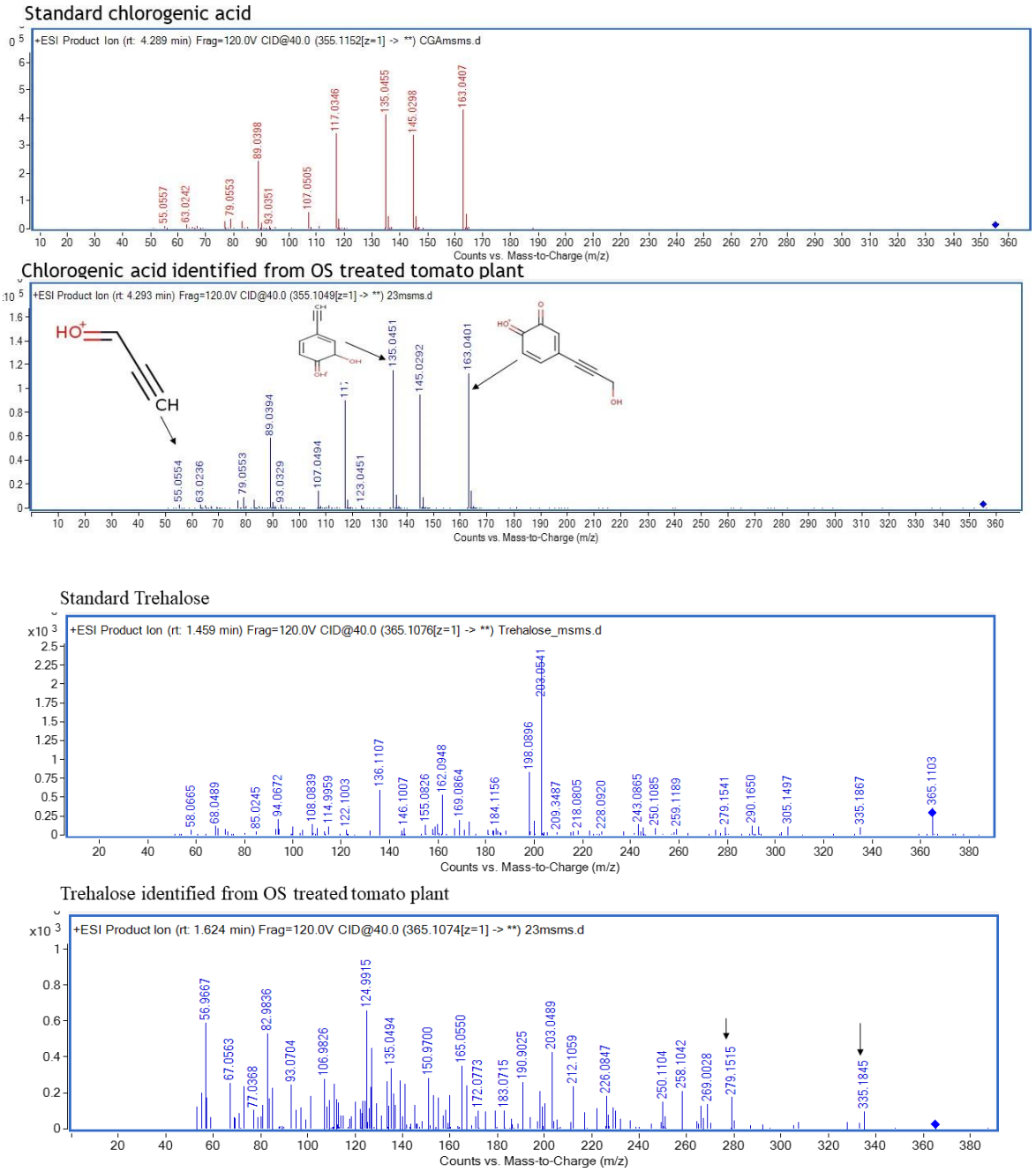


Figure 2.8: MS_MS confirmation of metabolites identified from the tomato leaves treated with *H. armigera* OS. The fragmentation pattern of identified metabolites were matched with the standard metabolites.

The accumulation of chlorogenic acid and trehalose in OS treated tomato leaves was confirmed by MS/MS fragmentation pattern with standard chlorogenic acid and trehalose (**Fig. 2.8**).

2.3.4 Modulation of chlorogenic acid biosynthetic pathway in tomato upon application of H. armigera OS

Among phenylpropanoids, chlorogenic acid (CGA) synthesis and accumulation is crucially important during plant-herbivore interaction (Kundu et al., 2018). Precursor as well as the intermediates of CGA biosynthesis pathway was significantly accumulated in the wounded tomato plants treated either with specifically OS_H or OS_{NH} and OS_{AD} compared to unwounded plants at 24 h. To evaluate the role of CGA biosynthetic genes in the accumulation of CGA and its intermediates, qRT-PCR of these genes was carried out. *CINNAMATE 4- HYDROXYLASE* (*SlC4H*), *HYDROXYCINNAMOYL-COA SHIKIMATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE* (*SlHCT*), P-COUMAROYL ESTER 3'- HYDROXYLASE (*SlC3H*), and *HYDROXYCINNAMOYL COA QUINATE HYDROXYCINNAMOYL TRANSFERASE* (*SlHQT*) were significantly induced after 24 h of across all the treatments (**Fig. 2.9**). The expression of *SlC4H* was significantly elevated at 4 h of $W+OS_{NH}$ (>10-fold) and $W+OS_H$ (>4-fold) treated plants than wounded tomato plants (**Fig.2.9**). Further, *SlC3H* was significantly up-regulated after 2 h (10-fold) in the $W+OS_{NH}$ treated plants compared to wounded tomato plants (**Fig. 2.9**). However, amongst the four genes, *SlHCT* and *SlHQT* are the major genes that encode for enzymes involved in final step of CGA biosynthesis. It was observed that after 24 h, the transcript level of both *SlHCT* (>15-fold) and*SlHQT* (>8-fold) were higher in response to $W+OS_H$ and $W+OS_{NH}$ treatment compared to justwounding or $W+OS_{AD}$ (**Fig. 2.9**). Higher accumulation of CGA and the expression of its biosynthesis genes in OS treated leaves compared to the wounded leaves, suggests a crucial involvement of CGA in the tomato plant defense against *H. armigera*.

2.3.5 Chlorogenic acid hinders the H. armigera larval growth

Metabolite and transcript profiling have revealed CGA as one of the significantly induced specialized defense metabolites in tomato. This led us to hypothesize that CGA could have an antibiosis effect against the generalist *H. armigera*. To examine the effect of CGA on larval growth, 1st instar *H. armigera* larvae were fed with different concentrations of CGA (50, 250, and 500 ppm) along with the artificial diet (control, without CGA). A significant reduction (about 30 to 40%) in the mass of larvae fed on artificial diet with CGA (250 and 500ppm) was recorded on 4th and 6th day (**Fig. 2.10**) compared to artificial diet without CGA. Also, dose-dependent growth inhibition of the larvae by CGA was noted from 2nd day and continued its effect till 6th day that could be one of the reasons for the retarded growth of *H. armigera* larvae.

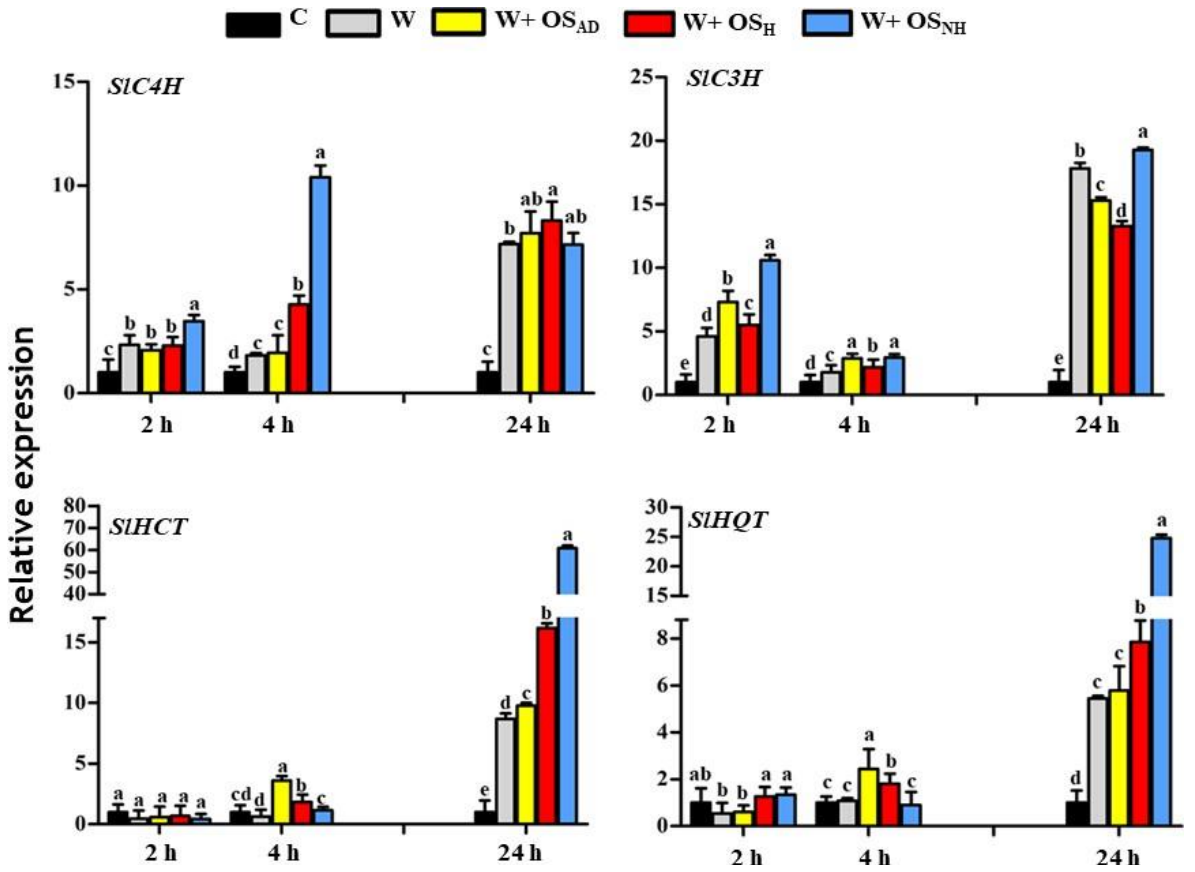


Figure 2.9: Influence of chlorogenic acid (CGA) biosynthetic pathway in tomato plant defense against *H. armigera* OS treatments. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control. Fold change was calculated by dividing sample values against control values. C4H- CINNAMATE-4-HYDROXYLASE, *SlC3H* (p-coumarate3-hydroxylase), *HCT- HYDROXYCINNAMOYL-COA:SHIKIMATEHYDROXYCINNAMOYLTRANSFERASE, HQT- HYDROXYCINNAMOYL COA QUINATE TRANSFERASE*. C- unwounded, W- wounded, W+OSAD - wounded and OS of *H. armigera* fed on artificial diet, W+OSH - wounded and OS of *H. armigera* fed on host (tomato) plant leaves, W+OSNH -wounded and OS of *H. armigera* fed on non-host (Capsicum) plant leaves.

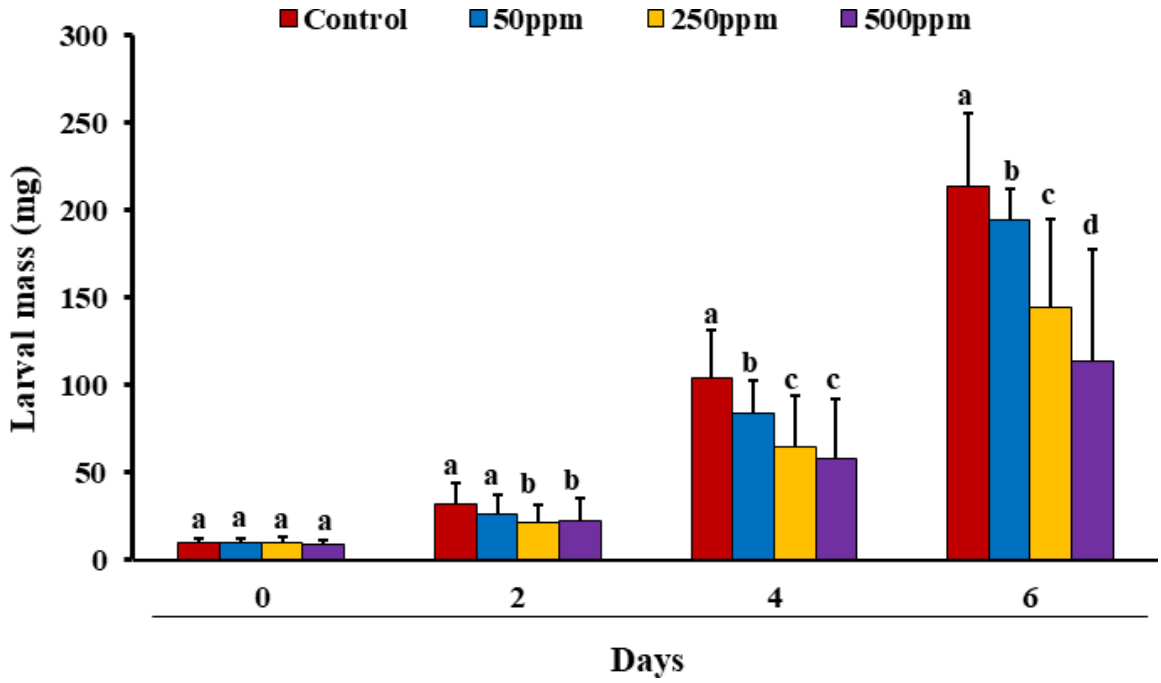


Figure 2.10: Impact of chlorogenic acid (CGA) on *H. armigera* growth. Dose-dependent effect of CGA on *H. armigera* larval growth. Each *H. armigera* 1st instar larva was pre-weighed, and those with equal mass were selected for the feeding (n=30). Larval mass was recorded after feeding oncontrol diet (artificial diet without CGA) and CGA added diet (50, 250, and 500 ppm- part per million)at various time intervals. Data shown are mean +/- SD. Bars represent the standard deviation of the means. Different letters (a, b, c, d) indicate significant ($P < 0.05$) difference.

2.4 Discussion:

During the plant-herbivore interaction and co-evolution process, insects have preferred to feed on some plant species and avoided others (Ehrlich and Raven, 1964). Undoubtedly, there may be several reasons for their preferences, such as accessibility of food, plants' nutritional value, and plants defense responses (Li et al., 2020). Considering the limited available resources, plants prioritize many metabolic pathways that will help them to defend against insect attacks. Signaling of plant defense-related pathways described to date are regulated by phytohormones (Erb et al., 2012). Based on several studies, it has also been suggested that generalist herbivores, such as *Spodoptera exigua* and *S. littoralis*, may enhance their fitness by activatingthe SA pathway to weaken JA-mediated resistance (Diezel et al., 2009; Erb et al., 2012). Insect-specific elicitors from the insect OS or oviposition fluids are often responsible for modulatingthe plant defense responses. The previous report suggests that feeding by tobacco hornworm (*Manduca sexta*) larvae elicits prominent ethylene (ET) and JA bursts (Halitschke and Baldwin,2004; Von

Dahl and Baldwin, 2007; War et al., 2012). As reported, JA biosynthetic genes, *SlLOX_D* and *SlAOS*, were significantly upregulated at 4 h after wounding and W+OS_H treatment to the tomato leaves. In comparison, *SlLOX_D* and *SlAOS* upregulation was lower when wounded leaves were treated with OS_{NH}. LOX_D is involved in the oxidation of linolenic acid, which leads to activation of defense through JA biosynthesis. Induced lipoxygenase activity has been found in tomato plants treated with *Spodoptera exigua* OS (Zebelo et al., 2014). Recently, Chen (Chen et al., 2019) have shown that OS of *Helicoverpa armigera* differentially modulates the transcript level of LOX_D in Arabidopsis plants. Similarly, we also observed the altered transcript level of LOX_D in all treatments. The expression of JA responsive gene pathogenesis related protein 12 was found to be attenuated in OS_H and OS_{NH} treated tomato plants. Salivary components of *H. zea* prevent nicotine induction in *Nicotiana tabacum* by directly inhibiting the wound signaling molecule JA and/or antagonizing its interaction with other (i.e. SA) signaling pathways (Musser et al., 2002). The suppression of JA responsive or signaling gene was also evident in SA upregulated *Arabidopsis* plant (Leon-Reyes et al., 2010), suggesting induced synthesis and signaling of SA could act as an antagonistto JA signaling. It is known that SA plays a central role in defense against biotrophic pathogenas well as herbivore *Eurydema oleracea* and acts as an antagonist of JA-mediated defense responses (Costarelli et al., 2020; Djamei et al., 2011). Interestingly, SA biosynthetic pathway(*SlPAL*) and responsive (*SlPR1* and *EDS*) genes expression was upregulated in OS_H and OS_{NH}compared to the wounding alone. Whereas *SlICS* gene, involved in SA biosynthesis through another route, was observed to be upregulated in wounded tomato leaves, suggesting the SA biosynthesis through *SlPAL* might be more important than through *SlICS* during *H. armigera*-tomato interaction. The *Spodoptera exigua* OS elevates the accumulation and signaling of SAin Solanaceous plants (Diezel et al., 2009; Musser et al., 2005). OS components are known to suppress as well as induce SA and JA signaling pathways (Chen et al., 2019; Halitschke et al.,2003; Roda et al., 2004; Schmelz et al., 2006; Wu et al., 2007). The uncharacterized small molecules (<3 kDa) from oral secretion of *S. littoralis* and *Pieris brassicae* were found to suppress wound-induced gene expression in *Arabidopsis* (Consales et al., 2012). Recently (Chen et al., 2019) have shown that the secretory protein HARP1 (*Helicoverpa armigera* R- like protein) from *H. armigera* oral secretion stabilizes JAZ degradation leading to suppressedJA signaling in *Arabidopsis*. Thus, it could be interesting to find OS components that suppressJA and activate SA to manipulate the plant defense. The amino acids serve as primary metabolites in plants for growth and defense. An altered level of the amino acids like tryptophan, glutamine and glutamate was evident in response to herbivore attack (Erb and Kliebenstein, 2020; Steinbrenner et al., 2011). The

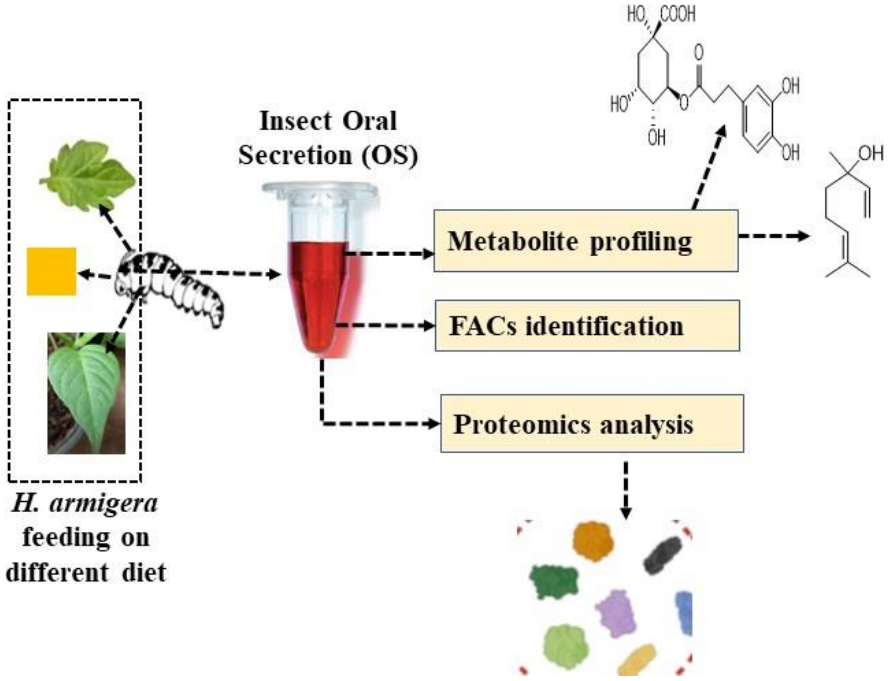
application of *H. armigera* OS on tomato plant resulted in a higher accumulation of primary metabolites like phenylalanine, tryptophan, tyrosine, and tyrosine could serve as precursor for the synthesis of specialized defensive metabolites. The steroidal glycoalkaloids and phenylpropanoid pathway metabolites involved in defense are mostly accumulated in Solanaceae plants after insect attack (Chowański et al., 2016a; Singh et al., 2021). The OS treatment on wounded tomato plants resulted in induced accumulation of phenolics and their precursors like cinnamic acid, p-coumaric acid, caffeoyl shikimic acid at an early time point (after 24 h), whereas the alkaloids like hydroxytomatine isomer 1 and acetoxy-tomatine isomer at late time point (after 72 h) in our study. Also, the induced transcript level of a gene like *PHENYLALANINE AMMONIA-LYASE* (*SlPAL*) involved in phenylpropanoid pathway was observed at early time point (after 4h) in response to OS_H and OS_{NH}. This suggests that in tomato plants phenylpropanoid pathway is the first preferred defense pathway in response to OS treatments. The regulation of transcript levels of genes involved in monoterpene biosynthesis and induced emission of volatile from *Spodoptera exigua* OS treated tomato plant is evident (Zebelo et al., 2014). Numerous studies have identified alkaloids and phenolics that affect insects growth and development (Gajger and Dar, 2021; Kumar et al., 2016). Several phenolic compounds like caffeoyl putrescine and CGA in Solanaceous plants were broadly explored upon herbivore treatment (Lee et al., 2017). In comparative metabolites analysis, several metabolites of the phenylpropanoid pathway including L-phenylalanine, chlorogenic acid, quercetin, and rutin were highly accumulated in tomato leaves in response to insects OS treatments. CGA was previously known to have anti-nutritive properties for the *S. frugiperda*, *S. exigua* (Kumar et al., 2016), and *S. litura* (Kundu et al., 2018). Further, chlorogenoquinones formed by oxidation of CGA, that bind to amino acids and proteins in insects and limiting the availability of amino acids required for growth and development, leading to reduced larval growth (Kundu et al., 2018; Kundu and Vadassery, 2019). Consistent with a higher accumulation of CGA, we also observed increased expression of CGA biosynthetic pathway genes in OS_H and OS_{NH} treated tomato leaves. Thus, CGA can be considered as a strong anti-nutritive, altering the herbivore feeding behaviour, growth, and survival. Along with phenylpropanoid pathway, the OS treatments lead to a higher accumulation of signaling sugars trehalose, which was evident with the induced expression of trehalose biosynthetic genes (*SlTPS1* and *SlTPP*). Trehalose after exogenous application on plants shown to possess elicitor and priming properties and improved protection against abiotic and biotic stresses (Hodge et al., 2013; Mostofa and Hossain, 2015; Muchembled et al., 2006; Reignault et al., 2001; Renard-Merlier et al., 2007; Shi et al., 2019; Singh and Shah, 2012; Tayeh et al., 2014). Also, trehalose

treatment plays an important role in gene expression regulation linked to the plant defense responses involving phytohormones and various phytoalexins synthesis (Aghdasi et al., 2008; Paul et al., 2008). Along with protection against insects, the role of trehalose in induced plant defense signaling has been reported earlier (Fernandez et al., 2010). Thus, trehalose also could influence the tomato plant defense against *H. armigera*.

In conclusion, current study investigated the impact of *H. armigera* OS on tomato defense responses. Application of OS on wounded tomato leaves modulated the expression of SA and JA biosynthesis and responsive genes as well as trehalose biosynthetic genes. Interestingly, plant metabolite analysis revealed enhanced accumulation of steroidal glycoalkaloids and phenolic metabolites in response to insect OS. Also, CGA, one of the key components of plant defense showed enhanced accumulation through phenylpropanoid pathway upon OS_H and OS_{NH} application. Additionally, dose-dependent feeding of CGA displayed retardation of the *H. armigera* larval growth. Overall, this study indicated that plant-based diet might have major role in altering the composition of herbivore insect oral secretion and their significant influence on modulating the plant defense in tomato.

Chapter 3

Integrated omics approach for analysis of oral secretion constituents from *Helicoverpa armigera*



3.1 Introduction

Chemical cues from insect oral secretions (OS), which include regurgitant and digestive fluids were perceived by plants to induce defense mechanisms (Acevedo et al., 2015). Further, OS constituents might interact with biomolecules of the plant and/or associated microorganisms. Interestingly, some plant biomolecules are known to influence the synthesis and secretion of insect OS (Paré et al., 1998; Peiffer and Felton, 2005a). For example, the amount of fatty acid-amino acid conjugates (FACs) is higher in OS of *Manduca sexta* than *Spodoptera exigua* when they feed on *Nicotiana attenuate* (Diezel et al., 2009). FACs are well-known elicitors in Lepidopteran insects, which are able to trigger direct as well as indirect plant defenses (Diezel et al., 2009). FACs activates the mitogen-activated protein kinase (MAPK) cascade that stimulated the jasmonic acid (JA) signaling pathway leading to subsequent synthesis of plant specialized metabolites and defensive proteins. The most potent FACs, volicitin (17-hydroxylinolenoyl-l-Gln), was detected in *S. exigua* OS (Alborn et al., 1997). The composition of Herbivore-Associated Molecular Patterns (HAMPs) in insects may be altered in response to diet, physiological state and parasitism. Importantly, plant-based linolenic acid has a significant impact on the synthesis of volicitin and is considered the backbone of FACs. For instance, insect larvae were devoid of volicitin if they fed on the fruits of *Physalis angulate*, which lack linolenic acid (De Moraes and Mescher, 2004). Along with FACs, many proteins and peptides have been well characterized from regurgitant of caterpillars. β-glucosidase and glucose oxidase (GOX) are the most studied proteins from the OS whereas the first peptide fragments of chloroplastic ATP synthase γ-subunit termed inceptins have also been identified in insect OS (Schmelz et al., 2006). The combination of proteomics and RNA sequencing approach helped to identify and characterize the set of salivary effector proteins from aphid (Thorpe et al., 2016). Similarly, by using a dual transcriptome-proteome based approach the salivary-secretory proteins from whitefly *Bemisia tabaci* and *Acyrthosiphon pisum* were identified (Carolan et al., 2009; H.-J. Huang et al., 2021). Further, the proteomic analysis of labial saliva of the generalist cabbage looper (*Trichoplusia ni*) suggested that the protein quantity in saliva is dependent on the host plant diet. Also, the salivary proteins are possibly involved in reactive oxygen species (ROS) scavenging in response to plant defenses (Rivera-Vega et al., 2018). Saliva of sucking insects is well characterized (Miles, 1999, 1972; Torsten Will, 2016), however few reports are available on the characterization of chewing insects' saliva. It has been assumed that caterpillar saliva has immunity, digestive, detoxifying properties (Rivera-Vega et al., 2017). In *Heliconius melpomene* adult saliva comprises active proteases which benefit to

digest pollen before ingestion (Harpel et al., 2015). Several salivary proteins were bug-specific, and some of them were involved in inducing plant defenses (H.-J. Huang et al., 2021).

Plant specialized metabolites often modulate interactions between plants and insects, which can substantially affect herbivores and their fitness. Further, these metabolites are signature cues of any host plant providing evidence about the identity, nutritional value, and physiological status to the feeding insect. Solanaceae family plants like tomato, eggplant, potato, Capsicum etc. are rich in phenolic compounds, glycoalkaloids, and defensive proteins such as proteinase inhibitors (Chowański et al., 2016b; Kennedy, 2003). Hence, based on this, we hypothesized that *Helicoverpa armigera* may secrete different enzymes, proteins or metabolites on plant feeding sites through OS. *H. armigera* is a polyphagous insect, feeds on wide range of host plants. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant range but also specific tissues of the host plant. To comprehend the impact of a plant-based diet on *H. armigera*, we analyzed the metabolites, FACs and proteins from OS of *H. armigera* fed on tomato as host (OS_H) and Capsicum as non-host (OS_{NH}) plants along with artificial diet (OS_{AD}).

3.2 Materials and methods

3.2.1 Insect rearing, collection and preparation of OS

Eggs of *H. armigera* (cotton bollworm) were obtained from the National Bureau of Agricultural Insect Resources (Bengaluru, India). The hatched 1st instar larvae (n=30) were reared on an artificial diet, and detached leaves of tomato and Capsicum plants. The artificial diet consists of chickpea (common host of *H. armigera*) seed powder and other components such as yeast extract, casein hydrolysate, sorbic acid, choline chloride, and vitamins. The detailed composition of the artificial diet is as described earlier (Nagarkatti and Prakash, 1974)and also in chapter 2. Insects were reared under the controlled conditions at 25 °C, 70% relative humidity, and a 14 h light/10 h dark cycle. The fourth instar larvae were taken for OS collection. The mouthpart of larvae was gently squeezed by hand, and the OS was collected using pipette in a 2 ml Eppendorf tube (Chen et al., 2019). Collected OS was centrifuged at 15000 rpm for 10 min at 4 °C. The supernatant was collected and stored at -80 °C for further use. OS of 10 larvae were pooled together and considered as a single biological replicate and such three biological replicates were used for the metabolite, FACs and proteomic analysis.

3.2.2 Liquid Chromatography- Quadrupole Time of Flight- Mass Spectrometry (LC-

QTOF-MS) based targeted metabolite profiling of H. armigera OS

For insect OS metabolite analysis, 20 µL of OS was mixed with 80 µL of extraction solvent (95% Methanol). The LC-QTOF-MS analysis was performed on Agilent 6530 Q-TOF (Agilent, USA) mass spectrometer connected to HPLC Prime Infinity II 1260 system (800 bar) according to (Vasav et al., 2020). The MS/MS fragmentation data was acquired at 10, 20 and 40 eV collision energy in positive mode. In the case of targeted metabolite analysis, the peak area of metabolites was determined using Agilent Mass Hunter Qualitative Navigator B.08.00, and Qualitative Workflow B.08.00 using the customizeddatabase created using Agilent personal compound database and library. The fold change of each metabolite was calculated by dividing mass feature peak area of specific compounds amongst OS_H, OS_{NH} and OS_{AD}. Individual metabolites were confirmed by comparing fragments generated by MS/MS data with standard compound or *in silico* fragments generated by Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) software (http://cfmid.wishartlab.com/) (Allen et al., 2015) and also according to reported experimentalfragmentation pattern (Li et al., 2020).

3.2.3 FACs analysis from OS of H. armigera

For FACs analysis, 20 µL of OS was mixed with 180 µL of LC-MS grade methanol (95%) and briefly vortexed. The extracts were homogenized in bath sonicator for 10 min. The OS-methanol extract was centrifuged at 14000 rpm for 15 min and collected the supernatant in fresh effpendorf and stored at -80°C for overnight (~16 h). The extract was centrifuged and filtered through 0.22 µm filter followed by 10 µL sample was injected. LC-QTOF-MS based data acquisition for FACs was carried out on Agilent 6530 Q-TOF (Agilent, USA) mass spectrometer connected to HPLC Prime Infinity II 1260 system (800 bar) according to Vasav et al., 2020. The MS/MS fragmentation data was acquired at 10, 20 and 40 eV collision energy in negative mode. The identification of peak area for FACs was done with Agilent Mass Hunter Qualitative Navigator B.08.00. The detection of major FACs in OS was performed by using m/z and fragmentation pattern reported by (Diezel et al., 2009; Krempl et al., 2021). Also, the fragmentation pattern of FACs were confirmed by comparing fragments generated by MS/MS data with *in silico* fragments generated by CFM-ID software (http://cfmid.wishartlab.com/).

3.2.4 Proteomic analysis of H. armigera OS by tandem mass spectrometry (LC-MS Triple TOF 5600)

To identify the variations in protein level of *H. armigera* larvae, the OS was collected from the synchronous fourth-instar larvae fed on artificial diet (OS_{AD}), tomato (host; OS_H) and *Capsium annuum* (non-host; OS_{NH}) leaves. Equal amount of protein (100 µg) from the OS_{AD}, OS_H and OS_{NH} were denatured at 80°C for 20 min followed by reduction with 150 mM dithiothreitol at 60°C for 15 min and alkylation with 200 mM iodoacetamide at ambient temperature in dark for 30 min. Further, protein digestion was carried out at 37°C for 16 h with 5 µg trypsin gold (Mass Spectrometry Grade, Promega Cat. No. V5280) after which digestion was quenched using hydrochloric acid. The digested peptides were desalted using ZipTip® pipette tips (Merck Millipore, USA) and 3.5 µg digest was injected onto a C18 reverse phase column (dimensions: 100 × 0.3 mm, 3 µm, 120 Å) of a microLC 200 liquid chromatography system (Eksigent Technologies, USA) coupled to a Triple TOF 5600 mass spectrometer (SCIEX, USA). Peptides were separated over a 120 min gradient of 5 to 40% acetonitrile in water with 0.1% formic acid at a flow rate of 7 µl/min. For label free quantification using Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS), spectral library was first generated by acquiring all samples in Information Dependent Acquisition (IDA) mode. SWATH-MS acquisitions were performed over a peptide mass range of 400 to 1250 m/z split into 34 overlapping windows of 25 Da each. Peptide fragmentation was performed using rolling collision energy. Nine replicate runs were acquired for of the three biological replicates of each treatment. The IDA data was searched against Uniprot and transcriptome database of *H. armigera* using ProteinPilot™ version 5.0 software. Trypsin was used for digestion and cysteine alkylating agent for fixed modification was selected as iodoacetic acid. A False Discovery Rate (FDR) of 1% was set for protein identification. The results generated were used in PeakView v2.2 software as spectral library and SWATH runs were processed using 50 ppm mass error, 4 min retention time window, 99% confidence and 1% FDR. The processed data was further exported to MarkerView™ v1.2.1 for quantitative and statistical analysis. The data across the runs was normalized using total area sum. Statistical significance was calculated using student's t-test and p-value <0.05 was considered as statistically significant. The proteins which showed fold change >1.3 fold with *p*-value <0.05 were considered to high abundance and <1.3 fold change with *p*-value <0.05 as low abundance.

3.3 Results

3.3.1 Differential accumulation of metabolites in OS of H. armigera fed on different diets

Metabolite data comparing the impact of different plant-based diet on insect OS is limited. *H. armigera* larvae prefer tomato plants (*Solanum lycopersicum* L.; host) as their diet compared to the Capsicum plants (*Capsicum annum* L.; non-host). This was also evident when *H. armigera* larvae fed on Capsicum plants, they showed delayed growth and development **(Fig. 3.1)**.

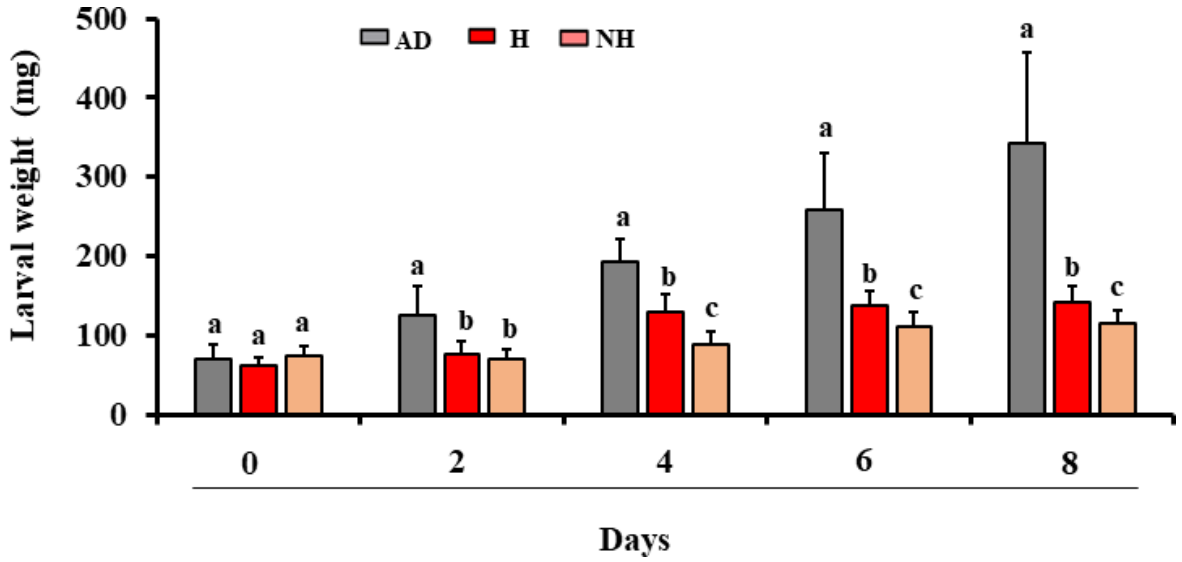


Figure 3.1: Effect of various diets on *H. armigera* larval growth. 1st instar larvae (n=30) were fed on artificial diet (AD), host-tomato (H) and non-host Capsicum (NH) leaves. Mass of *H. armigera* larvae were taken on 2, 4, 6, and 8 days after feeding on the respective diet. Data shown are mean +/ - SD. Different letters indicate significant difference ($p < 0.05$) with respective to artificial diet.

Non-targeted metabolic analysis identified total 1296 mass peaks in OS_{AD}, OS_{H} and OS_{NH}. Principal component analysis showed clear separation of all identified mass peaks from OS_{AD}, OS_{H}, and OS_{NH} contributed by PC1 (48.6%) and PC2 (41.4%) **(Fig. 3.2)**. Among identified number of mass peaks, 599, 634 and 505 were from OS_{AD}, OS_{H}, and OS_{NH}, respectively. Comparative analysis of OS metabolites resulted in identification of 75 metabolites that were common among OS_{AD}, OS_{H}, and OS_{NH}, 165 (75+90) were shared between OS_{AD} and OS_{H}, 112 (75+37) were common between OS_{AD} and OS_{NH}, and 240 (75+165) were shared between OS_{H}, and OS_{NH}. Interestingly, 397, 304, 228 distinct mass peaks were identified from OS_{AD}, OS_{H}, and OS_{NH}, respectively **(Fig. 3.3)**.

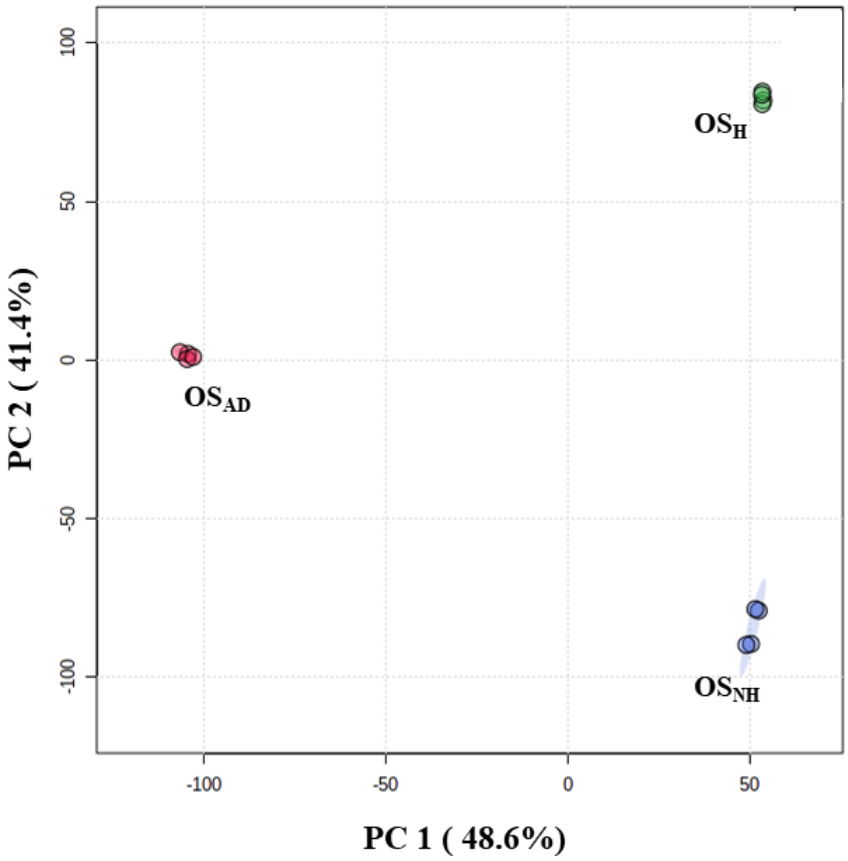


Figure 3.2: Principal component analysis of identified mass peaks from OS of *H. armigera* fed on different diet with ESI (+) mode. PCA was generated with help of Clustvis web server (Metsalu and Vilo, 2015).

Moreover, radar plot analysis of classes of identified mass peaks showed that phospholipids, alkaloids and terpenoids were higher in OS_{AD}, OS_{H}, and OS_{NH}, respectively **(Fig. 3.4)**. From identified mass peaks, on the basis of peak area, 45 significantly ($P < 0.05$) identified metabolites were confirmed by using MS/MS fragmentation pattern **(Table 3.1).** Out of these identified and confirmed metabolites, α-linolenic acid, samandarine, phytosphingosine-1-phosphate, 13 hydroxy-9-methoxy-10-oxo-11-octadecenoic acid, metanephrine, gentiatibetine were abundant in OS_{H}, while bolegrevilol, resolvin E1, oryzarol, etorphine, corchoroside A, gossyrubilone, O-geranylvanillin and stenostrol were prominent in OS_{NH}. The α-linolenic acid was also detected at moderate level in OS_{AD} and OS_{NH}. On the other hand, tocotrienol, sterebin D, dihydroxyacidissiminol, kanzonol K, chlorogenic acid metabolites were found OS_{H} and OS_{NH} **(Fig. 3.5)**. The comparative analyses show the differentially and diet-specific presence of metabolites in the *H. armigera* larval OS. MS/MS fragmentation of representative metabolites were shown **(Fig. 3.6)**

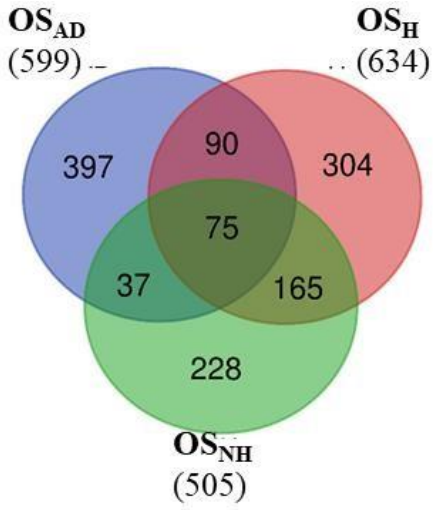


Figure 3.3: Venn diagram of overall metabolite identified from *H. armigera* OS fed on different diets - The venn diagram was created using http://bioinformatics.psb.ugent.be/webtools/Venn.

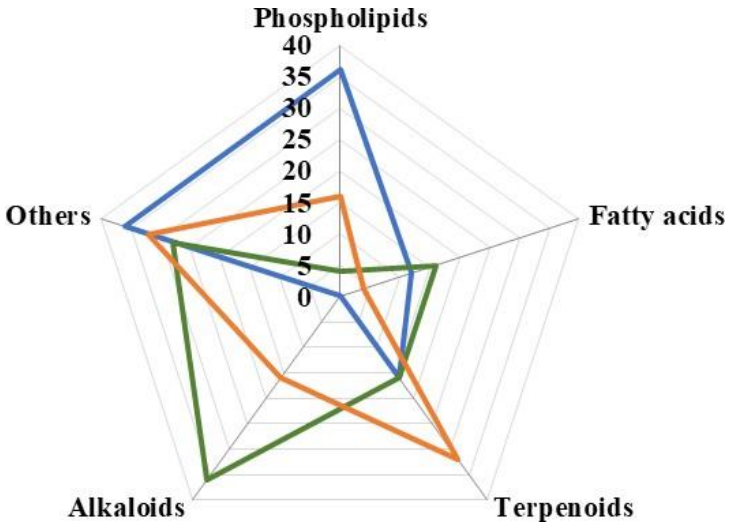


Figure 3.4: **Radar plot depicts the classes of metabolites unique in OS$_{AD}$, OS$_{H}$, and OS$_{NH}$ in terms of percentage.**

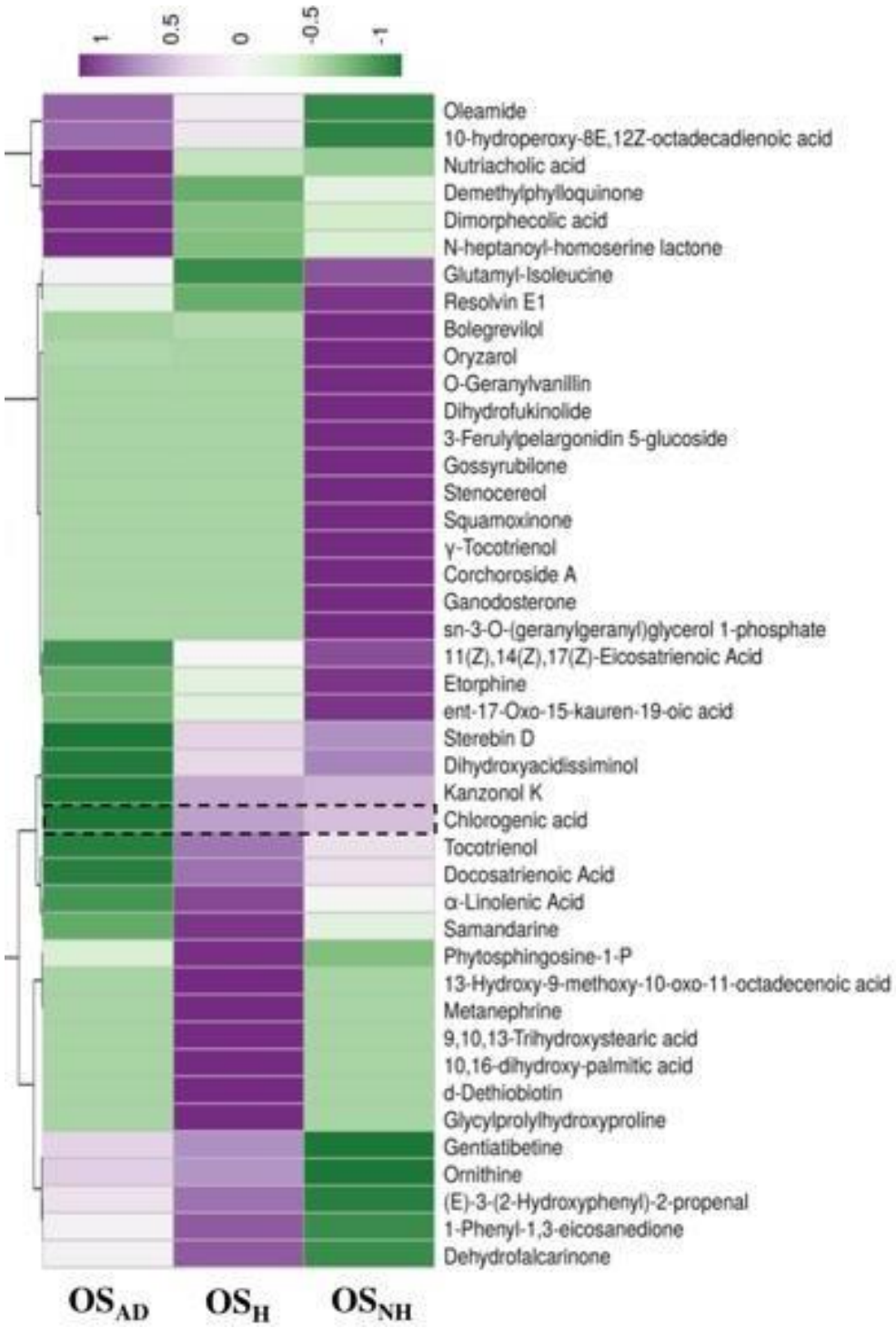


Figure 3.5: Heatmap of differentially accumulated metabolites in OS of *H. armigera.* Total area under peak of respective metabolite was considered and heatmap was generated with help of Clustvis web server, which is showed in terms of scale bar from +1 to -1.

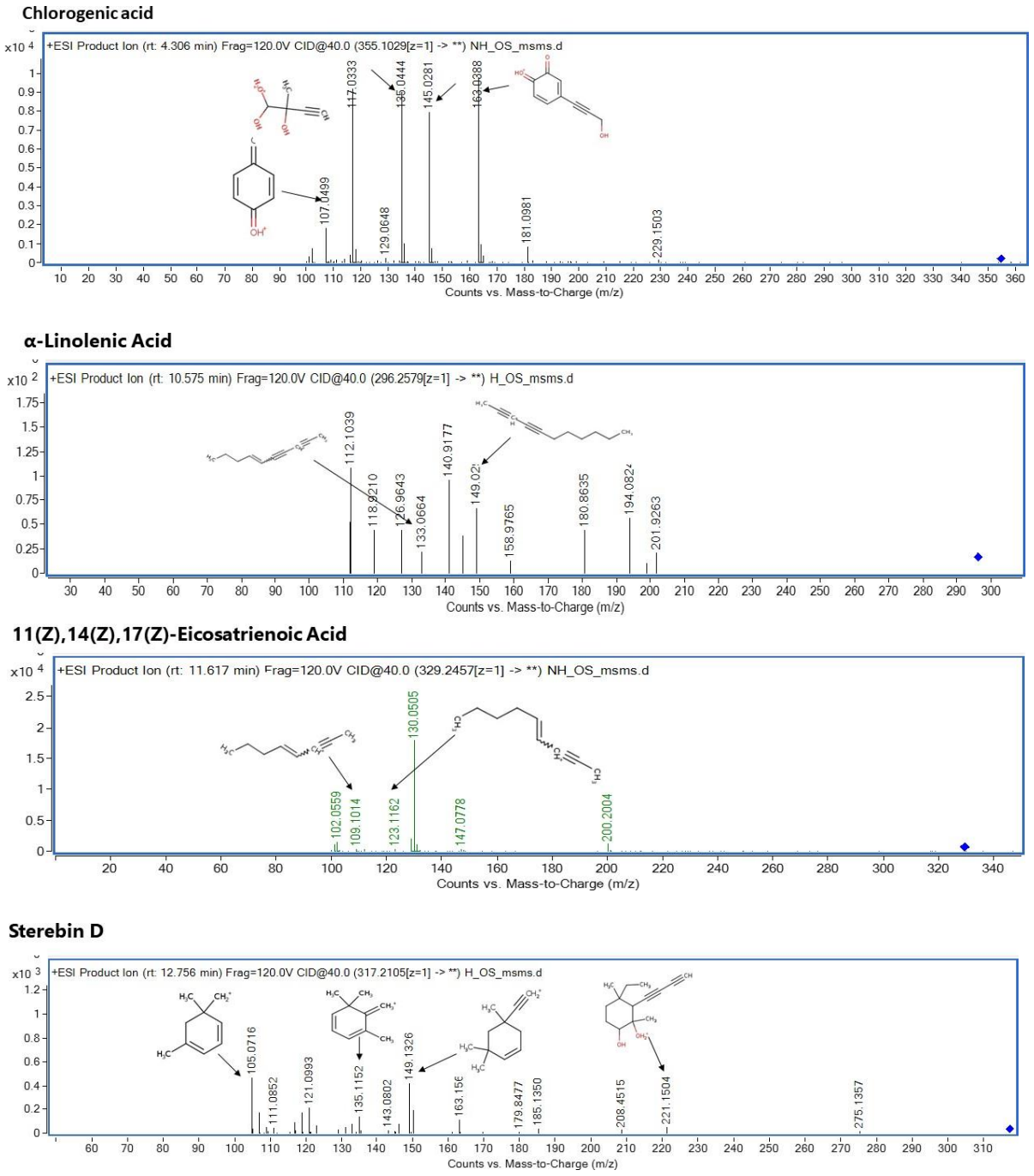


Figure 3.6: Representative LC MS/MS fragmentation pattern of identified metabolites from OS of *H. armigera.* Fragmentation pattern of identified metabolites was matched either with standard, *in silico* or experimental fragmentation. Matching mass peaks are indicated by arrow.

Table 3.1: Details of identified metabolites from OS of *H. armigera* larvae fed either on tomato (OS_H) or Capsicum (OS_{NH}) leaves, or artificial diet (OS_{AD})

Metabolite	Chemical Formula	m/z	Mass	Selected ion	Mass error (ppm)	RT (Min)	MS/MS Fragments
Tocotrienol[R]	$C_{26}H_{38}O_2$	405.28	382.28	[M+Na]+	0.2	9.90	119.08, 241.19
1-Phenyl-1,3-eicosanedione[R]	$C_{26}H_{42}O_2$	409.30	386.31	[M+Na]+	1.57	14.41	147.07, 263.23
Oryzarol[R]	$C_{26}H_{42}O_3$	425.30	402.31	[M+Na]+	0.65	10.30	147.07, 205.15
Etorphine[R]	$C_{25}H_{33}NO_4$	429.27	411.24	[M+NH4]+	-0.07	13.72	384.25, 412.24
Kanzonol K[R]	$C_{26}H_{28}O_6$	437.19	436.19	[M+H]+	-0.21	11.99	179.14, 237.62, 407.13
Glutamyl-isoleucine[R]	$C_{11}H_{20}N_2O_5$	261.14	260.14	[M+H]+	1.99	4.28	102.05,114.05, 169.08, 198.11, 244.11
α-Linolenic acid[R]	$C_{18}H_{30}O_2$	296.25	278.22	[M+NH4]+	-2.72	10.57	133.06, 149.02
11(Z),14(Z),17(Z)-Eicosatrienoic Acid[R]	$C_{20}H_{34}O_2$	329.24	306.24	[M+Na]+	-0.34	11.61	109.10, 123.11
Samandarine[R]	$C_{19}H_{31}NO_2$	328.22	305.23	[M+Na]+	-1.15	4.49	107.04, 317.21
Sterebin D[R]	$C_{18}H_{30}O_3$	317.21	294.22	[M+Na]+	2.11	12.75	105.07, 135.11, 149.13, 221.15
Dihydroxyacidissimino[R]	$C_{25}H_{33}NO_5$	445.26	427.23	[M+NH4]+	-0.65	10.02	169.05, 410.23
Docosatrienoic acid[R]	$C_{22}H_{38}O_2$	357.27	334.28	[M+Na]+	1.43	13.44	109.10, 123.11
ent-17-Oxo-15-kauren-19-oic acid[R]	$C_{20}H_{28}O_3$	317.21	316.21	[M+H]+	-1.66	13.01	135.18, 147.09, 163.14, 229.15
Bolegrevilol[R]	$C_{28}H_{40}O_4$	441.29	440.29	[M+H]+	1.53	8.12	187.14, 259.20, 423.28
(E)-3-(2-Hydroxyphenyl)-2-propenal[R]	$C_9H_8O_2$	166.08	148.08	[M+NH4]+	2.74	3.38	107.04, 131.04, 149.05
Gentiatibetine[R]	$C_9H_{11}NO_2$	166.08	165.07	[M+H]+	2.73	3.71	107.04, 120.08, 148.06
Oleamide[R]	$C_{18}H_{35}NO$	282.28	281.27	[M+H]+	1.63	17.30	156.13, 247.24, 256.25
Dehydrotomatine[R]	$C_{50}H_{81}NO_{21}$	1032.54	1031.54	[M+H]+	2.73	5.59	145.05, 414.33, 576.39
Dehydrofalcarinone[R]	$C_{17}H_{20}O$	263.14	240.15	[M+Na]+	-1.77	4.42	103.05, 117.07

Phytosphingosine-1-P[R]	$C_{18}H_{40}NO_6P$	397.30	397.30		1.9	11.01	124.11, 300.20, 380.30
10-hydroperoxy-8E,12Z-octadecadienoic acid[R]	$C_{18}H_{32}O_4$	335.21	312.33	[M+Na]+	0.8	12.88	109.06, 253.17
Prunitrin[R]	$C_{22}H_{22}O_{10}$	469.11	446.12	[M+Na]+	2.75	8.16	147.11, 237.07, 411.14
Ornithine[R]	$C_5H_{12}N_2O_2$	133.09	132.09	[M+H]+	2.08	1.16	102.06, 115.08, 116.07
Nutriacholic acid[R]	$C_{24}H_{38}O_4$	413.26	390.27	[M+Na]+	2.96	18.79	189.08, 341.22
Dimorphecolic acid[R]	$C_{18}H_{32}O_3$	297.24	296.23	[M+H]+	2.48	13.15	109.10, 123.11, 251.23, 279.23
Resolvin E1[R]	$C_{20}H_{30}O_5$	351.21	350.20	[M+H]+	-0.96	7.05	123.07, 135.30, 149.07, 201.00
Demethylphylloquinone[R]	$C_{30}H_{44}O_2$	437.34	436.33	[M+H]+	-2.75	16.02	135.11, 281.22
N-heptanoyl-homoserine lactone[R]	$C_{11}H_{19}NO_3$	231.17	213.13	[M+NH4]+	0.01	4.28	100.04, 156.07, 196.08
Ganodosterone	$C_{28}H_{40}O_2$	409.29	408.30	[M+H]+	-2.23	13.59	109.10, 373.26, 391.26
sn-3-O-(geranylgeranyl)glycerol 1-phosphate[R]	$C_{23}H_{41}O_6P$	445.27	406.30	[M+*K*]+	-1.71	10.02	123.05, 427.25
Corchoroside A	$C_{29}H_{42}O_9$	535.29	534.28	[M+H]+	2.38	9.74	113.02, 329.98
γ-Tocotrienol[R]	$C_{28}H_{42}O_2$	433.30	410.31	[M+Na]+	-2.19	15.49	119.08, 145.11, 151.04, 175.14
Squamoxinone[R]	$C_{37}H_{68}O_7$	663.45	624.49	[M+*K*]+	-2.26	17.87	495.26, 607.39
Stenocereol[R]	C28H46O2	437.34	414.35	[M+Na]+	1.9	16.02	135.11, 159.11, 241.23, 341.30
Gossyrubilone[R]	$C_{20}H_{25}NO_4$	344.18	343.18	[M+H]+	0.74	4.34	229.15, 239.13, 326.17
3-Ferulylpelargonidin 5-glucoside[R]	$C_{31}H_{29}O_{13}$	609.32	609.32	-	1.13	17.49	123.12, 147.08, 241.15
Dihydrofukinolide[R]	$C_{22}H_{32}O_6$	392.22	392.22	-	2.25	4.73	119.04, 375.18
O-Geranylvanillin[R]	$C_{18}H_{24}O_3$	289.17	288.17	[M+H]+	-1.53	11.59	107.08, 121.10, 243.25

d-Dethiobiotin[R]	$C_{10}H_{18}N_2O_3$	215.13	214.13	[M+H]+	2.57	2.10	113.96, 126.05, 172.96
Glycylprolylhydroxyproline[R]	$C_{12}H_{19}N_3O_5$	286.13	285.13	[M+H]+	1.85	1.88	112.07, 132.10, 140.07
10,16-dihydroxy-palmitic acid[R]	$C_{16}H_{32}O_4$	311.22	288.23	[M+Na]+	1.27	8.80	169.05, 211.11, 241.19
9,10,13-Trihydroxystearic acid[R]	$C_{18}H_{36}O_5$	333.26	332.26	[M+H]+	2.73	7.96	173.11, 297.24, 315.24
Metanephrine[R]	$C_{10}H_{15}NO_3$	215.13	197.10	[M+NH4]+	2.78	3.51	121.87, 123.10
13-Hydroxy-9-methoxy-10-oxo-11-octadecenoic acid[R]	$C_{19}H_{34}O_5$	342.24	342.24	-	1.73	4.79	141.06, 183.14
Chlorogenic acid[S]	$C_{16}H_{18}O_9$	355.10	354.10	[M+H]+	1.78	4.30	107.04, 117.03, 135.04, 145.02, 163.03

a RT: Retention time

b Fragmentation pattern matched to Standard (S) or Reference (R) from in-silico fragments generated by CFM-ID software (http://cfmid.wishartlab.com/) (Allen et al., 2014).

3.3.2 Differential accumulation of FACs in OS of H. armigera fed on different diets

Total seven types of glutamine conjugated FACs were detected in OS **(Fig. 3.7)**. Among the identified FACs, five were confirmed by MS/MS fragmentation pattern with the CFM-ID **(Fig. 3.8; Table 3.2)**. The FACs, N-17-Hydroxylinolenoyl-l-Gln (Volicitin), N-Linolenoyl-l-Gln and Tetradecanoyl-l-Gln were most abundant in OS_H and OS_{NH} as compared to OS_{AD}. Accumulation of another FAC, N-17-Hydroxylinoleoyl-l-Gln was highest in OS_{NH} followed by OS_H and OS_{AD}. Palmitoleoyl-l-Gln FAC was identified only in OS_H. Further, the N- Linoleoyl-l-Gln was not detected in OS_{AD} but found to be higher in OS_{NH} as compared to OS_H**(Fig. 3.7)**.

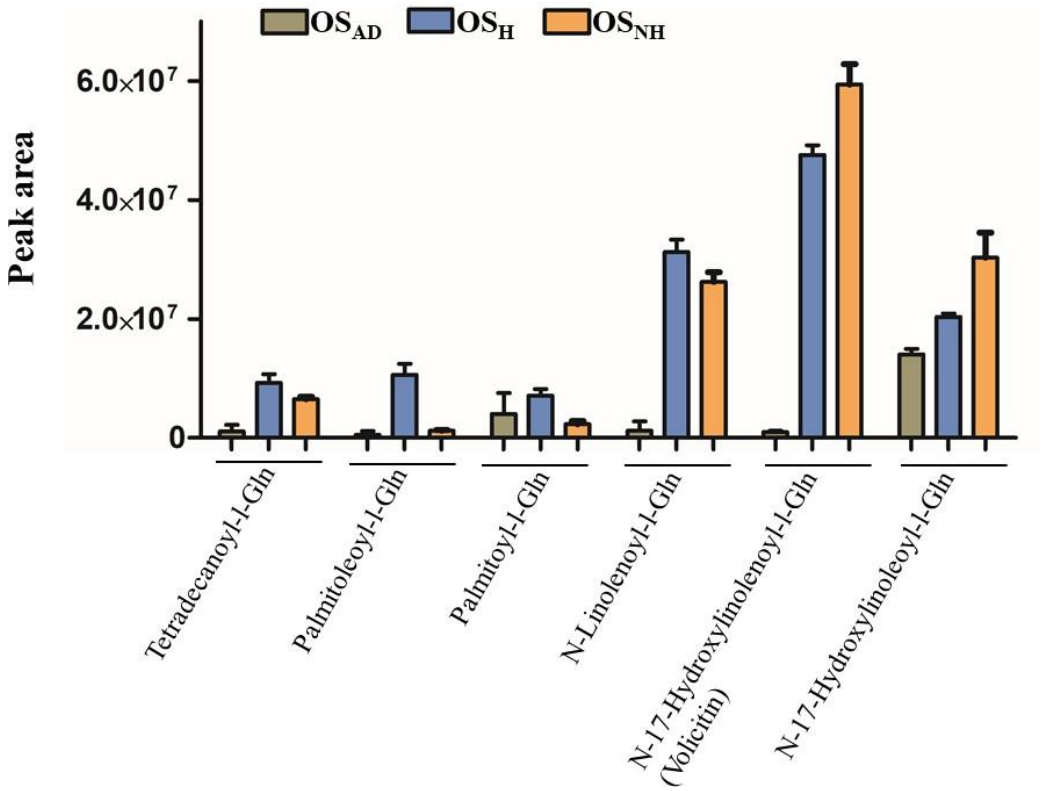


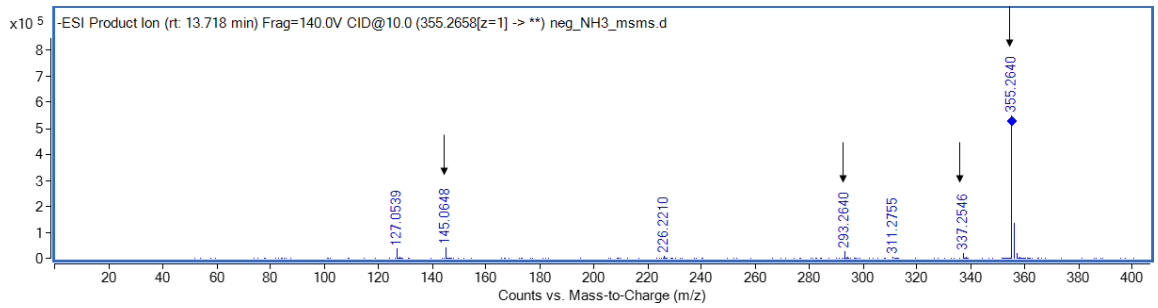
Figure 3.7: Differential level of identified FACs in OS of *H. armigera.*

Table 3.2: Details of identified FACs in the OS of *H. armigera*

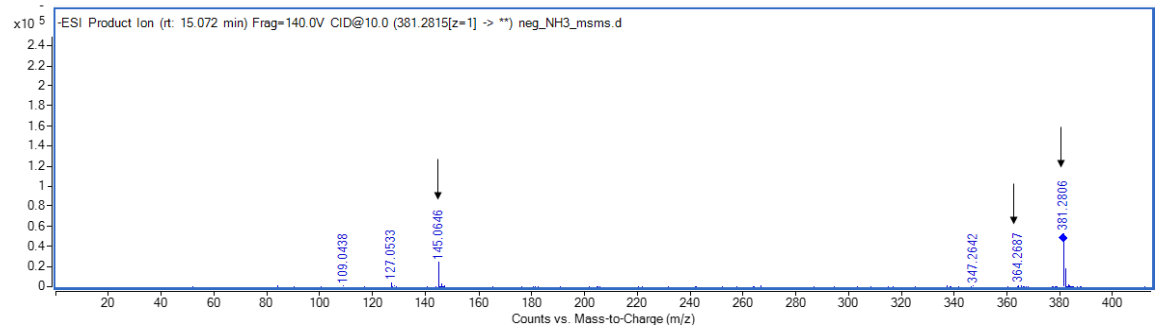
FACs	Chemical formula	m/z [M-H]	RT (Min)	MS/MS Fragmentation pattern
Tetradecanoyl-l-Gln	$C_{19}H_{36}O_4N_2$	355.265	13.71	355.264, 337.254, 293.264, 145.06
Palmitoleoyl-l-Gln	$C_{21}H_{38}O_4N_2$	381.281	15.07	381.280, 364.268, 145.06, 127.05
Palmitoyl-l-Gln	$C_{21}H_{40}O_4N_2$	383.29	15.41	383.297, 635.285, 321.295, 145.06
N-Linolenoyl-l-Gln	$C_{23}H_{38}O_4N_2$	405.28	13.81	405.280, 387.270, 145.06
N-Linoleoyl-l-Gln	$C_{23}H_{40}O_4N_2$	407.261	9.43	nd
N-17-Hydroxylinolenoyl-l-Gln (Volicitin)	$C_{23}H_{37}O_5N_2$	421.275	10.04	421.275, 403.265, 145.06
N-17-Hydroxylinoleoyl-l-Gln	$C_{23}H_{40}O_5N_2$	423.29	10.5	nd

***nd: not detected

Tetradecanoyl-l-Gln



Palmitoleoyl-l-Gln



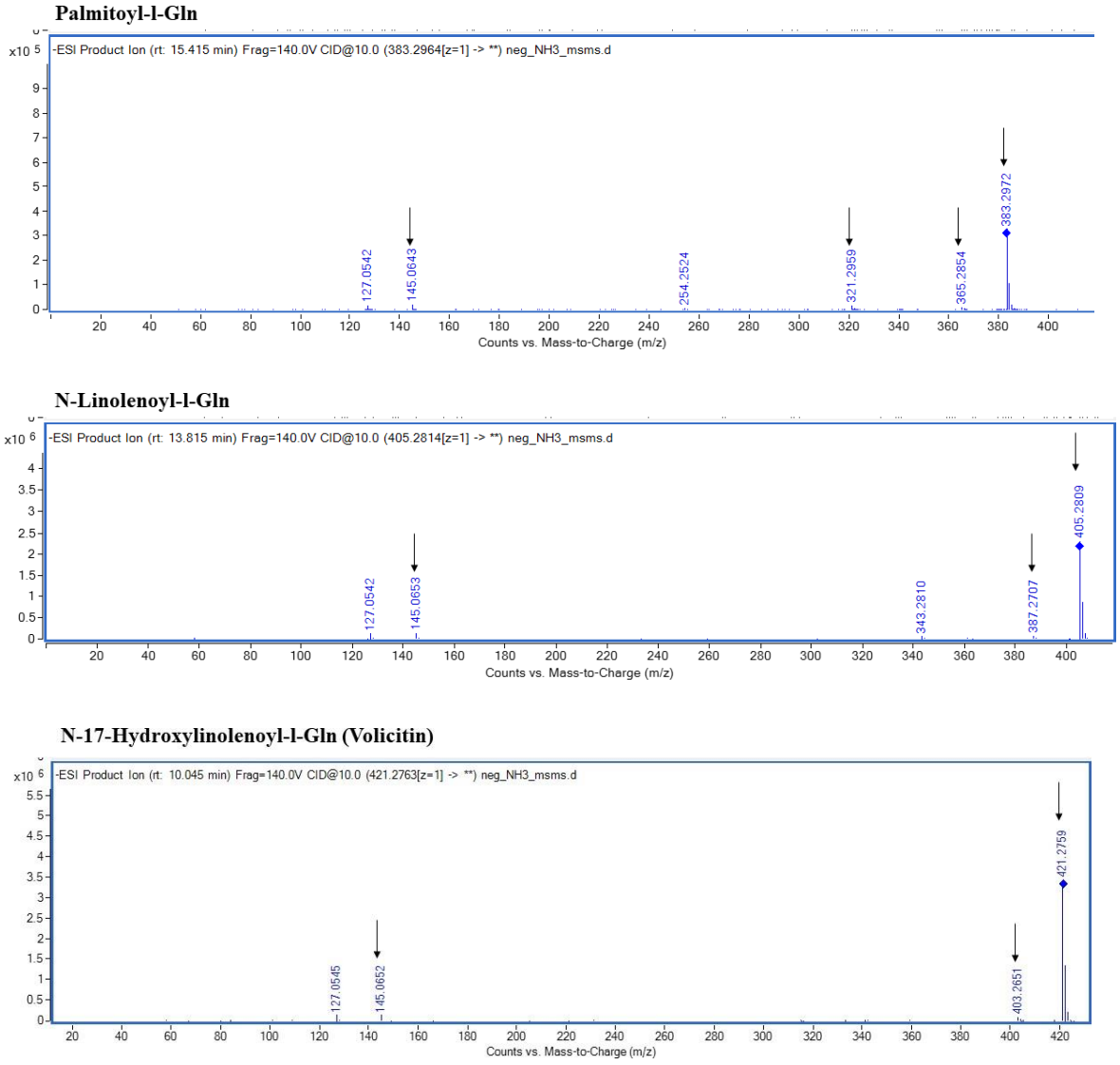


Figure 3.8: LC MS/MS fragmentation pattern of identified FACs in OS of *H. armigera.* Fragmentation pattern of identified FACs was matched either with *in silico* or with standard. Matching mass peaks are indicated by arrow.

3.3.3 Untargeted proteomics revealed differential accumulation of proteins in OS of H. armigera fed on different diets

The untargeted proteomic analysis of OS_{AD}, OS_{H}, and OS_{NH} generated spectral library of 221 proteins through IDA. Out of a total of 221 proteins, OS_{NH} and OS_{H} have 118 and 103 proteins, respectively. Among these 27 and 56 identified proteins were common and showed differential accumulation in OS_{H} and OS_{NH} as compared to OS_{AD} **(Fig. 3.9; Table 3.3; 3.4)**. In addition, 16 and 4 proteins were found in OS_{H} with high and low abundance, respectively. Similarly, in OS_{NH} 19 and 16 proteins were identified as high and low abundant respectively **(Fig. 3.10; Table 3.5-3.8)**. The highly accumulated proteins from OS_{H} and OS_{NH} belongs to digestive enzymes and some of them are uncharacterized. The proteins like isoforms of trypsin, aminopeptidase, carboxypeptidase, chitin deacetylase were abundant in OS_{H} as compared to OS_{NH} and OS_{AD}, however the peptidase, glycerol-3-phosphate acyltransferase, neutral lipase, CaMBD domain-containing protein, ATP-citrate synthase were more in OS_{NH}. Further, the lipases, peptidase S1, trypsin, polycalin, endonuclease were ubiquitously distributed in both OS_{H} and OS_{NH} as compared to OS_{AD}. The chymotrypsin, serine proteases, peptidase, peptidoglycan-recognition protein, cyclic nucleotide-binding domain-containing protein, beta-1,3-glucanase, along with some uncharacterized proteins were found to be less abundant in OS_{H} and OS_{NH}.

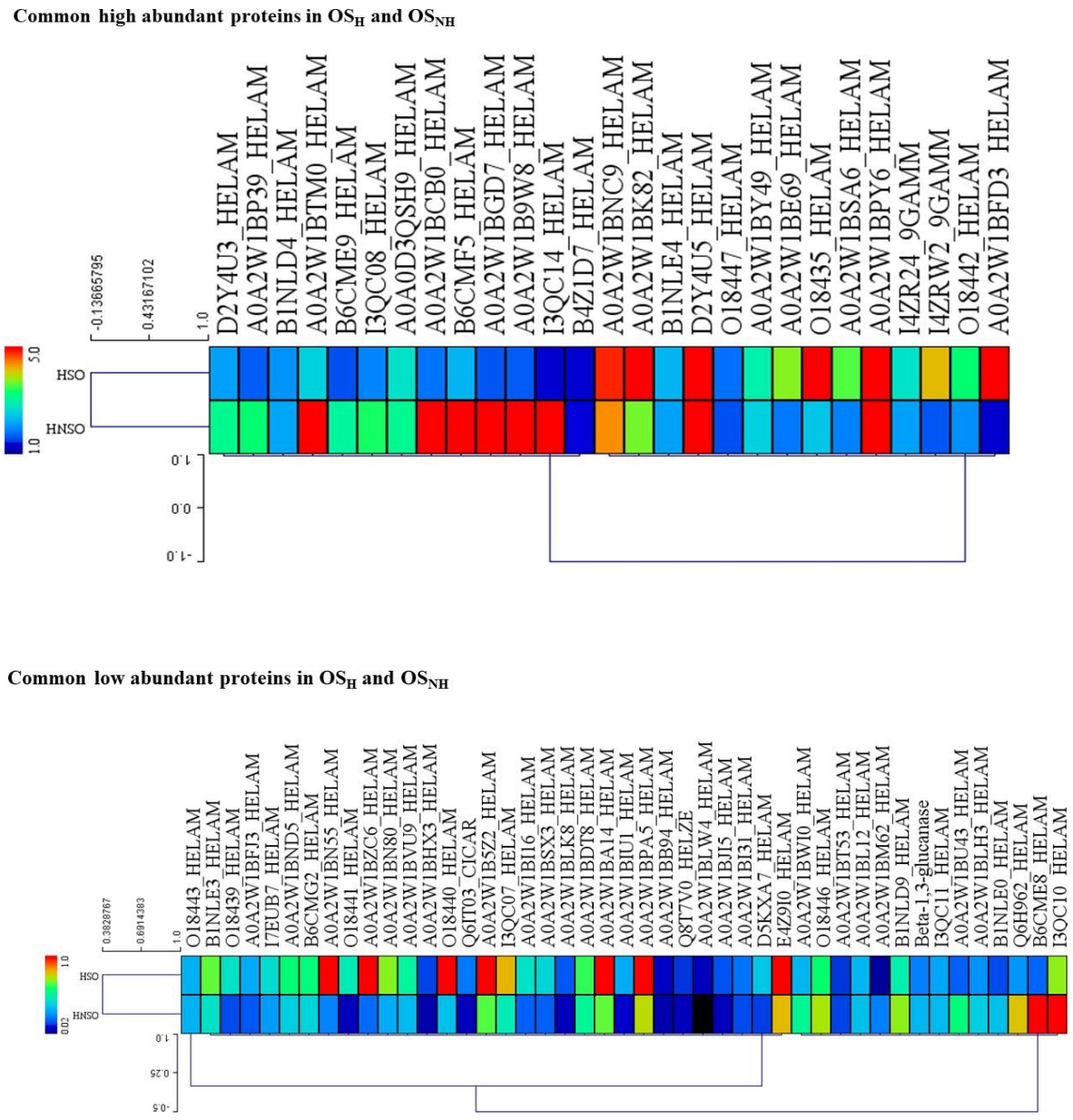


Figure 3.9: Heat map of common proteins identified in both OS_H and OS_{NH} of *H. armigera*. Protein abundance was found to be high (>1.3 fold; P value 0.05) and low (<1.3 fold; P value <0.05) when compared to OS_{AD}. Peak area of identified proteins were normalizedwith internal spiked protein (beta-galactosidase). MultiExperimental viewer 4.9 was used to create a heatmap to represent the fold change of the corresponding protein.

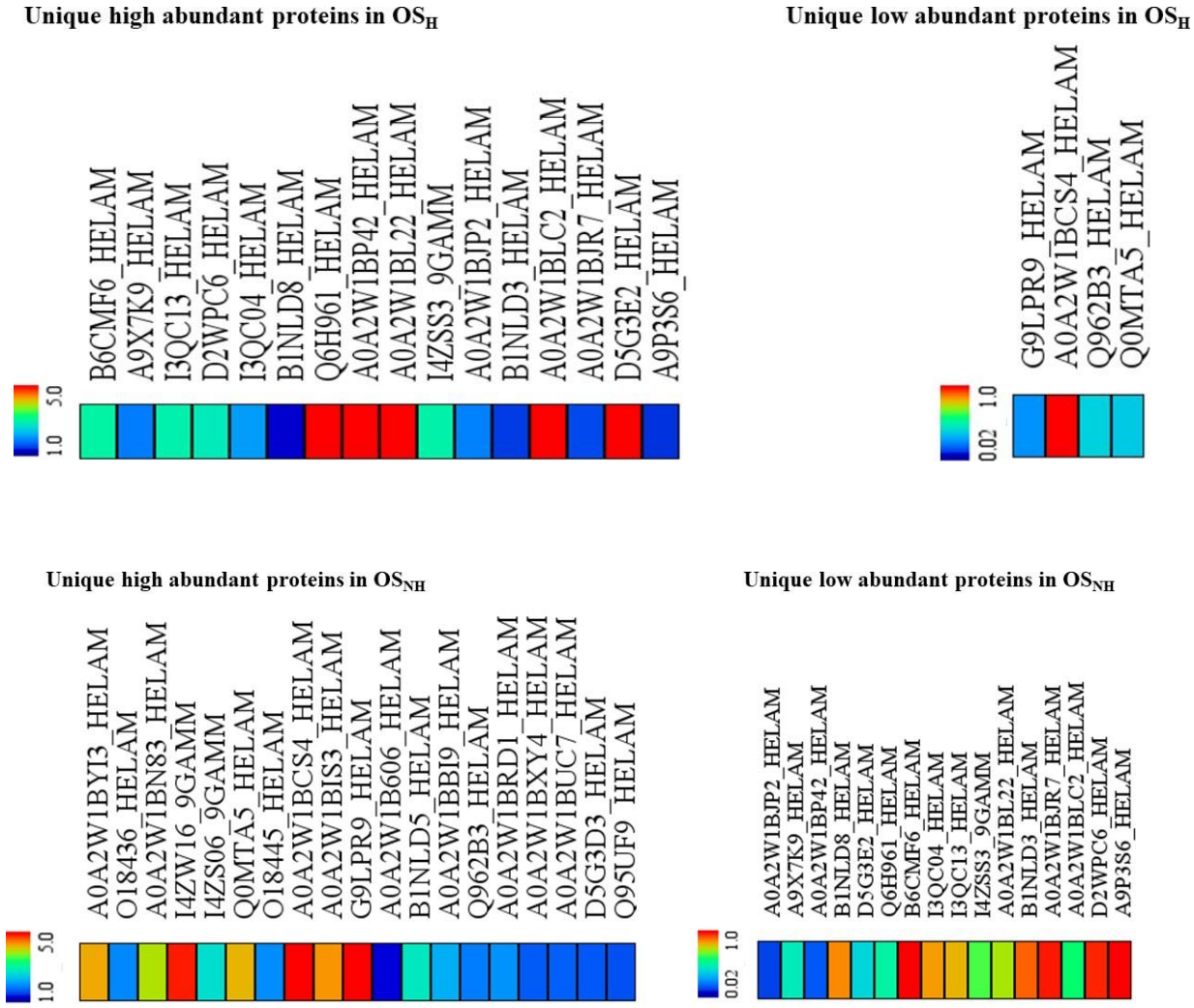


Figure 3.10: Heat map of unique proteins identified in OS_H and OS_{NH} of *H. armigera*

Protein abundance was found to be high (> 1.3 fold; P value <0.05) and low (<1.3 fold; P value <0.05). Peak area of identified proteins were normalized with internal spiked protein (beta-galactosidase). MultiExperimental viewer 4.9 was used to create a heatmap to represent the fold change of the corresponding protein.

Table 3.3: Details of common high abundant proteins identified from OS_H and OS_{NH} of *H. armigera*

Uniprot Protein ID	Fold change		Protein name	Coverage %
	OS_{NH}	OS_H		
A0A2W1BNC9_HELAM	4.36	4.79	Lipase domain-containing protein	70.69
D2Y4U3_HELAM	3.14	2.14	Carboxylesterase (Fragment)	39.32
A0A2W1BP39_HELAM	3.32	1.69	Peptidase S1 domain-containing protein	38.23
A0A2W1BK82_HELAM	3.68	11.63	Uncharacterized protein	82.68
B1NLD4_HELAM	2.19	2.05	Alpha-amylase	70.59
A0A2W1BTM0_HELAM	5.01	2.54	Peptidase S1 domain-containing protein	61.80
B1NLE4_HELAM	2.15	2.3	Protease	78.07
D2Y4U5_HELAM	5.97	10.45	Trypsin	67.79
B6CME9_HELAM	3.05	1.61	Trypsin (Fragment)	55.47
I3QC08_HELAM	3.38	1.95	I3QC08_HELAM	65.25
A0A0D3QSH9_HELAM	3.12	2.73	Polycalin	28.83
A0A2W1BCB0_HELAM	4.92	1.83	CaMBD domain-containing protein	1.37
B6CMF5_HELAM	10.94	2.29	Azurocidin-like serine proteinase (Fragment)	12.20
A0A2W1BGD7_HELAM	6.35	1.64	Prolyl-tRNA synthetase	0.76
O18447_HELAM	1.6	1.81	Serine protease	81.88
A0A2W1B9W8_HELAM	7.47	1.67	ATP-citrate synthase	1.29
I3QC14_HELAM	16.87	1.35	Neutral lipase	46.11
A0A2W1BY49_HELAM	2.54	2.96	Endonuclease	0.85
A0A2W1BE69_HELAM	1.86	3.73	Peptidase S1 domain-containing protein	56.08
O18435_HELAM	2.44	5.27	Trypsin-like protease	4.31
A0A2W1BSA6_HELAM	1.91	3.57	Peptidase S1 domain-containing protein	40.47
A0A2W1BPY6_HELAM	5.33	6.65	Uncharacterized protein	1.22
I4ZR24_9GAMM	2.13	2.75	Soluble lytic murein transglycosylase	1.71
I4ZRW2_9GAMM	1.64	4.14	FRG domain-containing protein	1.20
O18442_HELAM	2.01	3.32	Trypsin-like protease	75.48
B4Z1D7_HELAM	1.42	1.36	Alkaline phosphatase	1.68
A0A2W1BFD3_HELAM	1.33	10.49	NodB homology domain-containing protein	44.42

Table 3.4: Details of common low abundant proteins identified from OS_H and OS_{NH} of *H. armigera*

Uniprot ID	Fold change		Protein name	Coverage %
	OS_{NH}	OS_H		
B1NLE3_HELAM	0.45	0.66	Protease	54.60
O18439_HELAM	0.16	0.45	Diverged serine protease	83.98
O18443_HELAM	0.33	0.33	Chymotrypsin-like protease (Fragment)	70.64
A0A2W1BWI0_HELAM	0.53	0.34	Chymotrypsin-like protease (Fragment)	51.94
O18446_HELAM	0.72	0.59	Diverged serine protease	74.22
A0A2W1BFJ3_HELAM	0.18	0.32	Peptidase S1 domain-containing protein	54.29
I7EUB7_HELAM	0.29	0.43	Peptidoglycan-recognition protein	20.74
A0A2W1BND5_HELAM	0.39	0.58	Lipase domain-containing protein	54.72
B6CMG2_HELAM	0.41	0.57	Putative gram-negative bacteria-binding protein	79.72
A0A2W1BN55_HELAM	0.25	1.19	Metalloendopeptidase	22.38
O18441_HELAM	0.08	0.48	Trypsin-like protease	70.31
A0A2W1BZC6_HELAM	0.21	1.19	Peptidase S1 domain-containing protein	31.47
A0A2W1BN80_HELAM	0.32	0.69	Peptidase S1 domain-containing protein	76.95
A0A2W1BT53_HELAM	0.15	0.13	Cyclic nucleotide-binding domain-containing protein	2.26
A0A2W1BL12_HELAM	0.36	0.34	Uncharacterized protein	12.5
A0A2W1BVU9_HELAM	0.35	0.52	Cytochrome b561 domain-containing protein	3.03
A0A2W1BHX3_HELAM	0.06	0.15	Uncharacterized protein	35.33
O18440_HELAM	0.36	1.11	Trypsin-like protease	56.51
Q6IT03_CICAR	0.08	0.23	Kunitz proteinase inhibitor-1 (Fragment)	54.68
A0A2W1BM62_HELAM	0.24	0.04	C3H1-type domain-containing protein	1.57
A0A2W1B5Z2_HELAM	0.65	1.18	Lipase domain-containing protein	26.64
B1NLD9_HELAM	0.7	0.5	Protease	70.71
I3QC07_HELAM	0.48	0.8	Neutral lipase	32.24
Beta-1,3-glucanase	0.34	0.24	Beta-1,3-glucanase	77.60
A0A2W1BI16_HELAM	0.19	0.45	Uncharacterized protein	4.86
I3QC11_HELAM	0.35	0.31	Neutral lipase	17.61
A0A2W1BSX3_HELAM	0.19	0.41	Uncharacterized protein	4.25
A0A2W1BLK8_HELAM	0.08	0.18	Anaphase-promoting complex subunit 1	0.37

A0A2W1BDT8_HELAM	0.53	0.62	Mediator of RNA polymerase II transcription subunit 20	1.52
A0A2W1BA14_HELAM	0.66	1.04	Peptidase S1 domain-containing protein	31.76
A0A2W1BIU1_HELAM	0.1	0.32	Uncharacterized protein	7.79
A0A2W1BU43_HELAM	0.57	0.19	Fatty acyl-CoA reductase	1.33
A0A2W1BPA5_HELAM	0.74	0.98	Lipase domain-containing protein	51.34
A0A2W1BB94_HELAM	0.06	0.09	Nucleoside diphosphate kinase	5.88
A0A2W1BLH3_HELAM	0.39	0.27	Uncharacterized protein	1.48
Q8T7V0_HELZE	0.08	0.13	Cytoplasmic actin A3a2	4.78
A0A2W1BLW4_HELAM	0.01	0.08	Uncharacterized protein	45.80
B1NLE0_HELAM	0.36	0.16	Uncharacterized protein	19.57
A0A2W1BJI5_HELAM	0.08	0.17	Uncharacterized protein	50.13
A0A2W1BI31_HELAM	0.16	0.22	Uncharacterized protein	0.51
D5KXA7_HELAM	0.14	0.37	Carboxylic ester hydrolase	3.83
E4Z9I0_HELAM	0.79	1.12	Cytochrome c oxidase subunit 1 (Fragment)	6.07
Q6H962_HELAM	0.78	0.28	Carboxypeptidase	54.00
B6CME8_HELAM	1.12	0.2	Trypsin	31.90
I3QC10_HELAM	1.29	0.7	Lipase	3.95
A0A2W1BSK9_HELAM	0.19	0.35	A0A2W1BSK9_HELAM	5.60
A0A2W1BSW1_HELAM	0.89	0.78	Vps16_C domain-containing protein	2.33
I3QC05_HELAM	0.78	0.7	Neutral lipase	21.92
O18438_HELAM	1.06	0.64	Chymotrypsin-like protease	69.17
A0A2W1BJ38_HELAM	0.83	0.16	Metalloendopeptidase	9.29
A0A290U614_HELAM	0.85	0.19	Prophenoloxidase 1	4.09
A0A2W1BL45_HELAM	0.96	1.12	Peptidase S1 domain-containing protein	80.70
D7RZZ8_HELAM	1	0.09	Chymotrypsin	75.24
A0A2W1C098_HELAM	1.02	0.81	Ferritin	12.08
O18450_HELAM	0.99	1.04	Chymotrypsin-like protease	73.90
A0A2W1BS57_HELAM	0.99	0.47	Peptidase S1 domain-containing protein	12.71

Table 3.5: Details of high abundant proteins identified only in OS_H *H. armigera*

Uniprot ID	Fold change	Protein name	Coverage %
B6CMF6_HELAM	3	Inactive lipase (Fragment)	80.00
A9X7K9_HELAM	1.88	Lipase	17.12
I3QC13_HELAM	2.93	Neutral lipase	41.11
D2WPC6_HELAM	2.86	Chitin deacetylase 5b	50.76
I3QC04_HELAM	2.11	Neutral lipase	47.88
B1NLD8_HELAM	1.33	Protease	75.48
Q6H961_HELAM	6.74	Carboxypeptidase	22.66
A0A2W1BP42_HELAM	5.38	CYTOSOL_AP domain-containing protein	3.83
A0A2W1BL22_HELAM	11.6	COesterase domain-containing protein	1.07
I4ZSS3_9GAMM	2.97	Phenylalanine--tRNA ligase beta subunit	1.00
A0A2W1BJP2_HELAM	1.91	Peptidase S1 domain-containing protein	59.16
B1NLD3_HELAM	1.48	Alpha-amylase (Fragment)	26.66
A0A2W1BLC2_HELAM	79.129	EGF-like domain-containing protein	0.44
A0A2W1BJR7_HELAM	1.56	NodB homology domain-containing protein	74.36
D5G3E2_HELAM	8.01	Carboxylic ester hydrolase	1.63
A9P3S6_HELAM	1.43	Serine protease 4 (Fragment)	57.81

Table 3.6: Details of low abundant proteins identified only in OS_H

Uniprot ID	Fold change	Protein name	Coverage %
G9LPR9_HELAM	0.27	UDP-glycosyltransferase UGT47A2	1.30
A0A2W1BCS4_HELAM	1.22	Peptidase S1 domain-containing protein	47.83
Q962B3_HELAM	0.4	Aminopeptidase N	6.31
Q0MTA5_HELAM	0.38	HMG176	4.54

Table 3.7: Details of high abundant proteins identified only in OS_{NH} of *H. armigera*

Uniprot ID	Fold change	Protein name	Coverage %
A0A2W1BYI3_HELAM	4.24	Endonuclease NS domain-containing protein	26.85
O18436_HELAM	1.96	Serine protease 5	81.09
A0A2W1BN83_HELAM	3.89	Peptidase S1 domain-containing protein	24.22
I4ZW16_9GAMM	4.82	Glycerol-3-phosphate acyltransferase	0.7
I4ZS06_9GAMM	2.69	Tfp pilus assembly protein, pilus retraction ATPase PilT	3.76
Q0MTA5_HELAM	4.18	HMG176	4.54

O18445_HELAM	1.99	Chymotrypsin-like protease	70.16
A0A2W1BCS4_HELAM	9.38	Peptidase S1 domain-containing protein	47.83
A0A2W1BIS3_HELAM	4.33	Peptidase S1 domain-containing protein	21.87
G9LPR9_HELAM	9.87	UDP-glycosyltransferase UGT47A2	1.30
A0A2W1B606_HELAM	1.42	Peptidase S1 domain-containing protein	74.22
B1NLD5_HELAM	2.81	Sucrose-6-phosphate hydrolase	2.50
A0A2W1BBI9_HELAM	2.24	Uncharacterized protein	2.63
Q962B3_HELAM	1.89	Aminopeptidase N	6.31
A0A2W1BRD1_HELAM	2.03	General transcription and DNA repair factor IIH helicase subunit XPD	0.92
A0A2W1BXY4_HELAM	1.66	Endo/exonuclease/phosphatase domain-containing protein	2.21
A0A2W1BUC7_HELAM	1.7	Clathrin heavy chain	0.47
D5G3D3_HELAM	1.65	Carboxylic ester hydrolase (Fragment)	3.29
Q95UF9_HELAM	1.6	Aminopeptidase N	0.69

Table 3.8: Details of low abundant proteins identified only in OS_{NH} of *H. armigera*

Uniprot ID	Fold change	Protein name	Coverage %
A0A2W1BJP2_HELAM	0.15	Peptidase S1 domain-containing protein	59.16
A9X7K9_HELAM	0.47	Lipase	17.12
A0A2W1BP42_HELAM	0.18	CYTOSOL_AP domain-containing protein	3.83
B1NLD8_HELAM	0.85	Protease	75.48
D5G3E2_HELAM	0.41	Carboxylic ester hydrolase	1.63
Q6H961_HELAM	0.51	Carboxypeptidase	22.63
B6CMF6_HELAM	1.15	Inactive lipase (Fragment)	80.00
I3QC04_HELAM	0.83	Neutral lipase	47.88
I3QC13_HELAM	0.8	Neutral lipase	41.11
I4ZSS3_9GAMM	0.64	Phenylalanine--tRNA ligase beta subunit	1.00
A0A2W1BL22_HELAM	0.72	COesterase domain-containing protein	1.07
B1NLD3_HELAM	0.89	Alpha-amylase (Fragment)	26.66
A0A2W1BJR7_HELAM	0.96	NodB homology domain-containing protein	74.36
A0A2W1BLC2_HELAM	0.59	EGF-like domain-containing protein	0.44
D2WPC6_HELAM	0.95	Chitin deacetylase 5b	50.76
A9P3S6_HELAM	0.99	Serine protease 4 (Fragment)	57.81

3.3.4 Identification of secretory proteins in H. armigera OS

Targeted proteomic analysis of OS revealed the accumulation of HARP1 (*H. armigera* R-like protein) an effector protein along with other uncharacterized salivary secretory proteins (HARP3, HARP4, HARP5 and HARP6 (basis of nomenclature is given in chapter 4) in the OS. Further, peptide sequence analysis revealed that the secretory proteins are substituted with single or two amino acids. More than 3 peptides were detected for secretory proteins in OS **(Table 3.9)**. The SWATH analysis shows that the HARP1 is highly accumulated in OS_{NH} as compared to OS_{AD} (**Fig. 3.11**). However, the uncharacterized secretory protein with single amino acid substitution HARP4 is higher in OS_{H} as compare to OS_{AD} (**Fig. 3.11**). No significant changes were observed for the accumulation of other HARPs.

Table 3.9: Details of targeted secretory proteins identified in OS of *H. armigera.*
Single amino acid substitutions are highlighted in yellow.

Known/ Hypothetical protein name		**Number of peptides (95%)**	**Identified peptide sequence of secretory proteins**
HARP1		40	KSLILVAVLA, PAFRANMYQGAIR, NYYYKAPIAN, AVQYQDITYRGSSST, ISFIQAVEVGQTQWGQPSLR, GWGYYYMIEIWGR
HARP3		15	VDLNHGYARPDDILLYSN, AARVDLNHGYARP, APVANEVQSEDIAYSGSARITAIR, ATEVGQTQWAIPSVRSGGVGR, YSIEIWGR
HARP4		15	VDLSHGYARPDDILLYSNTVLR
HARP5 (HaOG211282)		27	QELQVADADEVPMSEMAVFIRK, KVLTADEEMPFVAPRNGMSLGNIGSSDR, LLSASTHSR, YTGSSSII, AYGSGQGATAR, VVEGYLGRNSITIQLQSAR, GFHYRIEIWGR
HARP6		22	NGVSLGNIGSSDRLLSASTHSR

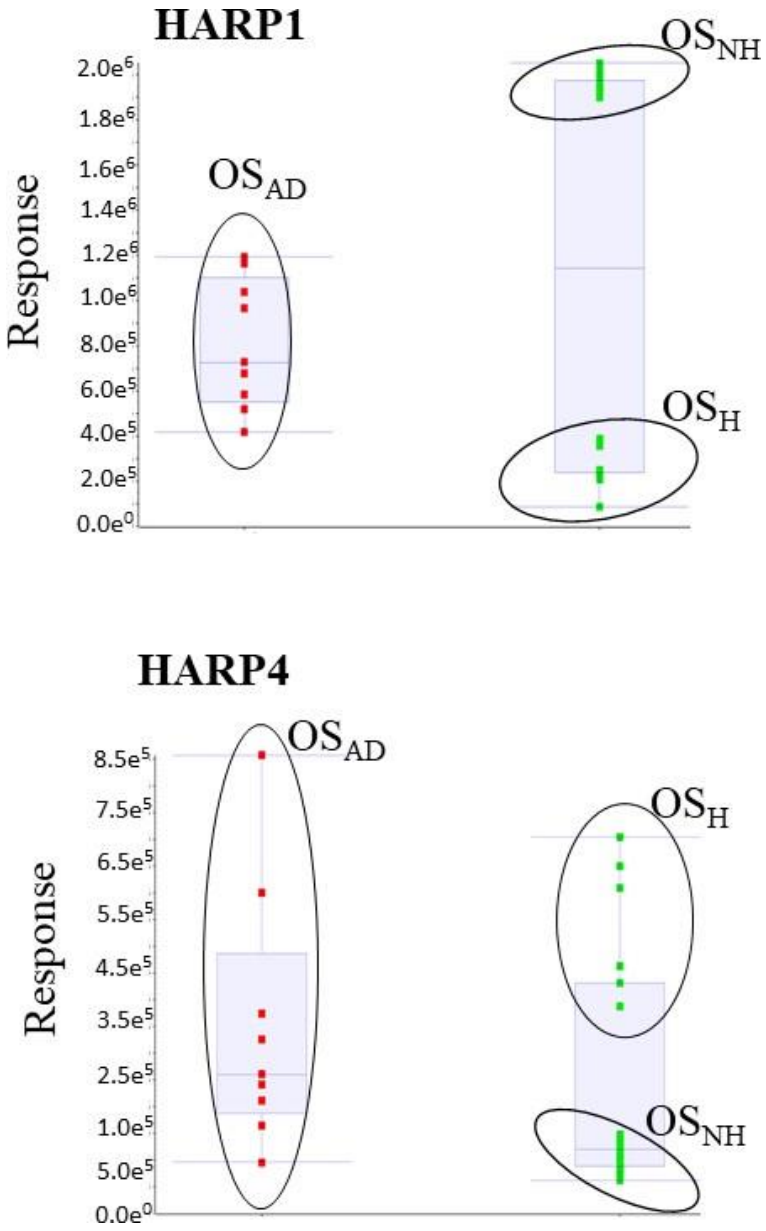


Figure 3.11: Differential accumulation of HARP1 and HARP4 proteins in OS of *H. armigera*. Box Whisker plot comparing the peak area and p-value of HARP peptides represented as response of OS_H and OS_{NH} against OS_{AD}.

3.4 Discussion

During the insect feeding, insect OS constituents are perceived in the host plant tissue that lead to activation of specific defense at local and systemic plant tissue to combat insect attack. It is increasingly accepted that herbivores could modulate host plants' natural defenses through OS components (Chen etal., 2019; Louis et al., 2013; Musser et al., 2002; Schmelz et al., 2006). Numerous studies haveidentified different chemical components in the insect OS comprising enzymes, proteins, and metabolites (Acevedo et al., 2017; Chen et al., 2019; Giri et al., 2006; Lawrence andNovak, 2004; Musser et al., 2002; Paré et al., 1998; Pohnert et al., 1999). However, most of these studies have been limited to induction of plant defense upon insect OS treatment when fed either on the host plant and/or artificial diet. Here, we have analyzed metabolite and proteincomposition of *H. armigera* OS fed on different diets (artificial diet, leaves of host or non-hostplant).

The nutritional quality of the plants positively modulates larval growth and development (Wang et al., 2020). There was a considerable reduction in larval growth when *H. armigera* larvae were fed on Capsicum and tomato leaves instead of artificial diet (Kallure et al., 2022b) Interestingly, metabolite profiling of *H. armigera* larvae OS was found to be influenced by various diets - artificial diet or leaves of tomato or Capsicum. The metabolites from different classes like terpenoids, phenylpropanoids and alkaloids were identified when insects were fed on plant leaves compared to the artificial diet. It is also intriguing to see numerous phospholipids accumulation in the OS_{AD}, and many of them were diet-specific. Further, samandarine, one of the steroidal alkaloids known to have toxic effect on herbivores, was found in OS_{H} (Daly et al., 2005). Another molecule, phytosphingosine-1-phosphate having role in plant signaling as well as in the stomatal closer during biotic and abiotic stress (Coursol et al., 2005; Huby et al., 2020; Zhang et al., 2014) was also detected in OS_{H}. Metanephrine, a phenolic metabolite derived from catecholamines identified from OS_{H} was found to be involved in alteration of insects' muscular contraction (Orchard and Lange, 1985). Moreover, a lipid peroxidation inhibitory diterpenoid, bolegrevilol, found in the OS_{NH} might be affectingthe lipid metabolism of insect after feeding (Hayashi et al., 1989). Sterebin, a melanogenesis inhibitor and chlorogenic acid, insect growth inhibitor (by reducing the availability of amino acids) both were found in OS_{H} and OS_{NH} (Kamauchi et al., 2014; Kundu and Vadassery, 2019).Corchoroside A found in OS_{NH} is one of the cardenolide glycosides. The cardenolide glycosidesare known to have cytotoxic activity (Moon et al., 2010). Resolvine, an active metabolite of

polyunsaturated fatty acids having role in inflammation was detected in OS_{NH} (Moro et al., 2016). Further, the terpenoids like gossyrubilone, *O*-geranylvanillin, and stenostrol a class of cholesterol and derivatives, are also identified only in OS_{NH}. The *O*-geranylvanillin was identified in *Chromolaena odorata* phenolic extract, which has shown antioxidant activity (Eze and Jayeoye, 2021). The tocotrienol, a member of vitamin E family identified in OS_H and OS_{NH}. The tocotrienol lowers cholesterol level by inhibiting hydroxy-methyl-glutaryl-coenzyme A (HMG-COA) reductase.

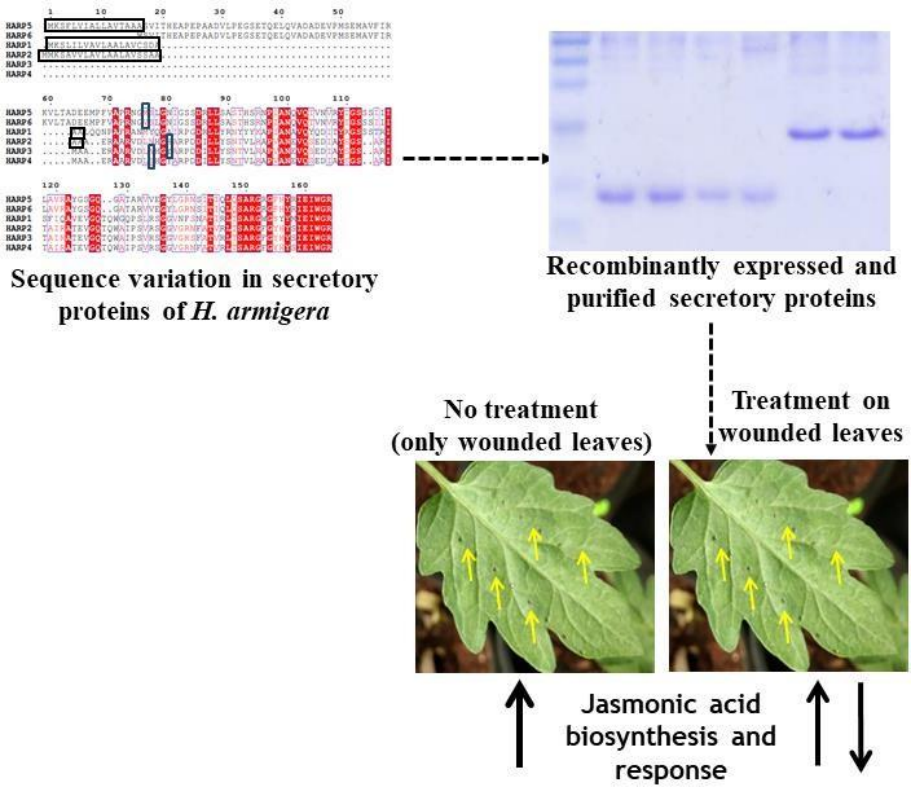
FACs found in OS of Lepidopteran larvae are known to induce defense responses in many plants. Although, glutamine and glutamic acid-conjugated fatty acids are well known but their abundance is mostly variable in herbivore species. Among them glutamine conjugated FACs are most abundant as compared to glutamic acid conjugation (Yoshinaga et al., 2010). Similarly, we have also detected accumulation of only glutamine conjugates (FACs) in the OS of *H. armigera*. Few reports suggest that the diet have a role on accumulation of FACs in insects (Ling et al., 2021; Paré et al., 1998; Yoshinaga et al., 2008). In *H. armigera* OS, the majority of metabolites and FACs are abundant in OS_{NH} and OS_H compared to OS_{AD}. The level of metabolites in the insect OS can be altered by repeatedly feeding on a plant or its tissues. The differential accumulation of bioactive molecules in the insect OS and release at feeding site might alter the plant defense responses.

Herbivore digestive system essentially enriched with enzymes for the digestion of food and metabolizing the plant constituents ingested during the feeding. Digestive proteases like trypsins chymotrypsins and other exopeptidases are known to cleave the plant proteins that led to availability of amino acid for growth and development of insects (Srinivasan et al., 2006). However, diet influences the accumulation of digestive enzymes in the insect OS (Zheng et al., 2022). For example, the higher concentration of tannin, gossypol content in diet downregulates the expression of peptidase, glycosyl hydrolases in *H. armigera* (Zheng et al., 2022). The salivary proteins of insect herbivore have role in detoxification and protection against host plant defense. The carboxylesterase, from *Aedes aegypti* and aminopeptidase in *Acyrthosiphonpisum* are found to be metabolize the xenobiotic compound and glutathione (Poupardin et al., 2014; Wu et al., 2021). Insects chitin deacetylase (CDA) have major role in moulting and pupation hence defects in their synthesis may lead to stunted growth and development. Induced accumulation of protease, carboxypeptidase and CDA in OS_H than OS_{NH} may be associated with improved *H. armigera* growth on tomato plant as compared to Capsicum. Further, the deficiency of glycerol-3-phosphate acyltransferase 1 declines triacylglycerol storage that led

to induced fatty acid oxidation in *Rhodnius prolixus* fat body (Alves-Bezerra et al., 2017). Here, we hypothesize that the induced accumulation glycerol-3-phosphate acyltransferase 1 in OS_{NH} may be essential for maintaining the triglycerol level in the insects. Additionally, peptidoglycan-recognition protein, beta-1,3-glucanase involved in insect immunity were found to be less abundant in OS_H and OS_{NH} (Pauchet et al., 2009; Wang et al., 2021). The secretory proteins from insect OS act as inducer or suppressor of plant defense (Musser et al., 2002). Recently, Chen et al., 2019 reported the HARP1 effector protein of *H. armigera* that suppress the wound induced plant defense in Arabidopsis and tobacco plant. Our proteomic analysis of *H. armigera* OS suggest that along with HARP1 many uncharacterized secretory proteins are also accumulated in OS. Hence, further detailed characterization of secretory protein from *H. armigera* will help in understanding about plant-insect coevolution. In the next chapter (Chapter 4), we have studied diversity in the HARPs present in *H. armigera* OS and their potential role in regulation of plant defenses.

Chapter 4

Identification and functional characterization of salivary secretory proteins from *Helicoverpa armigera*



4.1 Introduction:

Insect feeding damages the plant tissue that triggers the activation of plant defense (Koo and Howe, 2009). The synthesis and signaling of phytohormones like jasmonic acid (JA), salicylic acid (SA), ethylene is the central dogma for the induced plant defense against herbivores (N. Li et al., 2019). However, the chemical cues of herbivore oral secretion (OS) are known to modulate the activation plant defense. For example, OS derived compounds that activate the plant defense are known as elicitors whereas the molecules that disrupt the defense are known as suppressor or effectors. To adapt on different plants, insects potentially modulate the level of likely elicitors and/or effectors in their OS. The β-glucosidase enzyme in *Pieris brassicae* larval OSs induces release of volatiles from injured cabbage. Similarly, lipase and phospholipase C of *Schistocerca gregaria* and *Spodoptera frugiperda* induces the accumulation of oxylipin in Arabidopsis and protease inhibitors in corn, respectively (Acevedo et al., 2018; Schäfer et al., 2011). The first effector protein glucose oxidase (GOX) identified from *Helicoverpa zea* is known to inhibit the nicotine accumulation. Further, the increased weight of *Spodoptera littoralis* larvae fed on OS pre-treated leaves suggest that presence of effector like proteins in insect (Consales et al., 2012). In recent years many effector proteins like APC002, Mp10, Mp1, Mp2Armet, Me47, GroEL have been characterized from aphids, planthoppers and mites (Reviewed in (Kallure et al., 2022a). Effector protein NlSEF1 of brown planthopper (BPH), *Nilaparvata lugens* has the calcium binding domain that bind to induced cytosolic Ca2+ involved in defense signaling (Ye et al., 2017). Endo β 1–4 endoglucanase from *Nilaparvata lugens* degrades cellulose of plant cell wall resulting in reduced defense (Ji et al., 2017). Vitellogenin (Vg) an effector protein identified from small brown planthopper (*Laodelphax striatellus*, SBPH) interacts with WRKY transcription factor and attenuate the host rice defense (Ji et al., 2021). An effector Bt56 of *Bemisia tabaci* interacts with NTH202 KNOTTED 1-like homeobox transcription factor and interfere the JA/SA crosstalk in plants (Xu et al., 2019b). *Helicoverpa armigera* R-like protein 1 (HARP1) a secretory effector protein identified from OS interact with several JAZ proteins of Arabidopsis and prevent their degradation. The stabilization of COI1-JAZ interaction blocks the downstream JA signaling. Higher accumulation of HARP1 is evident in OS of *H. armigera* fed Arabidopsis or artificial diet supplemented with 0.1% gossypol (Chen et al., 2019). Identical to HARP1, REPAT38 from *S. exigua* interacts with JAZ proteins of host plants to hamper JA signaling. The secretion of effector like proteins through OS might help insects to feed on numerous plants by

modulating the defenses. *H. armigera* is a polyphagous insect, feeding on series of host plants. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant choice but also specific tissues of the host plant. Although *H. armigera* feeds on multiple plants still there are non-preferred crops like Capsicum, Arabidopsis. Our proteomic analysis of OS from *H. armigera* fed on tomato and Capsicum plants suggest differential accumulation of secretory proteins (Chapter 3). In this study we have selected six secretory proteins (HARP1 to 6) identified from OS of *H. armigera* for functional characterization. The sequence analysis of selected protein suggests their similarity with known effector like protein HARP1 from *H. armigera*. Further, we recombinantly expressed and purified the selected uncharacterized secretory proteins (HARP1 to 6). The application of recombinant HARPs on mechanically wounded tomato leaves shows differential expression of JA biosynthetic and responsive genes. However, no significant change was observed on the expression of SA biosynthetic and responsive genes.

4.2 Materials and methods:

4.2.1 In silico identification of effector/elicitor like proteins from generalist herbivore H. armigera

The transcriptome (Pearce et al., 2017) and proteomic data (Chapter 3) of *H. armigera* fed on various diets such as tomato, Capsicum (pepper), and artificial diet was analyzed to identify salivary secretory like proteins. Those sequence IDs annotated as probable salivary secretory protein was selected for further analysis. The selected sequences were run through the Signal P (Juan et al., 2019), TMHMM (Krogh et al., 2001), Target P (Juan et al., 2019), and WoLFPSORT (Horton et al., 2007) pipelines to predict effector/elicitor like properties.

4.2.2 Sequence and phylogenetic analysis of selected secretory proteins

The amino acid sequences of genes coding for salivary secretory proteins were compared with the HARP1 protein using the ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) program. The phylogenetic tree of Lepidopteran secretory proteins was constructed in MEGA5 (Tamura et al., 2011) with bootstrap value 1000.

4.2.3 Cloning and recombinant expression of salivary secreted proteins from H. armigera in bacterial expression vector

For functional characterization, the selected gene sequences without signal peptide were amplified from cDNA (using gene specific primer **Table 4.1**) and inserted into pET-28a vector. The HARPs + pET-28a constructs were transformed in shuffle T7 cells strain of *Escherichia coli* for recombinant expression. Thecells were grown at 37 °C till optical density at 600 nm

reached to 0.5 to 0.6. The culture was induced with 0.5 mM isopropyl 1-thio-D-galactopyranoside followed by incubation for 16 h at16 °C. The HARPs expressed shuffle cells were lysed in lysis buffer (50mM Tris-HCL, 100mMNaCl, 10% Glycerol, 10mM imidazole, pH 8.0). The lysate was centrifuged at 15000 rpm at 4

°C for 30 min and purification of recombinant proteins was carried out by affinity chromatography (Ni-NTA). The supernatant was passed through pre-equilibrated Ni-NTA resin. The bound recombinant HARPs (HARP1 to 6) were eluted with lysis buffer containing 250 mM of imidazole. The purity of recombinantly purified HARPs was checked on 15% SDS-PAGE and western blot using anti-His antibody.

4.2.4 Treatment of recombinant HARPs on mechanically wounded tomato leaves

Initially tomato plant leaves were mechanically wounded with pattern wheel. On the mechanically wounded leaves, 20 µg of recombinantly purified individual HARPs were applied and painted with brush. Similarly, mixture of all candidate HARPs (HARP1+HARP2+HARP3+HARP5, HARP4+HARP6) and *H. armigera* OS were applied on mechanically wounded tomato plants. The HARPs treated local leaves of tomato were collected at different timepoint (1, 4, 12 and 48 h) and snap frozen in liquid nitrogen.

4.2.5 Gene expression analysis by qRT-PCR of HARPs treated tomato leaves

The total RNA was isolated from tomato leaves by Spectrum Plant Total RNA kit (Sigma-Aldrich, USA). DNA-free RNA (1 µg) was used for cDNA synthesis using RevertAid First Strand cDNA Synthesis kit (Thermo scientific, USA). Quantitative real-time PCR (qRT-PCR) was carried out according to Shinde et al., 2017 and *SlActin* (Solyc11g005330) was used as housekeeping gene **(Table 4.2)**. PCR product specificity was confirmed by melting curve analysis. The generated threshold cycle (Ct) was used to calculate the gene expression of treated samples against control samples in terms of fold change (Schmittgen and Livak, 2008). Statistical analysis was carried out using Students t-test with $p<0.05$.

4.2.6 qRT PCR of selected HARPs in H. armigera fed on different diet

Total RNA was extracted from foregut, midgut and hindgut of 4th instar *H. armigera* larvae fed on artificial diet (AD), tomato (Host) and Capsicum (Non-host) plants using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with RNase-free DNAase I (Promega, Madison, WI, USA). 2 µg RNA was reverse-transcribed into cDNA with high-capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using TB Green Premix Ex Taq II (Tli RNase H Plus) (TaKaRa) with 7900HT Fast Real-Time PCR System with 384-Well Block Module (Applied Biosystems™). The expression values were

normalized using *β-actin* (Accession no.: AF286059) **(Table 4.3)**. For each biological replicate (15 insect), three technical replicates were analyzed.

Table 4.1: Primers used for recombinant expression of HARPs

Gene name	Forward primer	Reverse primer
HARP1	ATGGCTGCTCTGCAGCAGAACC	TTATCGGCCCCAGATTTCGATC
HARP2	ATGGCAGCTGAGCGCGCCGCC	TTATCGTCCCCAGATCTCGATGG
HARP5	ATGTCAGTGATCACGCACGAGG	TTAACGTCCCCAGATTTCAATCC

Table 4.2: Primers used for qRT PCR of tomato leaves

Protein name	Gene name	Forward primer	Reverse primer
Lipoxygenase	LOX_D	ACTCATCAGCACCGACATCG	ACTCTCCAGAAAGAACTCCTGC
12-oxophytodienoatereductase 3	OPR3	CAATAGATCATCTTGATGCCATG	CATATGCTACGTATCGTGGCTG
Protease inhibitor	PI-II	CTTCTTCCAACTTCCTTTG	TGTTTTCCTTCGCACATC
Threonine deaminase	TD	AACCCCCACCACCAACAGGT	AGCTCAAACACACGCGCTGGA

Table 4.3: Primers used for qRT PCR of HARPs

Gene name	Forward primer	Reverse primer
HARP1	GCCCCCATCGCGAACGCA	GTAGTAGCCCCAGCCGCGG
HARP5	ATGAGCTTGGGGAATATTGGGTC	CTGCCGGTATACCTCACGTTTA

4.3 Results:

4.3.1 Selection and sequence analysis of putative salivary effector like proteins from H. armigera (HARPs)

From the transcriptome sequences of *H. armigera* (Pearce et al., 2017) and our earlier proteomic data described in chapter 3, we identified the HARP1-like salivary secretory proteins. Three secretory proteins (Gene ID: HaOG211283, HaOG211284, and HaOG211285 (HARP2)) displayed 56 to 62% similarity with HARP1, whereas two others (Gene ID: HaOG211282 (HARP5) and HaOG211280) showed 38 to 42% similarity with HARP1 (**Table 4.4**). Two HARP2 isoforms named as HARP3 and HARP4, and one HARP5 isoform termed as HARP6, were PCR amplified along with selected HARPs **(Fig. 4.1)**. H80Y and N77S substitutions were detected in HARP3 and HARP4, respectively, whereas M76V substitution was found in HARP6 (**Fig. 4.1; Table 4.4).** The proteins with similar amino acid substitution

were detected in *H. armigera* OS (shown in chapter 3). Further bioinformatics analysis revealed that the proteins (Uniport ID: HaOG211285; HARP2) and (Uniport ID: HaOG211282; HARP5) both contain signal peptides, similar to HARP1 (**Fig. 4.2**). Target P and WolfPSORT predicted that the selected proteins were secretory (S1 reliability class) and extracellular (Extr: >18), respectively (**Table 4.5**). In all five uncharacterized proteins (HAPR2 to 6), domain analysis revealed the existence of a conserved multiprotein bridge factor (MBF2) domain similar to HARP1. Further, the phylogenetic analysis revealed that HARP1 with HARP2, HARP3 and HARP4 are in one clade may be due to their high sequence similarity whereas, HARP5 and HARP6 is in another clade (**Fig. 4.3**). Purified recombinant HARPs appeared as single protein band on SDS-PAGE and western blot (**Fig. 4.4**). Further, the approximate molecular weight of HARP1, HARP2, HARP3, and HARP4 were around 14 kDa while HARP5 and HARP6 were around 25 kDa was observed on SDS-PAGE and western blot (**Fig.4.4**). These masses were corroborated with the theoretical values of the respective HARPs. Based on these characteristics, we postulated that the uncharacterized salivary secretory proteins of *H. armigera* may have either elicitor or effector activity in modulating plant defense.

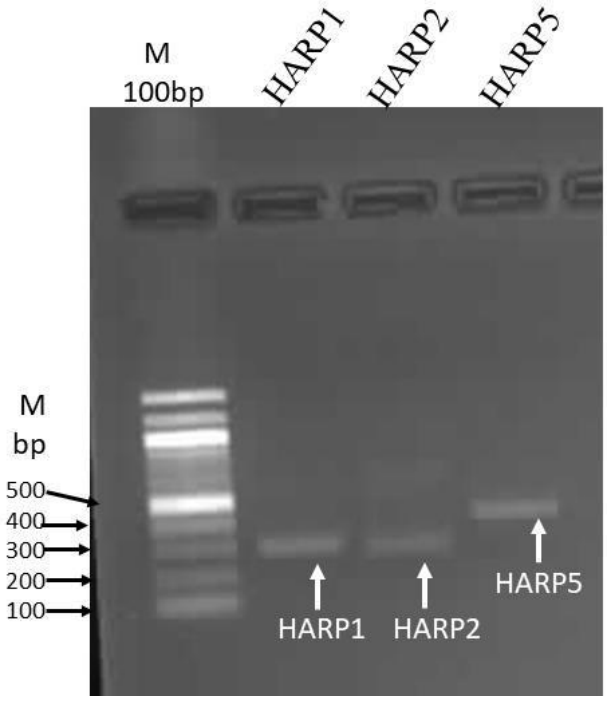


Figure 4.1: PCR amplification of genes coding for salivary secretory protein from *H. armigera.*

Table 4.4: The sequence similarity index of salivary secretory proteins identified from the transcriptome (Pearce et al., 2017) and proteome data (Chapter 3) of *H. armigera* with HARP1

Database	Accession number	Length (aa)	% Identity with HARP1	Highlighted Substituted amino acids
CSIRO	HaOG211280	270	38	
CSIRO	HaOG211281	112	34.82	
CSIRO	HaOG211283	107	61.68	
CSIRO	HaOG211284 (HARP4)	121	61.86	
CSIRO	HaOG211285 (HARP2)	119	61.02	NHGH SGSA
CSIRO	HaOG213605	136	38.02	
CSIRO	HaOG203518	162	37.82	
CSIRO	HaOG202635	136	36.75	
CSIRO	HaOG215392	146	35.45	
	HARP3	119	58.59	NHGY SGSA
	HARP4	119	57.58	SHGY SGSA
CSIRO	(HaOG211282) HARP5	164	40.20	NGMS
	HARP6	164	39.22	NGVS

*Blue coloured genes (Accession number) were selected for functional characterization
* The amino acid substitutions were highlighted in green colour

Table 4.5: *In silico* predicted features of salivary secretory proteins *H. armigera*

Gene ID	Signal Peptide	Target P	TMHs	TMHMM	Wolfpsort
HARP1	Present	S 1	TMHs: 0	Exp number of AAs in TMHs: 9.66	extr: 30
HaOG211280	Present	S 1	TMHs: 0	Exp number of AAs in TMHs: 6.48	extr: 19, plas: 7, E.R.: 5
HaOG211281	Absent	S 5	TMHs: 0	Exp number of AAs in TMHs: 0.02	extr: 12, cyto: 6, cysk: 6, nucl: 3, mito: 2
HaOG211282 (HARP5)	Present	S 1	TMHs: 0	Exp number of AAs in TMHs: 0.81	extr: 31
HaOG211283	Present	S 2	TMHs: 0	Exp number of AAs in TMHs: 2.63	extr: 29
HaOG211284 (HARP4)	Present	S 2	TMHs: 1	Exp number of AAs in TMHs: 17.78	extr: 30
HaOG211285 (HARP2)	Present	S 1	TMHs: 0	Exp number of AAs in TMHs: 16.88	extr: 31
HaOG213605	Present	S 1	TMHs: 0	Exp number of AAs in TMHs: 12.23	extr: 23, pero: 4, E.R.: 3
HaOG203518	Absent	-	TMHs: 0	Exp number of AAs in TMHs: 19.26	extr: 22, pero: 5, mito: 2
HaOG202635	Present	S 1	TMHs: 0	Exp number of AAs in TMHs: 10.09	extr: 22, pero: 5, E.R.: 2
HaOG215392	Present	S 1	TMHs: 1	Exp number of AAs in TMHs: 24.98	extr: 31

*Blue colored genes (Accession number) were selected for functional characterization

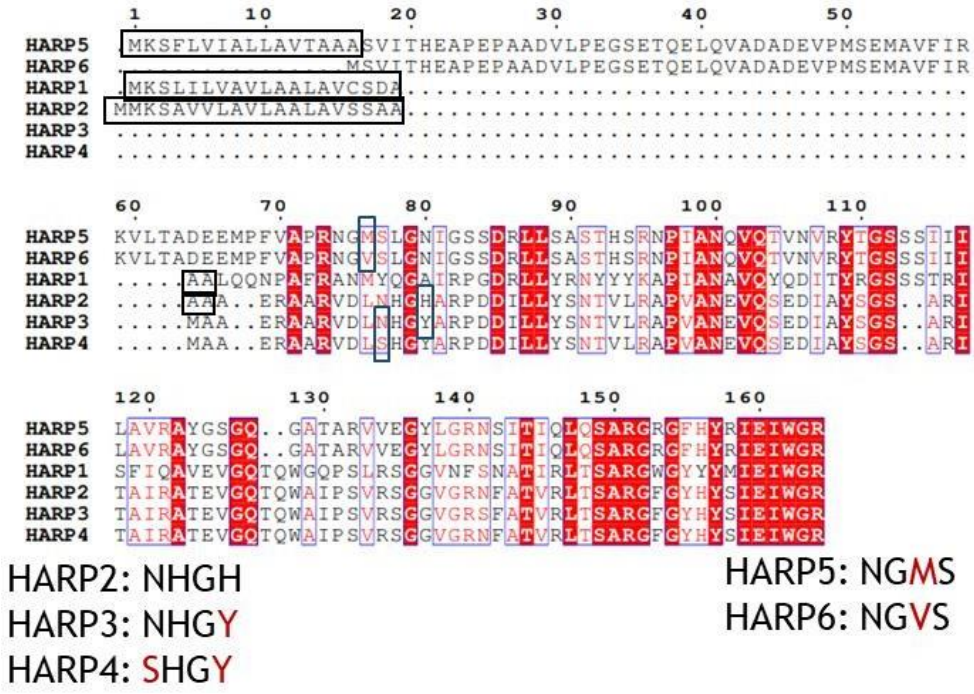


Figure 4.2: Multiple sequence alignment for uncharacterized salivary secretory proteins with characterized effector protein (HARP1) from *H. armigera*. The alignment was generated by ClustalW. Sequence in black box code for signal peptide (Predicted by Signal P4.0). The image is created by Espript 3.0 (Robert and Gouet, 2014). The single amino acid substitution was highlighted with blue vertical box.

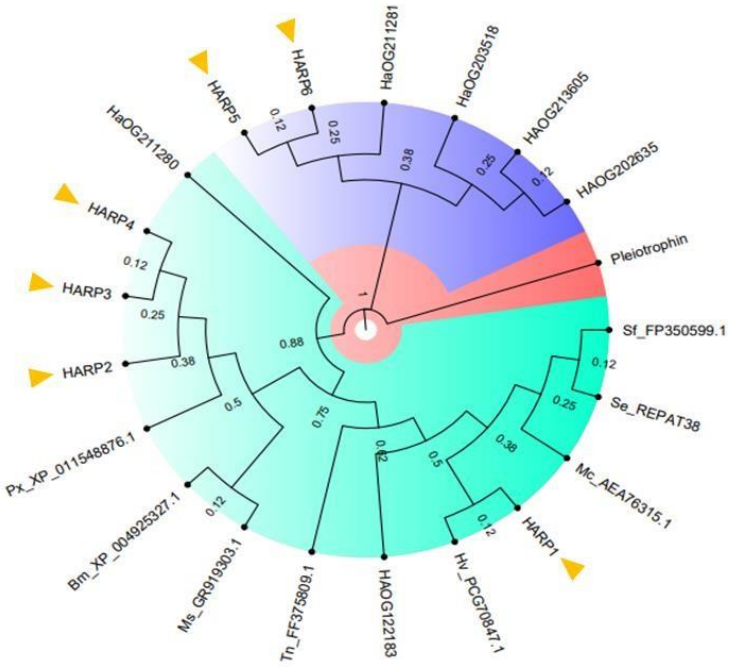


Figure 4.3: Phylogenetic tree of salivary secreted proteins from Lepidoptera insects. HARP1 (*H. armigera*) and the homologous proteins in *Heliothis virescens*, *Spodoptera*

frugiperda, *Spodoptera exigua*, *Agrotis ipsilon*, *Mamestra configurata*, *Trichoplusia ni*, *Hyphantria cunea*, *Manduca sexta*, *Bombyx mori*, and *Plutella xylostella* were analyzed. Orange colour marked proteins of *H. armigera* were selected for functional characterization.

4.3.2 Recombinantly purified HARP1-like proteins modulates JA biosynthesis and responsive gene expression in tomato plants

The phytohormone JA is involved in the activation of defense mechanisms against biotic threats, such as chewing insects (Wu et al. 2010). Chen et al. 2019, recently revealed that the homologous secretory proteins HARP1 and REPAT38 from lepidoptera insects influence JA signaling in Arabidopsis plants. In earlier proteomic study (Chapter 3) we observed the accumulation of uncharacterized HARP1 like secretory proteins in *H. armigera* OS. Based on this we hypothesized that the *H. armigera* secretory proteins as individual or in combination might differentially alter the wound induced defense in tomato leaves.

4.3.2.1 Impact of individual HARPs on wound induced JA biosynthesis and response in tomato leaves

In mechanically wounded leaves, overexpression of JA biosynthetic genes *SlLOX_D* (*LIPOXYGENASE*) (>10-fold; p-value <0.05) and *SlOPR3* (*12-OXOPHYTODIENOATE REDUCTASE 3*) (>17-fold; p-value <0.05) was observed at early timepoint (1 h) as compared to control (unwounded) leaves. Further, the higher expression of JA responsive genes *SlPI-II* (*PROTEASE INHIBITOR-II*) (>6-fold; p-value <0.05) and *SlTD* (*THREONINE DEAMINASE*) (>13-fold; p-value <0.05) was observed after 12 h of mechanical wounding in tomato leaves as compared to control leaves (**Fig. 4.5**). The treatment of HARP2, HARP4, HARP5 and HARP6 caused significant induction of *SlLOX_D* (>15-fold; p-value <0.05) after 1 h as compared to untreated wounded and HARP1-treated leaves (upto 11-fold) (**Fig. 4.6A**). The expression of *SlOPR3* (<13 fold; p-value <0.05) was reduced after 1 h treatment of HARPs except HARP2 as compared to wounded leaves **(Fig. 4.6B)**. However, no significant change was observed in both *SlLOX_D* and *SlOPR3* expression at 4, 12 and 48 h as compared to wounded leaves (**Fig. 4.6A and 4.6B**). On the other hand, JA-responsive genes showed alteredexpression pattern at 4 and 12 h timepoints against HARPs treatment (**Fig. 4.6C and 4.6D**). HARP2, HARP4, HARP5 and HARP6 treatment showed significant overexpression of *SlPI-II*at 4 h (>8-fold; p-value <0.05), and HARP1 treatment showed the same at 12 h (**Fig. 4.6C**). The wound induced level of *SlPI-II* was reduced in HARP2, HARP3, HARP5 and HARP6 treated tomato leaves after 12 h (**Fig. 4.6C**). Likewise, the transcript level of another JA responsive gene *SlTD* showed significant variation at 12 h timepoint. HARP1 treatment resulted in increased expression

of *SlTD*, whereas HARP3, HARP5 and HARP6 treatment reduced the level of *SlTD* (**Fig. 4.6D**). The expression of JA biosynthesis and responsive geneswere similar in HARPs treated tissue to that of mechanically wounded tissue after 48 h of treatment (**Fig. 4.6A-D**). Differential regulation of JA biosynthesis and response against individual HARP treatment suggest that, during feeding the HARPs may be secreted in combination.

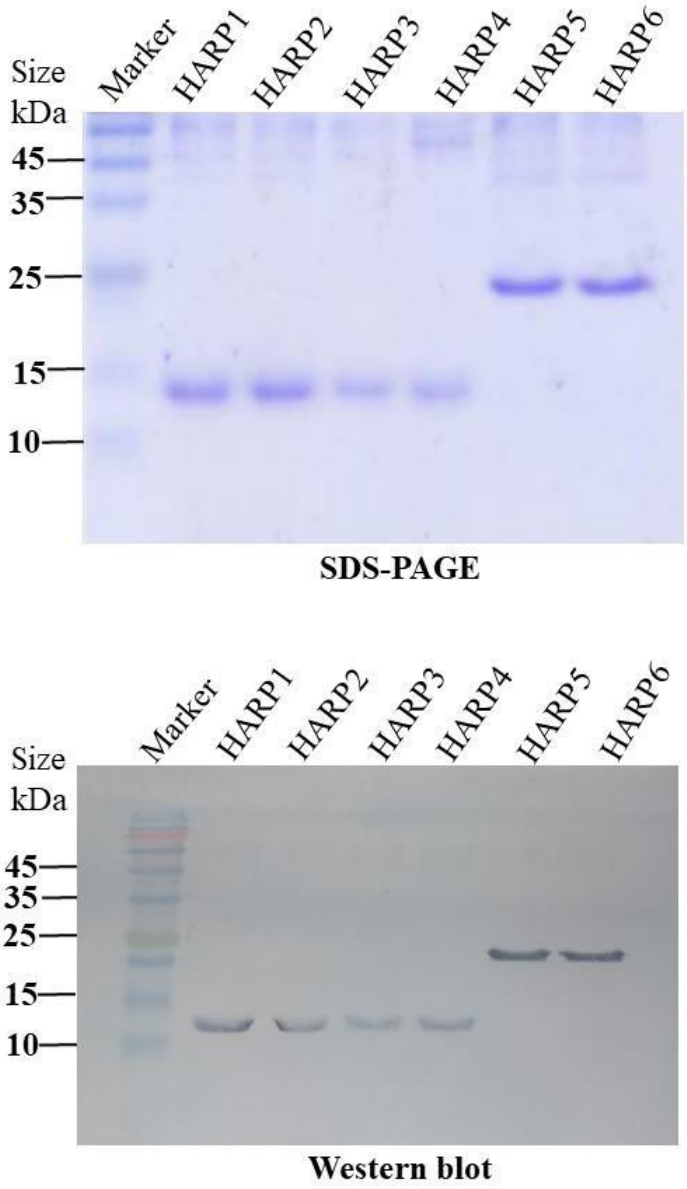


Figure 4.4: SDS-PAGE (15%) and western blot with anti-his antibody of recombinantly purified HARPs.

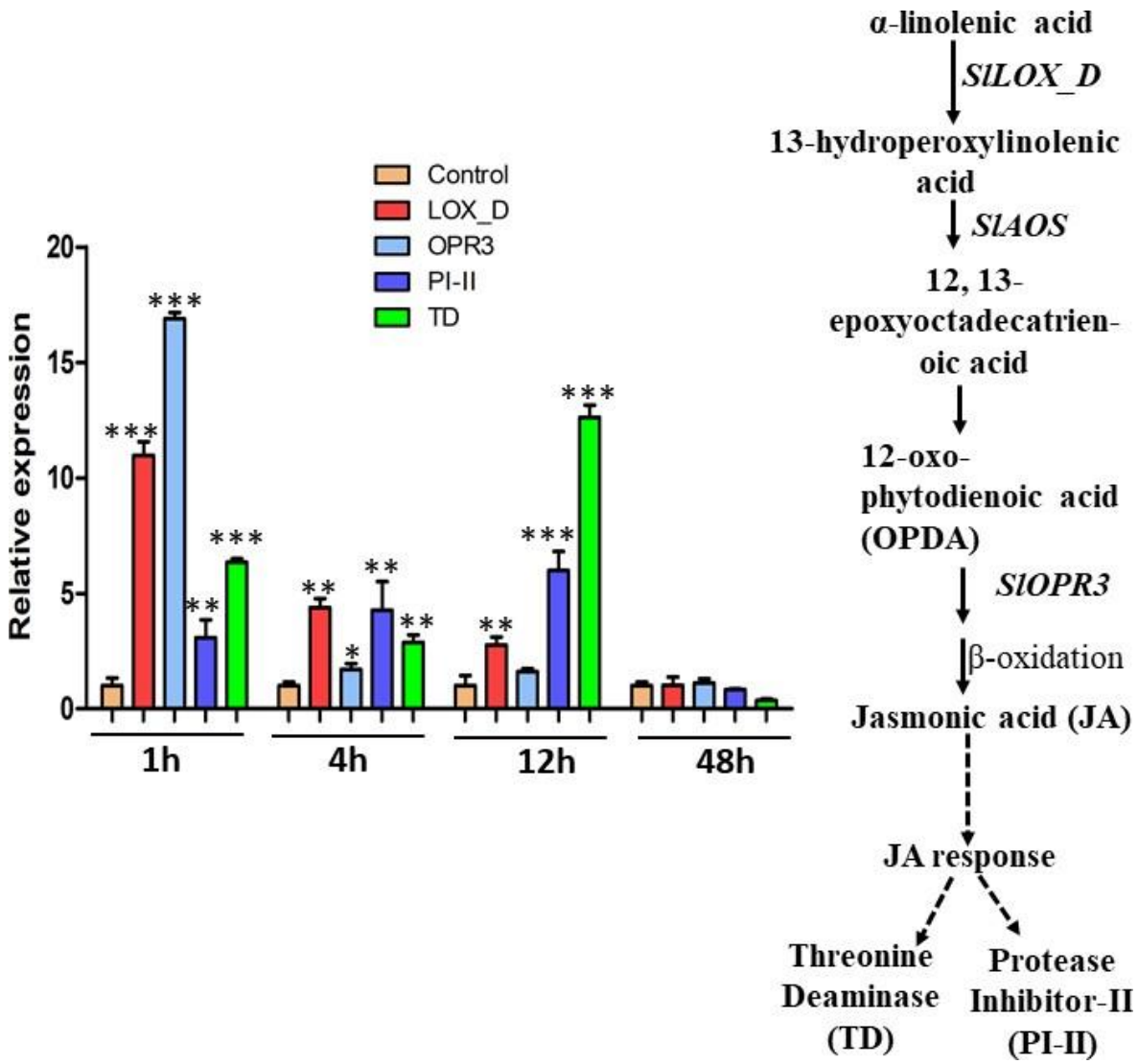


Figure 4.5: Impact of mechanical wounding on the expression of Jasmonic acid biosynthetic and responsive genes. qRT-PCR based relative expression of these genes were performed after 1, 4, 12 and 48 h of wounding using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Number of stars indicate significant difference (P < 0.05= *, <0.01=**, <0.001=***). *A. SlLOX (LIPOXYGENASE), B. SlOPR3 (12-OXOPHYTODIENOATE REDUCTASE 3), C. SlPI-II (PROTEASE INHIBITOR-II), D. TD (THREONINE DEAMINASE).*

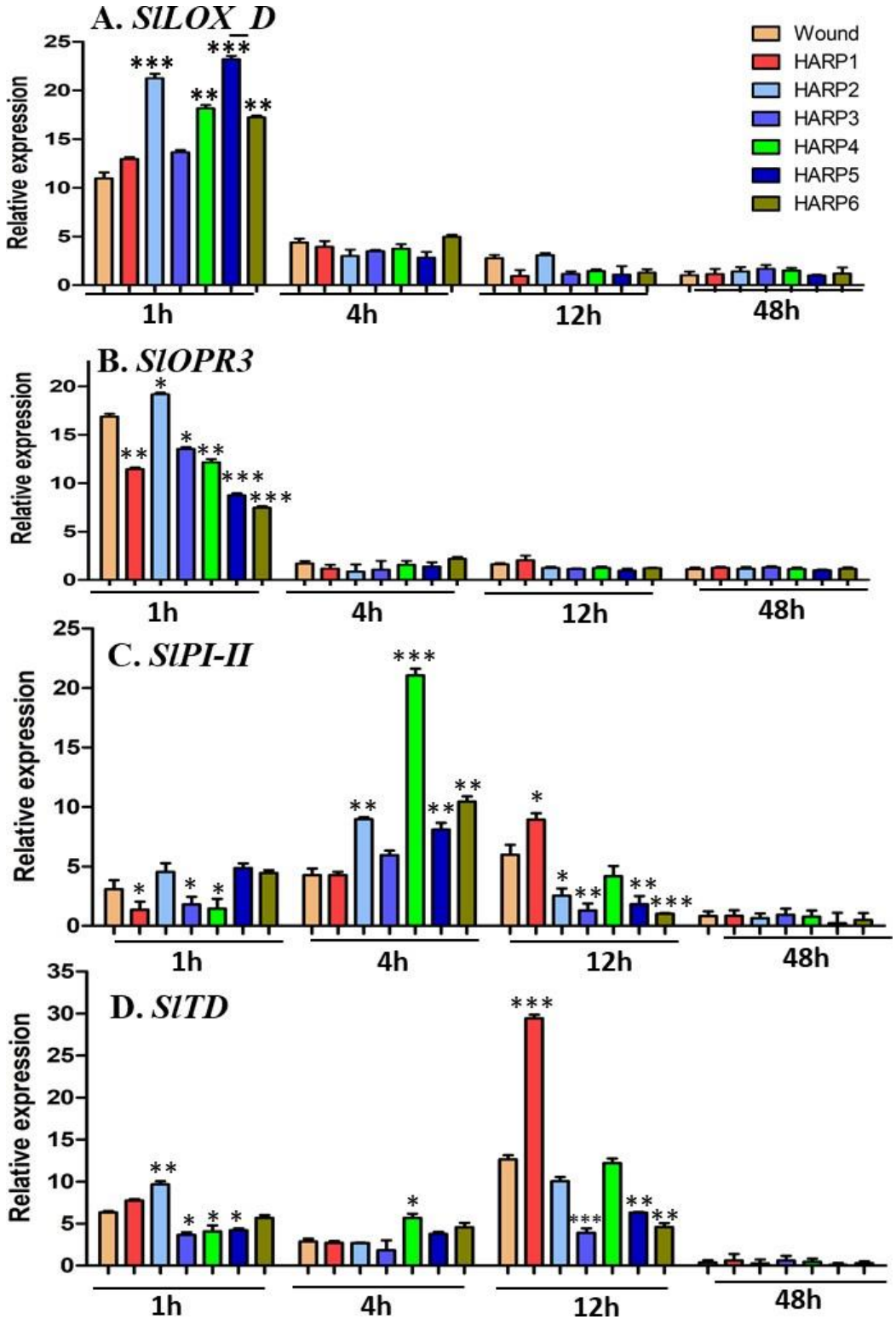


Figure 4.6: Expression profiles of Jasmonic acid biosynthetic and responsive genes in tomato leaves against individual HARPs treatment. qRT-PCR based relative expression of these genes were performed after 1, 4, 12 and 48 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three

technical replicates. Fold change was calculated by dividing sample values against control values. Number of stars indicate significant difference (P < 0.05= *, <0.01=**, <0.001=***). *A.SlLOX (LIPOXYGENASE), B. SlOPR3 (12-OXOPHYTODIENOATE REDUCTASE 3), C. SlPI-II (PROTEASE INHIBITOR-II), D. TD (THREONINE DEAMINASE)*.

4.3.2.2 Combination of HARPs differentially alter JA biosynthesis and responsive genes in tomato leaves

Differential expression of JA biosynthetic and responsive genes was observed in response to mixture of all six HARPs, HARP4+HARP6, HARP1+HARP2+HARP3+HARP5 and OS of *H. armigera*. The expression of *SlLOX_D* was increased in all the treatments after 1h as compared to wounded leaves. Highest expression of *SlLOX_D* was observed in HARP4+HARP6 treatment (>20 fold; p-value <0.05) after 1 h (**Fig. 4.7A**). However, this expression level was similar to wound response at 4 and 12 h. In case of *SlOPR3*, the expression was reduced in all the treatments as compared to mechanical wounding and the least expression was seen in HARP4+HARP6 treatment (<10-fold; p-value <0.05) (**Fig. 4.7B**). Similar to *SlLOX_D*, the expression of *SlOPR3* showed no significant variation at later timepoints across all the treatments **(Fig. 4.7A and 4.7B)**. The JA responsive genes showed variable expression pattern at different timepoints. In case of *SlPI-II*, treatment of all HARPs resulted in significant reduction in the expression at 1 and 12 h whereas, no significant change was observed at 4 h treatments (**Fig. 4.7C**). On the contrary, HARP1+HARP2+HARP3+HARP5 and OS showed substantial upregulation of *SlPI-II* (>5-fold; p-value <0.05) at 1 h and this expression was reduced at later timepoints as compared to wounding (**Fig. 4.7C**). However, HARP4+HARP6 showed a massive increase in the *SlPI-II* expression level at 4 h (>10-fold; p-value <0.05) which was reduced to normal level at 12 h. Similar to *SlPI-II*, *SlTD* also showed variation in the expression pattern against HARPs (**Fig. 4.7D**). Treatment of all HARPs, HARP4+HARP6 and OS reduced the *SlTD* level significantly at 1 h, however induced expression was observed after 4 h in HARP4+HARP6 treatment. The 4 h treatment of all HARPs, HARP1+HARP2+HARP3+HARP5 and OS resulted in reduced expression of *SlTD* **(Fig. 4.7D)**. At 12 h the expression level of *SlTD* was induced in response to HARP4+HARP6 treatment whereas the HARP1+HARP2+HARP3+HARP5 and OS-treated leaves showed significant reduction in *SlTD* expression as compared to wounded leave (**Fig. 4.7D**).

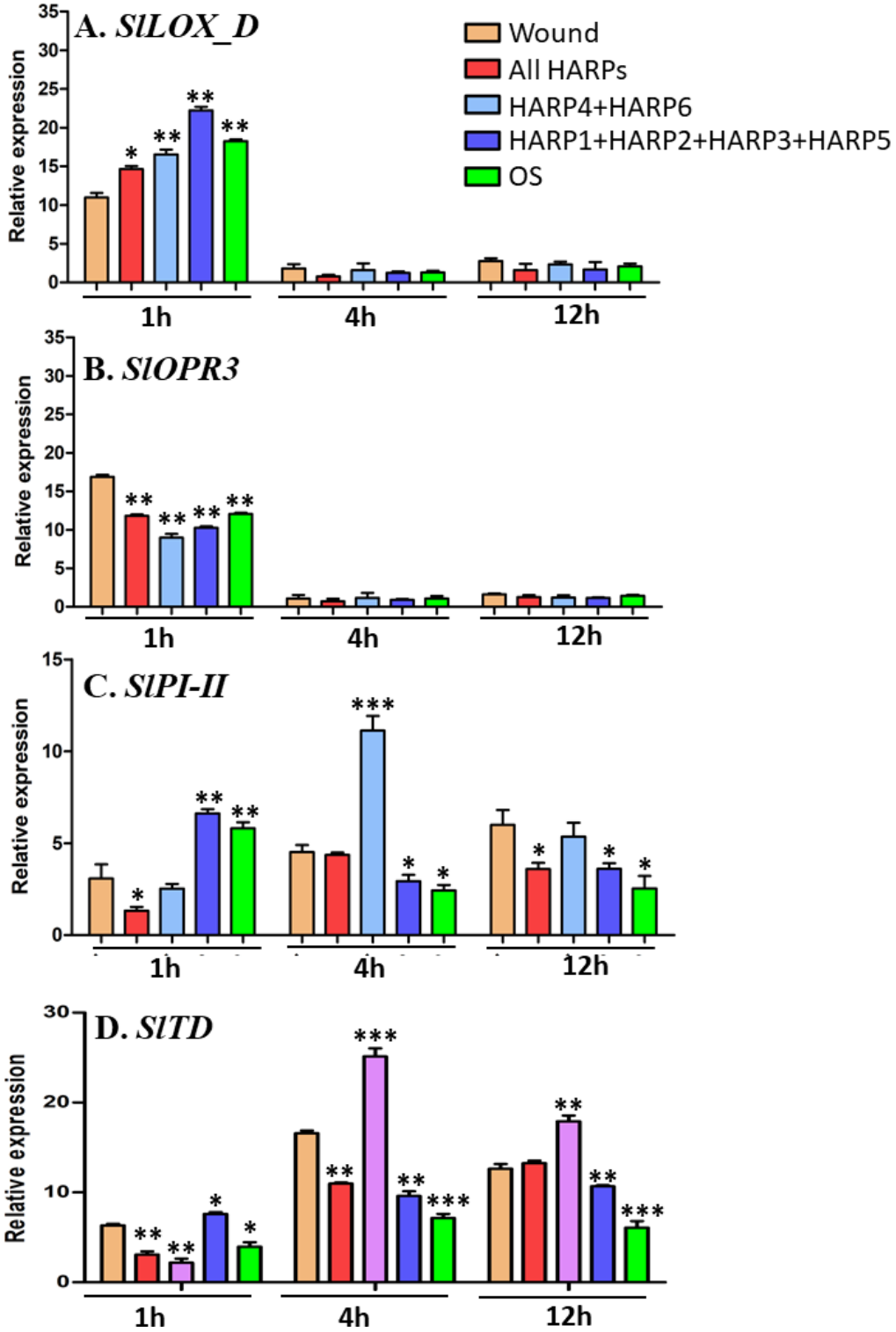


Figure 4.7: Expression profiles of jasmonic acid biosynthetic and responsive genes in tomato leaves against combination of HARPs treatment. qRT-PCR based relative expression of these genes were performed after 1, 4 and12 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control

values. Different stars indicate significant difference (P < 0.05= *, <0.01=**, <0.001=***). *A. SlLOX (LIPOXYGENASE), B. SlOPR3 (12-OXOPHYTODIENOATE REDUCTASE 3), C. SlPI-II (PROTEASE INHIBITOR-II), D. TD (THREONINE DEAMINASE).*

4.3.3 Unaltered expression of SA biosynthesis and responsive genes in HARPs treated tomato leaves

The crosstalk between the SA and JA pathways aids plants in initiating defenses based on the type of insect or pathogen attack (Koornneef and Pieterse, 2008; Thaler et al., 2012). In this study, we observed that the transcript levels of SA biosynthetic genes *SlPAL* and *SlICS* were similar in HARPs-treated and mechanically wounded tomato leaves. **(Fig. 4.8A and 4.8B)**. Similarly, in HARPs-treated tomato leaves, the expression of SA-responsive gene *SlPR1* was unaffected as compared to mechanically wounded leaves **(Fig. 4.8C)**.

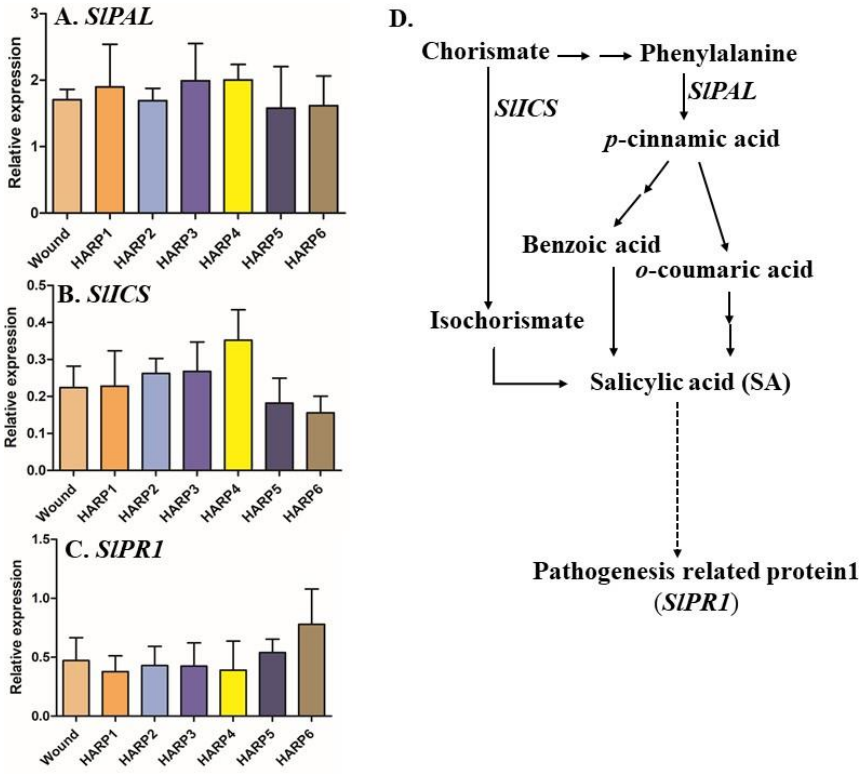


Figure 4.8: Expression profiles of salicylic acid biosynthetic and responsive genes in tomato leaves against individual HARPs treatment. qRT-PCR based relative expression of these genes were performed after 4h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. *A. SlPAL (PHENYLALANINE AMMONIA-LYASE), B. SlICS (ISOCHORISMATE SYNTHASE), C. SlPR1 (PATHOGENESIS-RELATED PROTEIN 1)*. D. Salicylic acid biosynthetic pathway.

4.3.4 Diet influences the expression of salivary secretory proteins in H. armigera

The secretory proteins are differentially accumulated in OS of *H. armigera* fed on different diet (Chapter 3). Further we observed dietary influence on transcript abundance of HARPs in different tissues like foregut, midgut and hindgut of *H. armigera*. The induced transcript level was observed for both HARP1 and HARP5 in all three tissues when fed on Capsicum (Non-host) as compared to tomato (host) leaves and artificial diet (AD) **(Fig. 4.9A and B).**

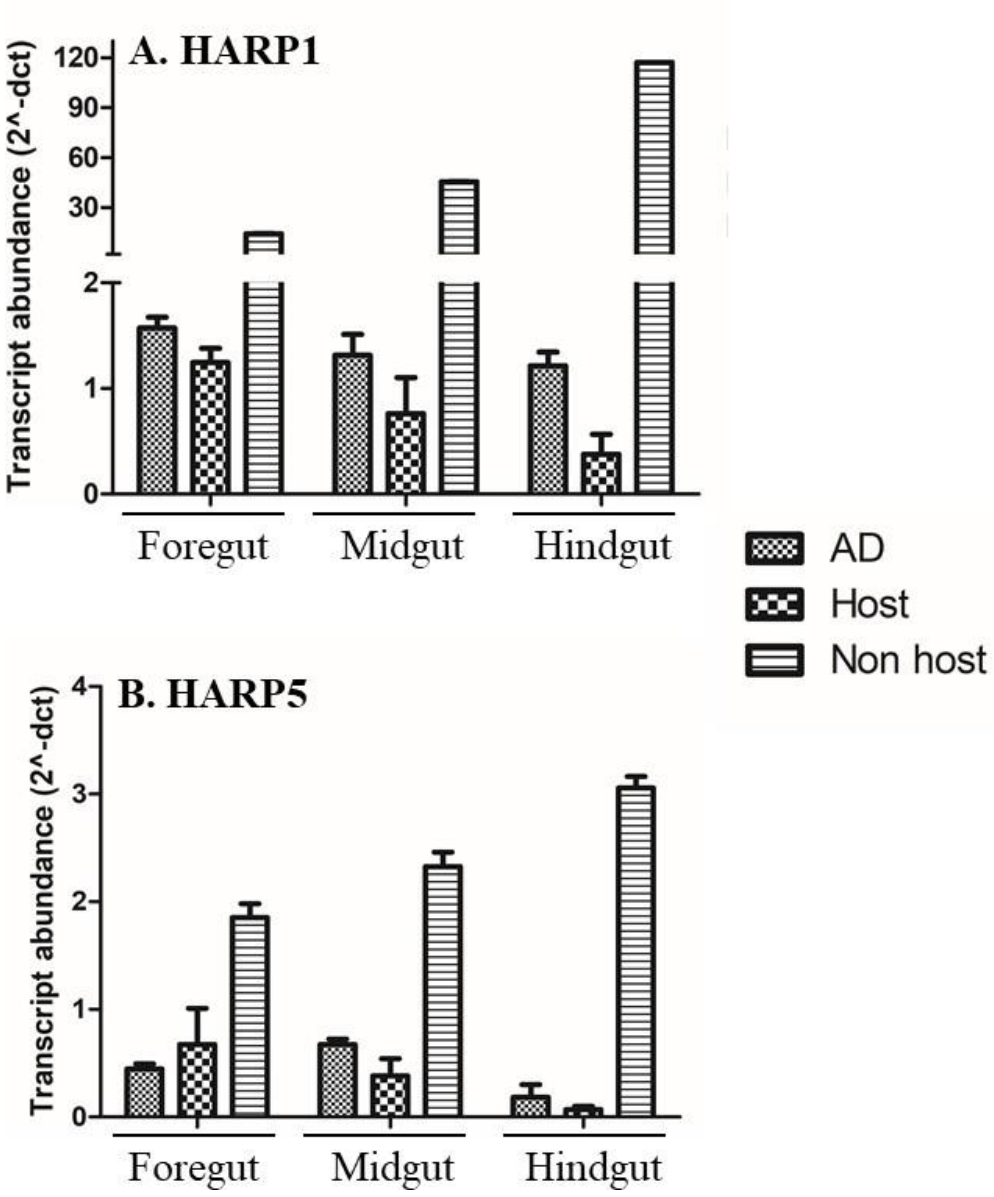


Figure 4.9: Transcript abundance of A. HARP1 and B. HARP5 in different tissue of *H. armigera* fed on artificial diet (AD), tomato (Host) and Capsicum (Non-host) plants. qRT-PCR based transcript abundance was calculated using as internal control *β-actin*.

4.4 Discussion:

Plants reprogram their gene expression patterns in response to environmental stresses, triggering chemical and physiological responses (Bostock et al., 2014). A plant's reaction to insect herbivore damage includes recognition of insect-derived molecules and wound induced defense response (Erb et al., 2012). Cross-talk between plant phytohormones like JA and SA as a key role in plant defense is well established (Koornneef and Pieterse, 2008; Thaler et al., 2012). The phytohormone JA is vital for controlling plant induced defense against insect herbivores (Consales et al., 2012). The plant defense system is stimulated by feeding damage and the detection of bioactive molecules from insect OS, such as FACs and glucosidase (*Pieris brassicae*). However, in addition to elicitor-like molecules, insects have devised a complex system to deal with induced plant defense by secreting effector-like proteins via the OS. Although many sucking insect effector-like proteins have been found, only a few have been identified in chewing insect. A recent salivary proteome analysis of two FAW strains (the"corn strain" and the "rice strain") fed on different host plants discovered several protein categories, including salivary proteins that could be involved in plant defense modulation (Acevedo et al., 2017).

H. armigera secretes HARP1-like effector proteins discovered from OS at feeding locations, according to Chen et al., 2019. The HARP1 stabilizes the JAZ-COI interaction, regulating JAZ degradation and resulting in JA signaling suppression. Because of the hampered defensive signaling, wound-induced PIs build-up was reduced. In *H. armigera*, our proteome and sequencing study of OS suggests the existence of uncharacterized HARP1-like proteins. The *SlLOX_D* gene, which encodes a chloroplast Lipoxygenase that may play a role in the octadecanoid defense-signaling system is up-regulated in leaves in response to wounding (Heitz et al., 1997). HARP2, HARP4, HARP5, and HARP6 treatment, on the other hand, resulted in a considerable induction of *LOX_D* at an early timepoint following wounding. For more than a decade, threonine deaminase (TD) has been used to assess the impact of wounding and elicitation of JA in potato (*Solanum tuberosum*) and tomato (Dammann et al., 1997; Hildmann et al., 1992; Samach et al., 1995). In tomato plants, abscisic acid and JA signaling are involved in wound-induced *SlTD* expression (Hildmann et al., 1992). In tomato leaves the wound-inducible PIs provide an appealing paradigm for understanding the signal transduction pathways (Yan et al., 2013). Plants recognize insect/herbivory-associated molecular patterns (HAMPs) to elicit and/or modify defense responses in a similar way to wounds (Arimura et al., 2011). When we applied recombinant HARP1 on wounded tomato plant, we noticed an

increase in the levels of two important plant defense proteins, *SlPI* and *SlTD*. GOX from lepidopteran insects has a dual role, acting as elicitor in tomato plants and effector in *Nicotiana tabacum* (Louis et al., 2013; Musser et al., 2002; Tian et al., 2012). (Schmelz et al., 2012) showed that a specialized herbivore reduces defense activation by transforming an elicitor into an antagonist effector. In tomato plants, uncharacterized HARPs such as HARP2 and HARP3 proteins which are substituted with a single amino acid suppress wound-induced *SlPI* and *SlTD*. Likewise, *H. zea* saliva ATPases have been known as wound-induced response suppressor in tomato plants (Wu et al., 2012). However, when combination of recombinantly pure HARPs are applied to damaged tomato plants, the expression of JA biosynthetic and responsive genes is modulated differently. In response to HARP4+HARP6, enhanced expression of *SlPI-II* and *SlTD* was observed, however the application of other HARPs in combination suppressed the wound-induced response in tomato plants. However, as time goes on, certain plants are able to overcome this suppression once they have adapted to recognize the insect's molecules (Hogenhout and Bos, 2011). When chemical signals are generated owing to wounding and insect attack in any location of the plant, specific receptor molecules in plants are activated and transduce the signaling cascade in plants, resulting in the activation of defense mechanisms (Mithöfer and Boland, 2008). From plants perspective, only a few receptors that interact with elicitor and effector molecules have been identified. It is necessary to gain a better knowledge of the influence of insect secretory proteins with single amino acid substitutions on plant defense. However, it is unclear how plants recognize the single amino acid substituted insect proteins as elicitors or effectors and modulates their defense mechanism. In addition, further characterization of insect proteins as elicitors or effectors, as well as their plant-interacting partner, will add to our understanding of plant-insect coevolution.

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Abstract

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Title of the thesis: Deciphering role of different diets on herbivore oral constituents and their role in modulating plant defense

Little is known about how different plant-based diets influence the insect herbivores' oral secretion (OS) composition and eventually the plant defense responses. We collected the OS from *Helicoverpa armigera* fed on the host tomato (OS_H), non-host *Capsicum* (OS_{NH}) plants, and artificial diet (OS_{AD}). Interestingly, the treatment of *H. armigera* OS_{AD}, OS_H and OS_{NH} on wounded tomato leaves showed a differential expression of genes involved in biosynthesis and/or signaling pathways of jasmonic acid (JA), salicylic acid (SA) chlorogenic acid (CGA), and trehalose. There was higher expression of genes involved in the phenylpropanoid biosynthesis pathway, which may lead to the increased accumulation of CGA and related metabolites. Specifically, high levels of CGA were detected after OS_H and OS_{NH} treatments in tomato leaves. The insect bioassays demonstrated that CGA significantly inhibits *H. armigera* larval growth. Further, we analyzed the metabolite accumulations in OS_{AD}, OS_H and OS_{NH} using Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry. We observed that OS_H and OS_{NH} samples were enriched with alkaloids and terpenoids, respectively; while OS_{AD} sample was abundant in phospholipids. Also, we found differential accumulation of fatty acid amino acid conjugates in *H. armigera* OS. Further, in proteomic analysis of *H. armigera* OS, we found the differential and diet specific accumulation of proteins in OS. The *in-silico* analysis of proteomics data gave insights on the accumulation of salivary secretory proteins, which possess high similarity with *Helicoverpa armigera* R-like protein 1 (HARP1 a known effector protein of *H. armigera)*. Also, we observed the presence of single amino acid substituted uncharacterized HARP1 like secretory proteins in OS. For functional characterization, we have recombinantly expressed and purified the HARP1-like secretory proteins (HARP1 to 6) of *H. armigera*. The application of recombinantly purified HARPs individually as well as in combination on wounded tomato plants shows the differential expression of JA biosynthesis ad responsive genes expression without affecting SA biosynthetic and responsive genes. We also observed the higher transcript abundance of HARP1 and HARP5 in foregut, midgut and hindgut of *H. armigera* fed on Capsicum (non-host) as compared to tomato (host) leaves and artificial diet. Overall, our work highlights the role of diet on differential accumulation of bioactive molecules in insect OS and the OS components differentially modulate the tomato plant defense.

• G.S. Kallure, A. Kumari, B.A. Shinde, A.P. Giri, Characterized constituents of insect herbivore oral secretions and their influence on the regulation of plant defenses, **Phytochemistry**, 193 (2022) ,113008.

• G.S. Kallure, A. Kumari, B.A. Shinde, V. T. Barvkar, A.P. Giri, Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense, **Plant Science**, 314 (2022), 111120.

List of papers with abstract presented (poster) at National/International conferences or seminars

Oral presentation of my research work at international conference on INSECT AND PLANT BIOLOGY:2021

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Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense

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Abstract:

Little is known about how different plant-based diets influence the insect herbivores' oral secretion (OS) composition and eventually the plant defense responses. We analyzed the OS composition of the generalist Lepidopteran insect, *Helicoverpa armigera* feeding on the host plant tomato (OS_H), non-host plant capsicum (OS_{NH}), and artificial diet (OS_{AD}) using Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry. Higher numbers and levels of alkaloids and terpenoids were observed in OS_H and OS_{NH}, respectively while OS_{AD} was rich in phospholipids. Interestingly, treatment of *H. armigera* OS_{AD}, OS_H and OS_{NH} on wounded tomato leaves showed differential expression of (i) genes involved in JA and SA biosynthesis and their responsive genes, and (ii) biosynthetic pathway genes of chlorogenic acid (CGA) and trehalose, which exhibited increased accumulation along with several other plant defensive metabolites. Specifically, high levels of CGA were detected after OS_H and OS_{NH} treatments in tomato leaves. There was higher expression of the genes involved in phenylpropanoid biosynthesis, which may lead to the increased accumulation of CGA and related metabolites. In the insect bioassay, CGA significantly inhibited *H. armigera* larval growth. Our results underline the differential accumulation of plant and insect OS metabolites and identified potential plant metabolite(s) affecting insect growth and development.

Keywords: Chlorogenic acid, Defense, Metabolites, Non-host, Oral secretion, Trehalose

Phytochemistry 193 (2022) 113008

Contents lists available at ScienceDirect

Phytochemistry

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

Characterized constituents of insect herbivore oral secretions and their influence on the regulation of plant defenses

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ARTICLE INFO

Keywords:
Effector
Elicitor
Herbivore insects
Oral secretion
Plant defense
Suppressor

ABSTRACT

For more than 350 million years, there have been ongoing dynamic interactions between plants and insects. In several cases, insects cause-specific feeding damage with ensuing herbivore-associated molecular patterns that invoke characteristic defense responses. During feeding on plant tissue, insects release oral secretions (OSs) containing a repertoire of molecules affecting plant defense (effectors). Some of these OS components might elicit a defense response to combat insect attacks (elicitors), while some might curb the plant defenses (suppressors). Few reports suggest that the synthesis and function of OS components might depend on the host plant and associated microorganisms. We review these intricate plant-insect interactions, during which there is a continuous exchange of molecules between plants and feeding insects along with the associated microorganisms. We further provide a list of commonly identified inducible plant produced defensive molecules released upon insect attack as well as in response to OS treatments of the plants. Thus, we describe how plants specialized and defense-related metabolism is modulated at innumerable phases by OS during plant-insect interactions. A molecular understanding of these complex interactions will provide a means to design eco-friendly crop protection strategies.

1. Introduction

For more than 350 million years, there have been ongoing dynamic interactions between plants and insects (Ehrlich and Raven, 1964; Mishra et al., 2015). Based on the host range of the insects, they have been classified as generalists or specialists (Ehrlich and Raven, 1964). Generalists feed on several plant species from different families, whereas specialists feed on one or more plant species of the same family. Another classification is based on their feeding strategies inflicting mechanical damage of different intensities on plants. Large herbivore insects are chewing insects that cause damage, with sharp and powerful mandibles evolved for munching, snipping, or tearing. Sucking insects have needle-like mouthparts (stylets) used to suck content from specific cells, such as phloem and xylem feeders, causing overall less physical damage. In all cases, oral secretions (OSs) of insects are bound to be encountered by the plants and potentially play an important role in insect-plant interactions (Acevedo et al., 2015; Alborn et al., 1997; Basu et al., 2018; Chung et al., 2013; Felton and Tumlinson, 2008; Lou and Baldwin, 2003; Ray et al., 2015; Reymond, 2013). Similarly, the process of digesting plant tissue might also affect these interactions (Lomate and Bonning, 2016).

Many plants respond to feeding damage by a rapid release of lipoxygenase pathway produced green leafy volatiles and by upregulating a more complex defensive response to OS-related herbivore-associated molecular patterns (Acevedo et al., 2015; Chen and Mao, 2020; Felton et al., 2014; Paré and Tumlinson, 1999; Qi et al., 2018; Schmelz, 2015; Yoshinaga, 2016). Early signaling responses to insect damage might start with calcium flux, variation of plasma membrane potential, reactive oxygen species production, and phosphorylation cascades (Farmer et al., 2020; Zebelo and Maffei, 2015), which can further lead to systemic signaling affecting parts of the plant distant from the damaged tissue. (Schilmiller and Howe, 2005; Turlings and Tumlinson, 1992; Zebelo and Maffei, 2015). Thus, specific defense preparation proceeds by signaling networks through modulating the levels of numerous kinases, transcription factors, phytohormones, specialized metabolites, and defensive proteins that might compromise plant growth (Erb and

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https://doi.org/10.1016/j.phytochem.2021.113008
Received 31 July 2021; Received in revised form 9 October 2021; Accepted 4 November 2021
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Table 1
Glucose oxidase (GOX) identified from insects' oral secretions/mouth parts and its potential/proposed functions in respective insects and host plants.

Insect sp.	Feeding on	Insect response	Plant response	References
Helicoverpa armigera *Menduca sexta*	Artificial Diet Tobacco Tomato Cotton Soybean Geranium	Host plant dependent Glucose oxidase activity	Suppression of plant defense	Eichenseer et al. (2010)
H. zea	Tomato Cotton Tobacco	Differential Glucose oxidase activity	Host plant affects herbivores oral secretion constitutes	Peiffer and Felton (2005)
H. armigera *H. assulta*	Hot pepper Tobacco Cotton	Higher glucose oxidase activity in generalist insect compared to specialized insect	Plant dietary component affect herbivory glucose oxidase activity	Yang et al. (2017)
H. armigera *H. zea* *H. assulta*	Artificial Diet	Higher glucose oxidase activity in generalist insect than specialist insect	Suppression of nicotine induction in plants	Zong and Wang (2004)
M. sexta *Spodoptera exigua*	Tobacco	Glucose oxidase activity is lower in *M. sexta* oral secretion than *S. exigua*	Modulation of cross-talk between Salicylic acid (SA), Ethylene (ET) and Jasmonic acid (JA) pathways	Diezel et al. (2009)
H. zea	Artificial Diet	Increased survival rate of insects	Suppression of induced resistance in *N. tabacum*.	Bede et al. (2006)
S. exigua	Artificial diet (differ carbohydrate/ protein)	Insect salivary GOX activity is diet-dependent	Not known	Babic et al. (2008)
H. zea	Wheat germ and casein-based artificial diet	Glucose oxidase is the main protein identified in insect saliva	Burst of Jasmonic acid and induction of late defense gene expression	Tian et al. (2012)
H. zea	Tomato, Soybean	Host plant dependent GOX activity for stomatal closure	Inhibits the release of herbivore-induced plant volatiles by stomatal closure	Lin et al. (2021)

Reymond, 2019). Insect OSs includes regurgitant and saliva with distinct origins and compositions. Regurgitants arise from the foregut and midgut while saliva is released from the labial gland through the spinneret (Eichenseer et al., 1999; Peiffer and Felton, 2005). The components of OS trigger or suppress plant defense and are known as elicitors or suppressors, respectively (Alborn and Schmelz, 2008; Louis et al., 2013; Musser et al., 2002). Few reports suggest that the synthesis and function of OS components might depend on the host plant or the feeding part of the plant. Different plant-based diets are known to influence the herbivore OSs constituents and, eventually, the plant defense responses. The fatty acid components of fatty acid amides are directly diet-related and have been suggested to affect insect feeding choice. Thus, as part of the diet of a herbivore, plants' defense proteins and metabolites of host plants may influence the OS composition (Acevedo et al., 2015).

Over 300 comprehensive reviews covering the nature and general features of plant-insect interactions have been published since 2010. As per our survey, only twelve reviews used the term OS in the title, abstract, or keywords. Among these Schmelz (2015) comprehensively reviewed the magnitude and direction of plant responses orchestrated through OS components. In particular, constituents of OSs of insects and their role in plant defense has been recently reviewed (Erb and Reymond, 2019; Stahl et al., 2018). The role of insect OS constituents in suppressing herbivore-specific induced defenses and activating inaccurate plant defense has also been highlighted (Felton et al., 2014; Hogenhout and Bos, 2011). From this information, we gain some insights that the most characterized OS components are proteinaceous molecules such as glucose oxidase (GOX), several aphid proteins (ApC002, MpC002, Bsp9, Me10, Me23, Bt56), proteases, lipases, and *Helicoverpa armigera* R-like proteins (HARPs), while small molecules include fatty acid amino acid conjugates (FACs), peptides, oligosaccharides, amino acids, fatty acids, sugars, etc. Hitherto, Chen and Mao (2020) summarized newly identified elicitors and effectors from insects and their target proteins in the plants.

Here, we have reviewed the most characterized components of insect OSs, which regulates the plant defenses that either benefit host plants or feeding insects. We highlight the factors that potentially influence the OS compositions, *viz.,* host plant (diet), and associated microorganisms of the insect and plant. We provide possible chemical, and biochemical markers that are common between natural insect feeding and OS treatments. Overall, we summarize the current updates on chemical cues that unravel the molecular dynamics of the plant-insect interactions and provide future perspectives in the area.

2. Characterized constituents of the OSs of insect herbivore

2.1. GOX – an enzyme from insect OS that regulates plant defense response

GOX was reported as one of the abundant proteins in the saliva of *Helicoverpa zea* Hübner (Noctuidae) during their active feeding stage and has been secreted into the wounded plant part (Eichenseer et al., 1999; Musser et al., 2006; Peiffer and Felton, 2005). In the presence of D-glucose, GOX catalyzes the production of D-gluconic acid and H_2O_2 (Eichenseer et al., 1999). A higher level of H_2O_2 is believed to be the main factor responsible for the altered plant defense by eliciting a salicylic acid (SA) burst and decreasing the jasmonic acid (JA) and ethylene levels (Diezel et al., 2009; Mittler et al., 2004). GOX has been recognized as a plant defense modulator in several plant species, including *Nicotiana attenuata* Torrey (Solanaceae), *Medicago truncatula* Gaertn. (Fabaceae), *Solanum lycopersicum* Linnaeus (Solanaceae), and *Arabidopsis thaliana* Linnaeus (Brassicaceae) (Bede et al., 2006; Diezel et al., 2009; Lè Ne Weech et al., 2008) (Table 1). Furthermore, GOX was first identified as a suppressor molecule from the saliva of *H. zea,* suppressing nicotine production and defense responses in the *N. tabacum* (Musser et al., 2002). Furthermore, the feeding and survival of larvae was superior when fed on GOX-treated tobacco leaves (Musser et al., 2002, 2005; Zong and Wang, 2004). The generalist behavior of insects might also be linked to the higher synthesis, activity, and stability of GOX (Eichenseer et al., 2010; Yang et al., 2017), consequently acting as a suppressor of plant defense so these insects can feed more on plants. Interestingly, GOX inhibits the release of herbivore-induced plant volatiles by stomatal closure in tomato and soybean plants (Lin et al., 2021), these responses are plant species-dependent. Possibly, volatiles are synthesized and build up in the intercellular spaces of the leaf, and diffuse to the atmosphere through guard cells.

GOX may also act as a primary elicitor (Fig. 1) by activating the

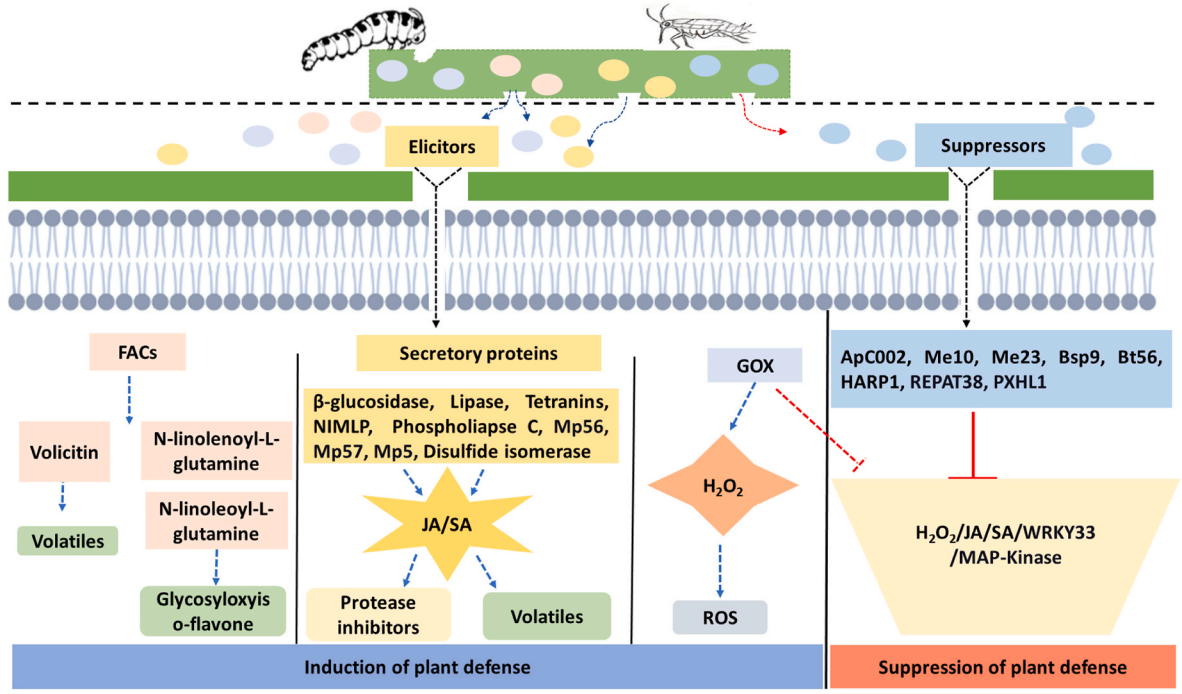

Fig. 1. Schematic presentation of identified bioactive molecules from the oral secretion/mouthpart of insects. Active molecules were characterized as inducer or suppressor of plant defense. Fatty acid amino acid conjugates (FACs) elicit volatile emission and isoflavonid synthesis. Proteinaceous elicitors induces Jasmonic acid (JA) and Salicylic acid (SA) biosynthesis and signaling leading to induce plant defense by protease inhibitors synthesis and release of volatiles. The Glucose Oxidase (GOX) induces reactive oxygen species (ROS) through hydrogen peroxide H_2O_2. Proteinaceous effectors either suppress the hydrogen peroxide H_2O_2, JA/SA biosynthesis and signaling or the interaction of transcription factors (WRKY) with kinases.

defense responses, which was first reported in tomatoes (Tian et al., 2012). Variable defense responses have been reported in other Solanaceae plants, including bell pepper (*Capsicum annuum* Linnaeus; Solanaceae), habanero pepper (*C. chinense*), and tomatillo (*Physalis philadelphica* Lamarck; Solanaceae) (Lin et al., 2020). These defense responses were possibly impacted by the availability of GOX substrates, D-glucose, in the host plants, emphasizing the importance of plant species and the quality of the host (Table 1). However, it is possible that in addition to the response to an oxidative H_2O_2 burst, some plants might have GOX-specific receptors that trigger an additional defensive response. However, no such receptors have thus far been identified.

The maximum quantity of GOX was reported from aggressively feeding larvae (Zong and Wang, 2004), but insect diets also seem imperative for the synthesis and secretion of GOX. For example, when *H. zea* larvae were reared on different host plants, varied amounts of GOX were detected in their salivary secretion (Peiffer and Felton, 2005). Overall, the quantity and quality of saliva constituents are inversely related to the quality of the host (Merkx-Jacques and Bede, 2004, 2005; Peiffer and Felton, 2005; Rivera-Vega et al., 2017). We can concluded that GOX synthesis and secretion are host-specific (Afshar et al., 2010; Merkx-Jacques and Bede, 2004, 2005; Peiffer and Felton, 2005), while they also vary among different caterpillar species depending on their feeding behavior (Eichenseer et al., 2010). Furthermore, to determine the dietary components essential for the GOX activity, lepidopteran insects were fed on their host plant and chemically defined artificial diets supplemented with sugars and other specialized metabolites (Hu et al., 2008). These comparisons have established that in general, proteins and carbohydrates could modulate GOX activity, insect growth, and development; however, phenolic components might have no impact on GOX activity (Babic et al., 2008; Hu et al., 2008; Simpson and Raubenheimer, 1993).

2.2. FACs – components of lepidopteran OS as elicitors of plant defense responses

Fatty acid amino acid conjugates (FACs) are among the most abundant OS bioactive molecules present in lepidopteran insects (Alborn et al., 1997; Yoshinaga et al., 2010), and are synthesized by the conjugation of fatty acid(s) and amino acid(s). A common feature among the discovered FACs is the conjugation of either L-glutamine or L-glutamic acid of insect origin with the linolenic acid or other free fatty acids derived from plant lipids. This chemical modification allows plants to distinguish herbivore attacks and to have specific defense responses (Paré et al., 1998). FACs are familiar in the Noctuidae family, but with variable quantities in different species (Mori et al., 2003). Several FACs, such as N-linoleoyl-L-glutamine, N-linoleoyl-L-glutamic acid, volicitin (N-17-hydroxylinolenoyl-L-glutamine), and N-hydroxylinolenoyl-L-glutamic acid were discovered from the OS of herbivore insects (Alborn et al., 2000; Halitschke et al., 2001; Mori et al., 2003; Paré et al., 1998; Pohnert et al., 1999; Tumlinson and Lait, 2005; Yoshinaga et al., 2010) (Table 2). Caeliferins, a family of sulfoxy fatty acids, have been identified from the OS of grasshopper *Schistocerca americana* Drury (Acrididae). Caeliferins trigger the release of terpenoid-like volatiles from maize seedlings (Alborn et al., 2007).

The most potent FAC, volicitin, was identified from *Spodoptera exigua* Hübner (Noctuidae) OS (Alborn et al., 1997), which contains two asymmetric carbons. Interestingly, synthetic volicitin with a D-glutamine conjugate did not show any activity (Alborn et al., 1997, 2000), suggesting that a structural configuration of amino acids has a significant role in the bioactivity of FACs. Similarly, hydroxylation on carbon 17 of linolenic acid is also important for bioactivity (Alborn et al., 2000). Most volicitin- and fatty acid amide-induced plant volatiles are terpenoids released in response to inducible upregulation of terpene synthases. In addition, FAC induction also often results in the release of indole which was found to be due to induced indole-3-glycerol phosphate lyase activity (Frey et al., 2000). Following this discovery, several other volatile components were also characterized after volicitin

Table 2
Different Fatty acid amino acid conjugates (FACs) identified from insects' oral secretions/mouth parts and potential/proposed functions.

Insect sp.	Feeding on	Molecule	Insect response	Plant response	Reference
M. sexta	Tobacco	FACs	FACs along with other molecules act as insect elicitors	Transcriptional and proteomic changes	Giri et al. (2006)
M. sexta	Tobacco	*N*-linolenoyl-L-Glu *N*-linoleoyl-L-Glu *N*-palmitoyl-L-Glu *N*-linolenoyl-L-Gln *N*-linoleoyl-L-Gln *N*-palmitoyl-L-Gln	Glu-Conjugate fatty acid is more abundant than Gln-conjugate fatty acid	Induced JA accumulation, and volatile release	Halitschke et al. (2001)
M. sexta	*Nicotiana attenuata*	*N*-linolenoyl-L-Gln *N*-linolenoyl-L-Glu Hydroxyoctadecatrienoic acid	Not known	Induced and suppressed suites of volatiles	Gaquerel et al. (2009)
M. sexta	Tobacco	FACs	18:3-Glu major elicitor in insect oral secretion	Induced JA biosynthesis and differential monoterpene emission	VanDoorn et al. (2010)
S. litura	Artificial Diet enriched with amino acid	Glutamine type FACs	Role in nitrogen assimilation and function as storage of glutamine in gut lumen	Not known	Yoshinaga et al. (2008)
Heliothis virescens, *S. exigua*, *S. frugiperda*, *S. littoralis*, *Epirrita autumnata*, *Operophtera*	Artificial Diet, *Betula pendula* leaves	Fatty Acid Amides	Amount of fatty acid conjugates is species specific	Not known	Pohnert et al. (1999)
S. exigua	Corn seedlings	Volicitin	L-glutamine conjugated volicitin identified from oral secretion	Emission of volatile compounds	Alborn et al. (1997)
H. subflexa	*Physalis angulata* fruit	Volicitin	Adaptive to dietary deficiency Reduced susceptibility to natural enemies	Differential accumulation of volatiles against fruit feeding caterpillar compared to leaf feeding and linolenic acid-treated leaves	De Moraes and Mescher (2004)
Not known	Not known	Synthetic volicitin	Not known	Increased emission of indole and sesquiterpene volatiles	Lawrence and Novak (2004)
S. exigua	Isotopically labelled corn seedlings	Volicitin	Chemical modification of ingested linolenic acid by insect	Triggered the release of plant volatile	Paré et al. (1998)
H. armigera *S. litura* *Mythimna separata* *Agrius convolvuli*	Cabbage Rice leaves Sweet potato	Volicitin related compounds	Species specific synthesis of volicitin related compounds in insect oral secretion	Not known	Mori et al. (2003)
Shistocerca americana	Maize	Caeliferins	Not known	Triggers release of terpenoid-like volatiles	Alborn et al. (2007)

treatment on damaged plants (Gaquerel et al., 2009) (Fig. 1). FACs of *S. litura* Fabricius (Noctuidae) N-linolenoyl-L-glutamine and N-linoleoyl-L-glutamine have been found to induce the accumulation of isoflavone 7-O-glucosides and isoflavone 7-O-(6″-O-malonyl-β-glucosides) in soybean (Nakata et al., 2016). From the perspective of the insects, activating the plant defense or attracting their enemies is an intriguing behavior. One plausible explanation is that FACs might function in nitrogen assimilation by regulating the supply of amino acids in the insects midgut (Yoshinaga et al., 2008). Thus, we can presume that insects have developed a mechanism to modify plant-based linolenic acid into the FACs, which plants perceive as messengers to activate defense mechanisms.

As mentioned earlier, in several insect species, FACs are synthesized with two different conjugates, glutamine (Gln) and glutamic acid (Glu). Bioassays with synthetic conjugates confirm that glutamine conjugates are more active in inducing plant volatiles than glutamic acid conjugates (Alborn et al., 2003). Furthermore, the diet of insects significantly influences the composition of these conjugates with OS of insects (Alborn et al., 1997, 2000, 2003; Halitschke et al., 2001). Importantly, plant-based linolenic acid has a significant impact on the synthesis of volicitin and is considered the backbone of FACs. For instance, insect larvae were devoid of volicitin if they fed on the fruits of *Physalis angulate*, which lack linolenic acid (De Moraes and Mescher, 2004). Furthermore, the impact of the closely related host on the synthesis and activity of FACs was analyzed. A single plant species may elicit discrete responses to different FACs, and a single FAC may trigger diverse responses to the related plant species (Xu et al., 2015).

Many plants, e.g., tomato, Arabidopsis, and cowpea, have been observed not to respond to FAC treatment. In contrast, FAC treatment had a strong effect on tobacco, eggplant, and corn plants, with upregulation of phytohormones and release of a suite of volatiles (Schmelz et al., 2009). A recent study, including wild species of tomato and other genera of Solanaceae, had demonstrated that the plant response to FACs does not follow phylogenetic relationships. Instead, responses to FACs are ancestral traits that may have been lost during the evolution or domestication of Solanaceae species (Grissett et al., 2020). Moreover, another prospect is the presence of the variable side chains, carboxylic acid and amine of N-linolenoyl-L-glutamic acid (18:3-GLU) and N-linolenoyl-L-glutamine (18:3-GLN), which mediate different defense responses in host plants (Alborn et al., 2003). It is possible that not yet known receptors in the host plants could relate the differential activity with FACs.

2.3. Secretory proteins in insect OS as elicitors and suppressor of plant defense

The proteins from herbivore OS have been characterized as elicitors and suppressors of plant defenses (Louis et al., 2013; Musser et al., 2002) (Table 3). The proteinaceous elicitor β-glucosidase from *Pieris brassicae* Linnaeus (Pieridae) OS activates volatile emission from cabbage leaves (Mattiacci et al., 1995). Lipase and phospholipase C from the OS of *Schistocerca gregaria* Forsskål (Acrididae) and *S. frugiperda* Smith

Table 3
Salivary proteins characterized as elicitor and suppressor from insects' oral secretions/mouth parts and potential/proposed function in respective insects and host plants.

Insect sp.	Feeding on	Molecules	Insect response	Plant response	Reference
Elicitors					
Pieris brassicae	Brussels sprouts	β-glucosidase	Attractive parasitic wasp	Activates volatile emission	Mattiacci et al. (1995)
Myzus persicae	Tobacco	Mp56 Mp57 Mp58	Decreased aphid reproduction	Activated plant defense responses	Elzinga et al. (2014)
M. persicae	Not known	Mp10 Mp42	Reduced aphid fecundity	Triggered defense response	Bos et al. (2010)
A. pisum	Fava beans	ACE1 ACE2	ACE1 and ACE2 work together to modulate *A. pisum* feeding and survival on plant	ACEs can hydrolyze systemin or other signal molecules that induce plant immune reactions	Wang et al. (2015b)
Schistocerca gregaria	*Arabidopsis*	Lipase	Not known	Elicit accumulation of oxylipin	Schäfer et al. (2011)
S. frugiperda	Rice and Maize	Phospho-Lipase C	Larval growth negatively regulated	Accumulation of protease inhibitors	Acevedo et al. (2018)
Tetranychus urticae	Kidney bean plants	Tetranins	Increased mortality of insects	Activates expression of SA and JA biosynthesis	Iida et al. (2019)
Nilaparvata lugens	Susceptible rice cultivar	NlMLP	Inhibition of NlMLP decrease feeding performance	Induces Ca^{2+} mobilization and JA signaling	Shangguan et al. (2018)
Laodelphax striatellus	Susceptible rice cultivar	Disulfide isomerase	Reduced feeding of insects	Induces JA signaling and callose deposition	Fu et al. (2021)
Plant-derived					
Not known	Tomato	Systemin	Not known	Induces oxidative bursts and accumulation of proteinase inhibitor	(Pearce et al., 1991; Wang et al., 2018)
Not known	*Arabidopsis*	PEPs	Enhanced resistance toward the pathogen *Pythium irregulare* and *Pseudomonas syringae*	Activates H_2O_2 synthesis and defensive gene	(Huffaker et al., 2013; Yamaguchi et al., 2006)
S. frugiperda	Cowpea or Maize	Inceptin	Found only in insects fed on leaf	Promoted JA signaling, ethylene production,	Schmelz et al. (2006)
M. sexta *Trichoplusia ni*	Tomato, *N. atteunata*	Threonine deaminase	Reduced the level of free threonine	Provides isoleucine (Ile) for biosynthesis of JA-Ile conjugates and activates specific defense responses	(Chen et al., 2005, 2007; Gonzales-Vigil et al., 2011)
Insect-associated microbes					
Macrosiphum euphorbiae-associated Buchnera aphidicola	*Arabidopsis*, Tomato	GroEL	Reduced aphid fecundity	ROS accumulation and Induced expression of pattern-triggered immunity early marker genes	Chaudhary et al. (2014)
Spodoptera littoralis associated bacteria probably	*Arabidopsis*	Porin-like protein	Act as an insect elicitor	Induces membrane potential changes and cytosolic Ca^{2+} elevations in Arabidopsis and *Vicia faba*	Guo et al. (2013)
Suppressors					
A. pisum	Fava beans	Armet	Promoted feeding on host plant	Suppression of host plant defense, induction of non-host plant defense,	Wang et al. (2015a)
Acyrthosiphon pisum	Fava beans	C002	Crucial for feeding of the pea aphid on host plant	Not known	Mutti et al. (2008)
Bemisia tabaci	Tobacco	Bt56	Promoted whitefly phloem-feeding on host plants	Induction of SA-signaling pathway	Xu et al. (2019)
M. persicae	Artificial Diet	MIF	Crucial for aphid survival, fecundity and feeding on host plant	Inhibition of expression of defense related genes and callose deposition	Naessens et al. (2015)
H. armigera	*Arabidopsis*	HARP1	Improved feeding of oligophagous insect on non-host plant	Inhibition of JA signaling	Chen et al. (2019)
M. persicae	Tobacco	MpC002 Mp55	Increased aphid fecundity	Suppression of plant defense	Elzinga et al. (2014)
B. tabaci	Tomato	Bsp9	Promoted performance and preference to host plant	Suppression of plant immune signaling	Wang et al. (2019)
N. lugens	Rice	NlSEF1		Reduce H_2O_2 production	Ye et al. (2017)
B. tabaci	Tomato	BtFer1	Promote the performance on host plant	Suppresses the ROS burst during feeding	Su et al. (2019)
M. euphorbiae	Tomato	Me10 Me23	Increased aphid fecundity	Ability to suppress *N. benthamiana* defense	Atamian et al. (2013)
M. euphorbiae	Tomato	Me47	Enhanced aphid colonization	Suppression of plant immunity	Kettles and Kaloshian (2016)
M. persicae	Tobacco	MpC002	Promotes *M. persicae* colonization on Arabidopsis	Modulation of defense	Pitino and Hogenhout (2013)
Tetranychidae sp	Tobacco	Tu28, Tu84, Te84	Promote the reproductive performance of *T. urticae*	Suppression of SA defense	Villarroel et al. (2016)
Apolygus lucorum	Tobacco	A16	Promote the performance on host plant	degrade toxic oxidation products produced during feeding	(Dong et al., 2021)
Plant-derived					
H. zea	Tomato	Apyrase	Secrete ATP hydrolyzing enzymes that suppress plant defense	Suppresses the defensive genes regulated by the jasmonic acid and ethylene	Wu et al. (2012)
Insect-associated microbes					
	Arabidopsis	SAP11			Sugio et al. (2011)

(*continued on next page*)

Table 3 (*continued*)

Elicitors					
Insect sp.	Feeding on	Molecules	Insect response	Plant response	Reference
Aster yellows phytoplasma in Macrosteles quadrilineatus			Increased susceptibility to phytoplasma insect vectors	Changes leaf morphogenesis and reduces the plant defense responses	
Aster yellows phytoplasma in M. quadrilineatus	Arabidopsis	SAP54	Promotes Insect Colonization	Degrade MADS-domain transcription factors, suppresses the flower development	MacLean et al. (2011)
Aster yellows phytoplasma in *M. quadrilineatus*	Arabidopsis	SAP05	Promotes insect colonization	Control several plant developmental pathway	Huang et al. (2021)
Tomato yellow leaf curl virus in *B. tabaci*	Tobacco	C2	Promotes survival and reproduction of *B. tabaci*	Suppression of plant defenses by interacting with plant ubiquitin and blocks JA signaling	Li et al. (2019)
Tomato yellow leaf curl China virus in *B. tabaci*	Arabidopsis	βC1	Enhanced performance of the vector whiteflies	Suppresses terpene synthesis by interacting with MYC2 transcription factor	Li et al. (2014)
Cucumber mosaic virus (CMV) in *M. persicae*	Arabidopsis	2b	Manipulate host's appeal to insect vectors	Blocks JA signaling	Tungadi et al. (2017)

(Noctuidae) have been found to elicit the accumulation of oxylipin and protease inhibitors in *Arabidopsis* and corn, respectively (Acevedo et al., 2018; Schäfer et al., 2011) (Fig. 1). Another group of elicitor proteins called tetranins from *Tetranychus urticae* Koch (Tetranychidae) activates SA and JA biosynthesis in kidney beans and eggplants (Iida et al., 2019). The secretory protein NlMLP of the sucking insect *N. lugens* Stål (Delphacidae) induces Ca^{2+} mobilization and JA signaling in *Oryza sativa* Linnaeus (Poaceae) (Shangguan et al., 2018). Induced plant defenses have been observed in *N. tabacum* against individual secretory proteins Mp56, Mp57, and Mp58 from *Myzus persicae* Sulzer (Aphididae) aphids. However, the synthesis and secretion of these proteins was mostly dependent on the host plants (Elzinga et al., 2014). The transient expression of the secretory enzyme disulfide isomerase of the brown planthopper *Laodelphax striatellus* Fallén (Delphacidae) induces JA signaling and callose deposition in *N. benthamiana* (Fu et al., 2021).

In addition to elicitors from OS, the suppressor-like proteins have also been identified (Table 3). The protein C002 has been identified from the mouthpart of *Acyrthosiphon pisum* Harris (Aphididae) while feeding on phloem sap of host plant Fava beans (*Vicia fava* Linnaeus; Fabaceae). Knockdown of the *ApC002* gene in *A. pisum* resulted in altered feeding behavior; specifically, the aphids were unable to assess the phloem cells. (Elzinga et al., 2014; Mutti et al., 2008). The functional characterization of *Macrosiphum euphorbiae* Thomas (Aphididae) saliva resulted in the identification of Me10 and Me23 as effector proteins that suppress the plant defense of *N. benthamiana*, allowing aphids to increase their population (Atamian et al., 2013). Aphid effectors were demonstrated to be fast evolving and provided aphid-host specificity by promoting colonization on specific plant species (Pitino and Hogenhout, 2013). Other salivary proteins, Armet, ACE1 and ACE2, from *A. pisum* were reported as suppressor proteins. The induced transcriptional response due to Armet proteins in tobacco plants benefits insects. Knockdown of these suppressors altered the feeding performance of aphids on plants. These proteins are crucial for the adaptation of *A. pisum* to different plants, as they modulate the required transcriptional responses in both host and nonhost plants. Higher transcript levels of Armet, ACE1 and ACE2 were observed in *A. pisum* when fed on a plant-based diet as compared to an artificial diet (Wang et al., 2015a, 2015b). Thus, these findings signify the importance of the plant diet on the alteration of the synthesis and secretion of effector-like proteins by the aphids. The migration inhibitory factor protein from *M. persicae* saliva was identified as another critical component for the improved aphid survival, fecundity, and feeding on different host plants. The transient expression of migration inhibitory factors in *N. benthamiana* suppressed the transcript levels of defense genes and reduced callose deposition (Naessens et al., 2015). Functional characterization of two salivary secretory proteins, Bsp9 and Bt56, from *Bemisia tabaci* Gennadius (Aleyrodidae), have revealed that both the proteins modulate the defense signaling in tomato and tobacco, respectively. Bsp9 improves the feeding of *B. tabaci* on tomato plants by suppressing the activation of WRKY33 and MAP-kinase interactions, while Bt56 modulates SA signaling in tobacco through a KNOTTED 1-like transcription factor (Wang et al., 2019; Xu et al., 2019). Stink bugs also activates MPKs pathway by releasing their saliva to the developing soybean seeds that modifies the seed cell wall and may activate defense metabolic pathways (Giacometti et al., 2016, 2020). The overexpression of effector protein RpC002 in barley resulted in enhanced susceptibility toward *Rhopalosiphum padi* Linnaeus (Aphididae) and not against *M. persicae*. Reduced transcript levels were observed for defense signaling genes in RpC002 transgenic barley lines (Escudero-Martinez et al., 2020). *In silico* analysis of spider mites (*Tetranychidae* sp.) has identified Tu28 and Tu84 and its homolog Te84, which can account for suppression of SA defense. Transient expression of three of these proteins in tobacco plants promoted the reproductive performance of *T. urticae* (Villarroel et al., 2016). In the brown planthopper (*N. lugens*), secretory calcium-binding protein-1 (NlSEF1) functions as a suppressor. The recombinant NlSEF1 protein was reported to reduce H_2O_2 production in rice. Furthermore, knockdown of NISEF1 in *N. lugens* resulted in reduced feeding performance, leading to higher mortality (Ye et al., 2017). Recently, two other effector proteins were reported that target the ROS pathway to promote their performance on host plants. Salivary ferritin, BtFer1 from the whitefly *B. tabaci*, suppress the ROS burst during feeding on tomato plants (Su et al., 2019). The mirid bug (*Apolygus lucorum* Meyer-Dür; Miridae) releases salivary gland effector A16 in the host cells to interfere with plant susceptibility (Dong et al., 2021). Thus, synthesis and induced levels of novel suppressors may be beneficial for the insects feeding on different host and nonhost plants.

Few effector-like proteins have been identified from chewing insects. Recently, the effector *Helicoverpa armigera* R-like protein 1 (HARP1) was identified in OS (Chen et al., 2019). HARP1 protein has stabilized JAZ proteins involved in JA-mediated defense responses in Arabidopsis. The overexpression of HARP1 in the nonhost plant *N. benthamiana* helped *Plutella xylostella* Linnaeus (Plutellidae) to improve their feeding performance. The induced accumulation of HARP1 protein was found in *H. armigera* OS collected from insects feeding on Arabidopsis plants compared to artificial diet (Chen et al., 2019), suggesting that diet could play an important role in the biosynthesis and secretion of suppressor-like proteins in insects. Similar to HARP1, REPAT38 from *S. exigua* interacts with JAZ proteins of host plants to impede JA signaling. Hence, the secretion of effector-like proteins through OS could help insects to feed on multiple plants by modulating the defenses (Fig. 1). Many HARP1-like proteins are conserved in lepidopteran insects; however their functional role has yet to be elucidated.

To minimize the feeding performance of insects on multiple plants, the role of dietary components and their significance on the accumulation of effector-like proteins in OS needs to be explored. The use of

knockdown and RNAi to silence the expression of suppressor transcripts in insects can help to reduce the insect infestation. Similarly, understanding the digestive physiology of insects will also provide new strategies for the management of insect pests (Lomate and Bonning, 2016). Few proteinaceous elicitors have also been identified from the OS of herbivores; hence studying the differentially accumulated elicitor-like protein from insects will help to engineer plants with enhanced defense against herbivores.

3. Host plants and insect-associated microbes alter the OS composition of insects that differentially tweaks the plant defense responses

3.1. Plant-derived peptides and metabolites in the OS of insects responsible for regulating plant defense

The OS typically contains proteins, peptides, oligosaccharides, fatty acids, and a combination of primary and specialized metabolites. Several of these compounds have plant origin and are known to be involved in the regulation of plant defense responses (Table 3). For example, sheath saliva of brown marmorated stink bug (*Halyomorpha halys* Stål; Pentatomidae) elicited the JA-inducible defense gene proteinase inhibitor 2 (*Pin2*), but this induction was observed only when sheaths had been collected from tomato plants, indicating their plant origin (Peiffer and Felton, 2014). Systemin and HypSys (18–20 amino acid) peptides induce defense responses in Solanaceous plants through the JA signaling pathway (Pearce et al., 1991; Wang et al., 2018). Twenty-three-amino acid plant elicitor peptides (Peps), plant ATPase-derived inceptin (11-amino acid) peptide, and protein apyrase (446-amino acid) are found in the OS of chewing insects. These peptides/proteins are known to function as effectors. Peps and inceptins lead to activation of the defense response in the Fabaceae and Cruciferae families via the wound-inducible JA signaling pathway, while apyrase suppresses the JA-dependent signaling in tomato (Schmelz et al., 2006, 2007; Wu et al., 2012). In *N. attenuata,* threonine deaminase (TD) has been demonstrated to provide isoleucine (Ile) for the biosynthesis of JA-Ile conjugates that activate specific defense cascades against insect infestation (Kang et al., 2006). Moreover, feeding of *Manduca sexta* Johannsen (Sphingidae) and *Trichoplusia ni* Hübner (Noctuidae) on tomato plants resulted in the accumulation of active TD in the insect gut without a regulatory domain that continuously catalyzes the cleavage of threonine (Chen et al., 2005). TD inhibits the growth of *M. sexta* not only by reducing the level of free threonine but also by producing the toxic metabolite α-ketobutyrate (Chen et al., 2005, 2007; Kang and Baldwin, 2006). Furthermore, it was found that the chymotrypsin-like proteases of lepidopteran insect origin are responsible for proteolytic cleavage of the regulatory domain of the duplicated TD paralog (TD2), leading to an active form of the enzyme that depletes threonine levels (Gonzales-Vigil et al., 2011). Studies have also reported that *Spodoptera littoralis* Boisduval (Noctuidae) OS contains β-galactofuranose polysaccharides of unknown origin (either from the plant, insect, or associated microorganism), which act as a prominent elicitors of defense responses in Arabidopsis and soybean. These OS polysaccharides are responsible for early events viz. membrane depolarization, elevation in cytosolic Ca^{2+} ions, and generation of reactive oxygen species in plants (Arimura, 2021; Uemura et al., 2020).

3.2. Influence of microbial associates on insect OS composition and response to plant defense

Symbiotic microbes are important for herbivores because they deliver amino acids (Douglas Angela, 2015), help in digestion (Visôtto et al., 2009), and detoxify specialized metabolites (Hammer and Bowers, 2015; Mason et al., 2015). The ability of insects to exploit some host plants depends, at least in part, on their association with a specific microorganism (Hosokawa et al., 2007; Tsuchida et al., 2004). However, plant metabolites also influence the microbiota of the digestive systems of insects and hence the OS of the insects (Grunseich et al., 2019; Shikano et al., 2017). Evolutionary forces that shape plant-insect interactions may possibly have also impacted the insect microbial interactions (Noman et al., 2020).

There is an increasing amount of evidence wherein insect-associated microbes have influenced plant defense in numerous ways. Recently, Yamasaki et al. (2021) showed that JA biosynthesis and signaling are induced by *S. litura* OS devoid of bacterial isolates; however, their presence activates SA biosynthesis and signaling. Microbes present in the insect OS directly come in contact with plant wounds during insect feeding (Chung et al., 2013). Bacterial symbionts in the OS of Colorado potato beetles (*Leptinotarsa decemlineata* Say; Chrysomelidae) elicit SA-regulated defense. Colonization of these bacteria seems beneficial for insects, as SA upregulation leads to JA downregulation and increases the insect performance (Chung et al., 2013). Depending on the host plants, variable plant defense responses were observed with similar bacterial isolates (Acevedo et al., 2017). Furthermore, some microbe-derived molecules can also modulate plant defenses (Table 3). Similar to GroEL and porin-like proteins, elicitors from insect-associated microbes induce early defense responses in host plants (Chaudhary et al., 2014; Guo et al., 2013). Herbivore-associated fungi and viruses can also directly induce a higher level of defense in several plants (Chen and Mao, 2020; Tan et al., 2018). *Wolbachia* sp. via their host (western corn rootworm) downregulated numerous defense-related genes (Barr et al., 2010). However, some suppressor molecules have been reported from vector-borne insect pathogens, such as SAP11, SAP54, and SAP05 (aster yellows phytoplasma), C2 and βC1 protein (tomato yellow leaf curl China), and 2b protein (cucumber mosaic virus) (Huang et al., 2021; Li et al., 2014, 2019; MacLean et al., 2011; Sugio et al., 2011; Tungadi et al., 2017). SAP11, βC1, C2 and 2b proteins target the synthesis and signaling of the JA pathway and increase the performance of host plants. The phytoplasmic effector SAP54 promotes insect colonization by controlling plant reproduction through degradation of MADS-box proteins. However, SAP05 controls several plant developmental pathways for its benefit by degrading of developmental regulators (Huang et al., 2021). These molecules suppress plant defense, which is beneficial to the host insects feeding on those plants.

In addition to releasing effectors, insect-associated microbes can also indirectly trigger plant defense by influencing insect synthesis of biologically active molecules. For example, the *H. zea* gut-associated bacterium *Enterobacter ludwigii* Hoffmann (Enterobacteriaceae) induces salivary GOX and triggers tomato plant defense (Wang et al., 2017). Similarly, bacteria present in the gut of herbivores has been estimated to play a role in the synthesis of FACs (Spiteller et al., 2000). However, the biosynthesis rate was very slow. Overall, the knowledge emerging from recent studies, including insect-microbe-plant interactions has emphasized the importance of herbivore-associated microbes in modulating plant defense responses (Noman et al., 2020).

3.3. Plant transcriptional reprogramming leading to the induction of common protein and metabolite defensive markers upon insect feeding and OS application

Plant defense is continuously modulated by transcriptional reprogramming by altering the biosynthesis and signaling of phytohormones such as JA, SA and ET in plants (Bodenhausen and Reymond, 2007; Erb and Reymond, 2019; Heidel-Fischer et al., 2014; War et al., 2018). Inducible defense is fascinating, as it provides plants with a flexible and less costly affair. These inducible defenses consist of defense proteins and specialized metabolites, which plants prioritize over growth to defend against herbivores. Inhibitors of protease and amylase are essential plant defensive proteins against various insect pests, including lepidopteran, hemipteran, and coleopteran (Ahn et al., 2007; Jadhav et al., 2016; Parde et al., 2012; Tamhane et al., 2005). Additionally, various studies have reported ribosome-inactivating proteins, vegetative

Table 4
Specialized metabolites from different plants identified against herbivore insect pests.

Host Plant	Insect Pest	Secondary Metabolite	References
	Sucking insects	Only against sucking insects	
Wild tomato (*Solanum habrochaite*)	Silver leaf whitefly (*Bemicia tabaci*), Spider mites (*Tetranychus urticae*)	7-epizingiberene, R-curcumene	Bleeker et al. (2011, 2012)
Arabidopsis (*Arabidopsis thaliana*)	Asian citrus psyllid (*Diaphorina citri*)	(E)-β-caryophylene	Alquézar et al. (2017)
Rice (*Oryza sativa*)	Brown plant hopper (*Nilaparvata lugens*)	2-heptanone, 2-heptanol, (+)-limonene, (*E*)-linalool oxide, Linalool, α- curcumene	Ye et al. (2020)
Cucumber (*Cucumis sativus*)	Spider mites (*T. urticae*)	Cucurbitacin-C	Balkema-Boomstra et al. (2003)
Arabidopsis (*A. thaliana*)	Green peach aphid (*Myzus persicae*)	Indole- Glucosinolates	Barth and Jander (2006); Kim and Jander (2007)
Pepper (*Capsicum annuum*)	Two-spotted spider mite (*T. urticae*)	Flavonoid-*O*-glucoside, Linalool, (*E*)-ß-ocimene	Zhang et al. (2020)
	Chewing insects	**Only against chewing insects**	
Maize (*Zea mays*)	Fall armyworm (*Spodoptera frugiperda*), African cotton leafworm (*S. littoralis*)	2-ß-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one	Glauser et al. (2011)
Cassava (*Manihot esculenta*)	Cassava burrower bug (*Cyrtomenus bergi*)	Cyanogenic glucosides	Bellotti and Arias V, (1993)
Bitter almond (*Prunus dulcis*)	Flat headed woodborer (*Capnodis tenebronis*)	Amygdalin, Prunasin	Garrido Vivas and Malagón (1990)
Arabidopsis (*A. thaliana*)	Flea beetle (*Phyllotreta nemorum*)	Dhurrin	Tattersall et al. (2001)
Poplar (*Populus tremula*)	Gypsy moth (*Lymantria dispar*)	2-methylbutyronitrile, 3-methylbutyronitrile	Irmisch et al. (2014)
Neem (*Azadirachta indica*)	Cotton bollworm (*Helicoverpa armigera*)	Azadirachtin-A	Dawkar et al. (2019)
Norway spruce (*Picea abies*)	European spruce bark beetle (Ips typographus)	D-lemonene, eucalyptol	Schiebe et al. (2012)
Norway spruce (*P. sitechensis*)	White-pine weevil (*Pissodes strobe*)	Dehydroabictic acid	Robert et al. (2010)
Wild tobacco (*Nicotiana attenuata*)	Tobacco hornworm (*Manduca sexta*)	17-hydroxygeranyllinalool glucoside	Heiling et al. (2010)
White mustard (*Sinapis alba*)	Flea beetle (*P. cruciferae*)	Sinalbin	Bodnaryk (1991)
Arabidopsis (*A. thaliana*)	(S) Cabbage large butterfly (*P. brassicae*)	Kaempferol-3, 7-dirhamnoside	Onkokesung et al. (2014)
Citrus (*Citrus maxima*)	Leafcutter ant (*Atta cephalotes*)	Limonene	Cherrett (1972)
Winter cress (*Barbarea vulgaris*)	Flea beetle (*P. nemorum*)	Hederagenin cellobioside, Oleanolic acid cellobioside	Kuzina et al. (2009)
Winter cress (*B. vulgaris*)	Diamond black moth (*Plutella xylostella*)	3-O-[O- β-D-glucopyranosyl-(1–4)-β-Dglucopyranosyl]-hederagenin	Agerbirk et al. (2003); Shinoda et al. (2002)
Wild tobacco (*N. attenuata*)	Spotted cucumber beetle (*Diabrotica undecimpunctata*), Beet armyworm (*S. exigua*), Pallid-winged grasshopper (*Trimerotropis* spp.)	Nicotine	Roda et al. (2004); Steppuhn and Baldwin (2007)
Potato (*S. tuberosum*), Wild potato (*S. chacoense*)	Guatemalan potato moth (*Tecia solanivora*), Colorado potato beetle (*Leptinotarsa decemlineata*)	α-solanine, α-chaconine	Karlsson et al. (2013) Sinden et al. (1986)
Pepper (*C. annuum*)	Oriental leafworm (*S. litura*)	Rutin, Vanillic acid Sinapic acid, Syringic acid	Movva and Pathipati (2017)
Tomato (*S. lycopersicum*)	Tobacco hornworm (*M. sexta*), Stinkbugs (*Podisusm aculiventris*), Soybean looper moth (*Pseudoplusia includens*), Fall armyworm (*S. frugiperda*), Cabbage looper (*Trichoplusia ni*), Corn earworm (*Heliothis zea*), Beet armyworm (*S. exigua*), Tobacco budworm (*H. virescens*)	Chlorogenic acid, Rutin, Tomatine	Bloem et al. (1989); Stamp and Osier (1998); Traugott and Stamp (1997)
	Sucking and chewing insects	**Against both sucking and chewing insects**	
Rice (*O. sativa*)	(C) Lawn armyworm (*S. mauritia*), (C) Rice skipper (*Parnara guttata*), (S) Brown plant hopper (*N. lugens*)	p-coumaroylputrescine, Feruloylputrescine	Alamgir et al. (2016)
Nightshade potato (*S. demissum*)	(C) Colorado beetle (*L. decemlineata*), (S) Potato leafhopper (*Empoasca fabae*)	Demissine	Harborne (1988)
Maize (*Z. mays*)	(C) First-brood european corn borer (*Ostrinia nubilalis*),	Dihydroxy-7-methoxy1,4-benzoxazin-3-one-glucoside	Niemeyer (1988)

(*continued on next page*)

Table 4 (*continued*)

Host Plant	Insect Pest	Secondary Metabolite	References
	Sucking insects	Only against sucking insects	
	(S) Maize plant louse (*Rhophalosiphum maydis*)		
Oilseed rape (*Brassica napus*)	(C) Field slug (*Deroceras reticulatum*)	Glucosinolates	Glen et al. (1990)
Cabbage (*B. oleracea*)	(S) Cabbage medium butterfly (*Pieris rapae*)	Glucosinolates	Agrawal and Kurashige (2003)
Watercress (*Nasturtium officinale*)	(C) Amphipod (*Gammarus pseudolimnaeus*), (C) Physid snail (*Physella* sp.), (S) Limnephilid caddisflies (*Hesperphylax designates*) (S) *Limnephilus* sp.	Glucosinolates	Newman et al. (1992)
Wild potato (*S. berthaultii*)	(S) Green Peach aphid (*M. persicae*)	O-acylsugars	Neal et al. (1990)
Wild tobacco (*N. attenuata*)	(C) Tobacco hornworm (*M. sexta*)	O-acylsugars	Luu et al. (2017)
Wild tomato (*S. galapagense, S. cheesmaniae, S. pimpinellifolium and S. pennellii*)	(S) Silver leaf whitefly (*B. tabaci*), (C) Tomato leaf miner (*Tuta absoluta*, *T. urticae*) (S) Thrips species	O-acylsugars	Alba et al. (2009); Leckie et al. (2012); Lucini et al. (2015); Rakha et al. (2017); Vilela De Resende et al. (2006)

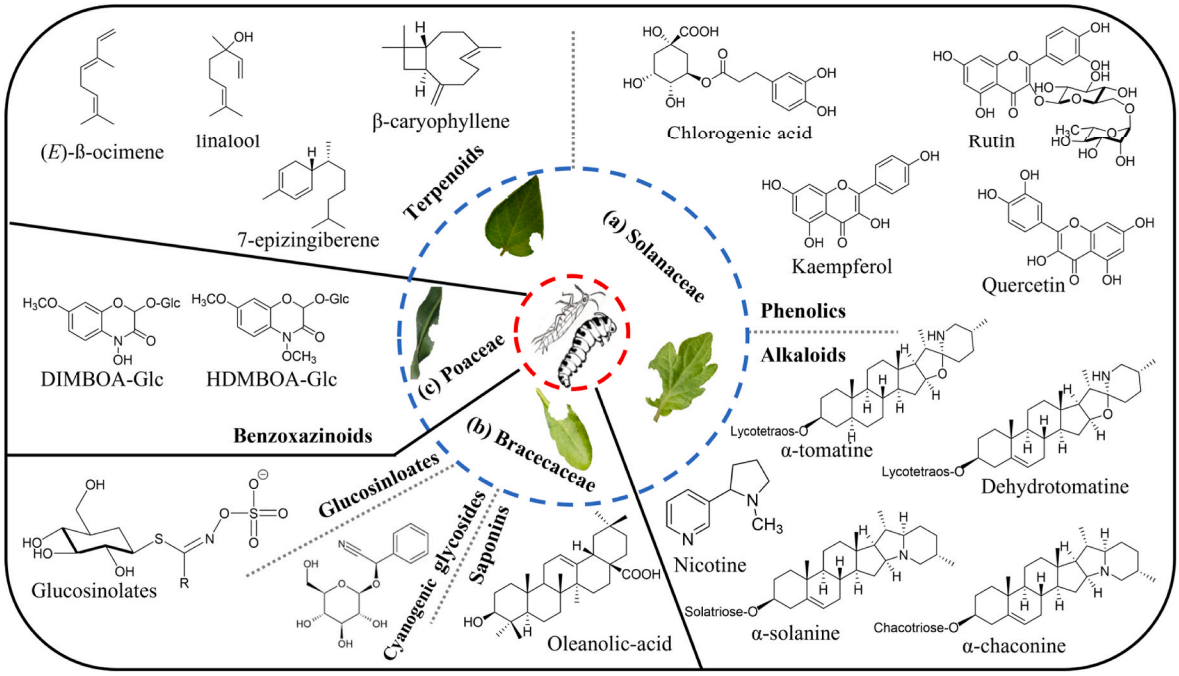

Fig. 2. Representative plant defensive metabolites (potential markers) induced upon insect feeding and application of insect OS on wounded plants. Specific group of metabolites are induced in plants belonging to particular families. (a) Solanaceae; terpenoids, phenolics, alkaloids (b) Bracecaceae; glucosinolates, cyanogenic glycosides, saponins and (c) Poaceae; benzoxazinoids.

insecticidal proteins, pathogenesis-related proteins of various plant origins viz. maize, apple, and Sambucus nigra Linnaeus (Adoxaceae) have insecticidal activity against different insect pests (Gatehouse et al., 1990; Shahidi-Noghabi et al., 2008; Stirpe, 2013; Zhu et al., 2018). Furthermore, lectins have a protective function against several insect pests (Vandenborre et al., 2011). Induced accumulation of lectins and hevein-like protein was observed not only upon different insect feeding but also in response to OS in *N. tabacum* and Arabidopsis, respectively. However, mechanical wounding did not affect lectin accumulation (Reymond et al., 2000; Vandenborre et al., 2009a, 2009b). Moreover, identical but lower levels of proteins involved in defense, primary metabolism and transcriptional regulation were observed in native tobacco (*N. attenuata*) against *M. sexta* feeding compared to OS treatment (Giri et al., 2006). Altogether, these reports suggested that the above-mentioned proteins (particularly protease inhibitors, pathogenesis-related 10, and lectins) are commonly induced in plants by various insect pests and thus can be considered plant defensive protein markers.

Apart from proteins, plants also produce several specialized metabolites (Table 4) targeting herbivore biological systems, such as nervous, digestive, and endocrine organs (Mishra et al., 2015; War et al., 2018). The response of plants to insect herbivory can be general or insect species-specific. For example, the increase and consistent release of volatiles such as linalool, (E)-ßocimene, (E)-2,4-hexadiene, β-caryophyllene, (E,E)-α-farnesene, α-humulene, 7-epizingiberene, and R-curcumene were reported in Solanaceae plants in response to *Heliothis*

virescens Fabricius (Noctuidae) feeding, or OS, and does not differ from the response to feeding by the spotted spider mite (Fig. 2) (Zhang et al., 2020). Similarly, cucurbitacin is a commonly occurring triterpenoid in cucurbits that affects sap-sucking spider mite's growth (Kaushik et al., 2015). Thus, plant volatiles viz. terpenoids are common and vital cues in plant-insect interactions; however, these molecules could be plant-family-specific (Fig. 2).

Alkaloids are widely distributed specialized nonvolatile molecules found in more than 20% of vascular plants (Bhambhani et al., 2021). Various reports have shown that alkaloids such as nicotine, α-tomatine, dehydrotomatine, α-solanine, and α-chaconine are induced in Solanaceous plants in response to different chewing insect pests (Fig. 2) (Steppuhn et al., 2004; Steppuhn and Baldwin, 2007). Furthermore, phenolic compounds, including phenolic acids, chlorogenic acid, anthocyanins, polyphenols, lignins, coumarins, tannins, stilbens, and flavonoids, accumulate in plants against herbivore attack (Bernards and Båstrup-Spohr, 2008; Vogt, 2010). These metabolites have toxic effects on chewing and sucking herbivores (Bernards and Båstrup-Spohr, 2008). The induced levels of two phenolamides, *p*-coumaroylputrescine and feruloylputrescine, have been reported in *O. sativa* leaves treated with *Mythimna loreyi* Duponchel (Noctuidae) OS (Shinya et al., 2016). Moreover, winter cress (*Barbarea* sp.) plants produce a varied amount of saponin-aglycones viz. oleanolic-acid cellobioside, and hederagenin cellobioside, important in providing resistance to flea beetles (*Phyllotreta vittula* Redtenbacher; Chrysomelidae) (Kuzina et al., 2009). Brassicaceae plants accumulate 3-O- [O- β-D-glucopyranosyl-(1–4)-β-D-glucopyranosyl-hederagenin, a special saponin that is detrimental to *P. xylostella* (Agerbirk et al., 2003). In response to tissue damage, Brassicaceae family members release volatile isothiocyanates as a result of enzymatic degradation of glucosinolates. Species-specific glucosinolate composition will give different volatile profiles, but as with green leafy volatiles, this is the result of mixing a substrate with an enzyme rather than an active induced release (Fig. 2) (Singh, 2017; Sun et al., 2020). In-line with this approach, benzoxazinoids are indole alkaloids found in most of Poaceae family members that are toxic to many chewing herbivore insects (Fig. 2) (Dafoe et al., 2011; Maag et al., 2016; Niculaes et al., 2018; Tzin et al., 2017; Wouters et al., 2016). The specialized metabolites appear to be specific to feeding guilds (Table 4). These specialized metabolites and their biosynthetic pathway enzymes/genes can be considered potential markers viz. alkaloids and phenolics from Solanaceae, cyanogenic glucosides and glucosinolates from Bracecaceae, and benzoxazinoids from Poaceae during plant-insect interactions (Fig. 2). Overall, the studies suggest that early reflection in transcriptional reprogramming of specialized metabolite biosynthesis is necessary for the plant to defend against herbivores. Hence, monitoring the phytohormone and specialized metabolite biosynthesis and signaling can be used as markers (at the level of gene expression, proteins/activities, and metabolites) for obtaining insights into plant-insect interaction studies by designing various mimicry experiments to unravel the complexities.

4. Conclusions and future perspectives

It is exciting to identify and characterize molecular-level messengers of plant-insect interactions. These messengers might belong to plants, insects or associate organisms and are small (metabolites), complex (conjugates, peptides, oligosaccharides), or large (proteins) molecules. Insect OS is a reservoir for such molecules and plays a critical role in this communication. To date, several such molecules from insect OS have been demonstrated to regulate plant defense responses. In this review, we appraise multiple pieces of evidence put forth by researchers for several molecules to unravel their specific functions as activators (elicitors) or suppressors across plant species. In some cases, both roles are identified for the same molecules, which needs to be further examined. Interestingly, the role of these spy-like molecules in manipulating defense signals provokes the speculation of new molecular war strategies in plant-insect interactions. Nonetheless, several of these molecules are further modified through various mechanisms in plants and insects aiming to generate active or more effective signals. It has been suggested, but not conclusively shown, that the synthesis and function of insect OS components might depend on the host plant or associated microorganisms. We have certain indications that diet influences the composition of OS concerning to elicitors or effectors. We bring a few points where further studies are needed in the area:

- Role of GOX as an elicitor or suppressor or it varies from plant-to-plant systems
- FAC molecular complexity, biosynthesis, and exact role-specific FACs in the regulation of plant defense mechanisms
- Role of diet in OS composition and bioactive molecules in plant-insect interactions
- Understanding the mechanisms of OS proteins in the regulation of the plant defense machinery at innumerable levels
- Efforts to identify robust markers for studying plant-induced defense mechanisms across various conditions to mimic herbivory
- Discovery of novel functional molecules from insect OS

Thus, we describe how plant-specialized and defense-related metabolism is modulated at innumerable phases by OS during plant-insect interactions. Studies emphasizing diet composition and its impact on the herbivore OS are still in their infancy. Numerous prospects now exist to characterize insect OS using genomic, proteomic, and metabolomic tools. The identified effectors could be an alternative environmentally friendly measure for insect pest management. Finally, a detailed understanding (i) of the constituents of herbivore OS, (ii) their perception, and (iii) counterdefense mechanisms in plants will help to design eco-friendly crop protection strategies.

Declaration of competing interest

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

Acknowledgements

This work was funded by Department of Biotechnology (GAP312326) and Council of Scientific and Industrial Research, Government of India, New Delhi (MLP101526). AK is supported by Department of Biotechnology under Ramalingaswami Fellowship program (BT/HRD/35/02/2006). G.S.K is supported by Department of Science and Technology, New Delhi, under the INSPIRE fellowship (IF150067) and BAS by University Grant Commission, New Delhi, India under the scheme Dr. D. S. Kothari Postdoctoral fellowship (BL/17–18/0429).

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Plant Science 314 (2022) 111120

Contents lists available at ScienceDirect

Plant Science

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Dietary influence on modulation of *Helicoverpa armigera* oral secretion composition leading to differential regulation of tomato plant defense

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ARTICLE INFO

Keywords:
Chlorogenic acid
Defense
Metabolites
Non-host
Oral secretion
Trehalose

ABSTRACT

Little is known about how different plant-based diets influence the insect herbivores' oral secretion (OS) composition and eventually the plant defense responses. We analyzed the OS composition of the generalist Lepidopteran insect, *Helicoverpa armigera* feeding on the host plant tomato (OS_H), non-host plant capsicum (OS_{NH}), and artificial diet (OS_{AD}) using Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry. Higher numbers and levels of alkaloids and terpenoids were observed in OS_H and OS_{NH}, respectively while OS_{AD} was rich in phospholipids. Interestingly, treatment of *H. armigera* OS_{AD}, OS_H and OS_{NH} on wounded tomato leaves showed differential expression of (i) genes involved in JA and SA biosynthesis and their responsive genes, and (ii) biosynthetic pathway genes of chlorogenic acid (CGA) and trehalose, which exhibited increased accumulation along with several other plant defensive metabolites. Specifically, high levels of CGA were detected after OS_H and OS_{NH} treatments in tomato leaves. There was higher expression of the genes involved in phenylpropanoid biosynthesis, which may lead to the increased accumulation of CGA and related metabolites. In the insect bioassay, CGA significantly inhibited *H. armigera* larval growth. Our results underline the differential accumulation of plant and insect OS metabolites and identified potential plant metabolite(s) affecting insect growth and development.

1. Introduction

Herbivorous insects interact with every plant distinctly and have been categorized as generalists or specialists based on their degree of dietary specialization [1]. Whereas, generalist feeds on several plant species from different families, and specialist feeds on one or related plant species of the same family. Chemical cues from insect oral secretions (OS), which include regurgitant and digestive fluids were perceived by plants to induce defense mechanisms [2]. Further, OS constituents might interact with biomolecules of the plant. Recently, Chen and Mao [3] and Kallure et al. [4] reviewed several insects OS molecules that interact with plant metabolites and proteins. For example, *Helicoverpa armigera* R-like protein 1 (HARP1) and REPAT38 from *Spodoptera exigua* interacts with JAZ proteins of host plants to impede JA signaling [5]. Several aphid proteins (ApC002, MpC002, Bsp9, Me10, Me23, Bt56) are also known to interact with plant biomolecules, for example, Bsp9 a salivary protein of *Bemisia tabaci* improves the feeding on tomato plants by suppressing the activation of WRKY33 and MAP-kinase interactions. Further, to modulate the plant defense the Mp1 from *Myzus persicae* and Bt56 from *B. tabaci* interact with VPS52 and NTH202 proteins respectively [6,7]. Interestingly, some plant biomolecules are known to influence the synthesis and secretion of insect OS [8,9]. For instance, a significant metabolic change is observed in insects when fed on different host plants [10]. Host plants have affected the expression of detoxifying enzymes of *Myzus persicae* along with numerous changes in the sugar production and metabolism of protein and lipid [10]. Several insects contain highly specialized active molecules in their OS. For example, glucose oxidase (GOX), fatty acid amino acid conjugates (FACs), HARP1, and inceptins are major constituents of insect OS that are well characterized [5,8,11,12]. These

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https://doi.org/10.1016/j.plantsci.2021.111120
Received 30 June 2021; Received in revised form 8 November 2021; Accepted 14 November 2021
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active molecules are recognized by plants and trigger (as an elicitor) or suppress (as an effector) plant defense [11,13]. Wounding alone upregulates jasmonic acid (JA) pathway significantly [14–17], whereas the application of insect OS fine-tunes the defense responses with a high degree of specificity to the individual insect attack [12,18–20].

Apart from plant defensive proteins, plants produce numerous specialized metabolites upon insect attack as chemical defense mechanism *viz,* alkaloids, terpenoids and phenolic compounds [21–24]. Further, infestation by *Manduca sexta* and *H. zea* on tomato plant exhibited differential metabolic profiling. For example, amino acids and phenolics are induced mainly by *H. zea,* whereas the level of nitrogen and carbon transporters are altered by *M. sexta* [25]. These plant specialized metabolites often modulate interactions between plants and insects, which can substantially affect herbivores and their fitness. Further, these metabolites are signature cues of any host plant providing evidence about the identity, nutritional value, and physiological status to the feeding insect. Moreover, the altered defense in rice against OS of *Mythimna loreyi* and *Parnara guttata* suggested that the presence of various elicitors in insect OS that are involved in the regulation of plant defense signaling [26]. However, studies addressing how different plant-based diets influence herbivores OS metabolites and their impact on the plant defense responses are still limited [2,3,27,28].

Helicoverpa armigera (Hübner) is a polyphagous insect, feeding on a wide range of host plants. Yet, *H. armigera* larvae have preferences for their diet not only by the host plant range but also specific tissues of the host plant. To comprehend the impact of a plant-based diet on *H. armigera*, we analyzed the metabolites of *H. armigera* OS fed on tomato as host (OS_H) and capsicum as non-host (OS_{NH}) plants and artificial diet (OS_{AD}). We quantified the expression of several defense marker genes on mechanically wounded tomato leaves treated with OS_H, OS_{NH}, and OS_{AD}, which are known to mimic insect infestation [19,26]. Further, we performed both non-targeted and targeted metabolite analyses of OS-treated tomato leaves to correlate the phytohormone (JA) and salicylic acid (SA) responses with defense metabolites and, subsequently, the effect of the selected metabolite(s) on larval growth is studied. Our work suggests that when fed on different diets, differential occurrence of molecules was observed in *H. armigera* OS, which could have been influenced by plant metabolites (dietary content). Analysis of differentially accumulated plant metabolites was found to be correlated with known plant defense response. One of the candidate metabolite identified in this study, chlorogenic acid (CGA), reduced larval growth of *H. armigera* and was also found in OS when fed on the host and non-host plants.

2. Materials and methods

2.1. Insect rearing, collection and preparation of OS

Eggs of *H. armigera* (Cotton bollworm) were obtained from the National Bureau of Agricultural Insect Resources (Bengaluru, India). The hatched 1st instar larvae (n = 30) were reared on detached leaves of tomato (Host), detached leaves of capsicum (Non-host) plants, and on an artificial diet. The artificial diet is consists of chickpea (common host of *H. armigera*) seed powder and other components such as yeast extract, casein hydrolysate, sorbic acid, choline chloride, and vitamins. The detailed composition of the artificial diet is as described earlier [29]. Insects were reared under the controlled conditions at 25 °C, 70 % relative humidity, and a 14 h light/10 h dark cycle.

The fourth instar larvae were taken for OS collection. The mouthpart of larvae was gently squeezed by hand, and the spitted out OS was collected using pipette in a 2 mL Eppendorf tube [5]. Collected OS was centrifuged at 15000 rpm for 10 min at 4 °C. OS of 10 insects were pooled together and considered as a single biological replicate and such three biological replicates were used for the metabolite analysis. The supernatant was collected and stored at −80 °C for further use.

2.2. Treatment of H. armigera OS on tomato leaves and tissue collection

OS was collected from the host, non-host plant, and artificial diet fed *H. armigera* larvae, and diluted as 1:2 with phosphate buffer. One-month-old tomato plants were used for the study. The tomato leaves (fully expanded 3rd and 4th leaf from the main shoot-apex) were mechanically wounded with a pattern wheel and immediately 10 µl of diluted OS was applied. Two leaves of each plant and such two plants were considered as one biological replicate and such three replicates were analyzed independently in the present study. Control (unwounded), wounded and OS treated leaves were collected at various time points and snap frozen in liquid nitrogen and kept at −80 °C for further experiments. The experiment was repeated two times. For gene expression analysis, samples were collected after 2, 4, and 24 h. For metabolite analysis, samples were collected at 4, 24, 48, and 72 h after OS treatment.

2.3. Gene expression analysis by qRT-PCR

Total RNA was isolated from tomato leaves by Spectrum Plant Total RNA kit (Sigma-Aldrich, USA). RNA obtained was treated with DNase I (Thermo Fisher Scientific, USA) and its quantity and quality was determined using NanoDrop 2000 (Thermo Fisher Scientific, USA). DNA-free RNA (1 µg) was used for cDNA synthesis using RevertAid First Strand cDNA Synthesis kit (Thermo scientific, USA). Gene-specific primers (Supplementary Table 1) were designed using GeneRunner software (http://www.generunner.net/) and sequences were obtained from the Sol genomic database. Quantitative real-time PCR (qRT-PCR) was carried out according to Shinde et al. [30] and *SlActin* (Solyc11g005330) was used as housekeeping gene. PCR product specificity was confirmed by melting curve analysis. The generated threshold cycle (Ct) was used to calculate the gene expression of treated samples against control samples in terms of fold change [31].

2.4. Liquid Chromatography- Quadrupole Time of Flight- Mass Spectrometry (LC-QTOF-MS) based targeted metabolite profiling

Collected snap frozen leaf samples were grounded to fine powder and weighed prior to extraction. Metabolites from 100 mg of fine-grounded leaf powder were extracted with 200 µL of extraction solvent (1:2 w/v, tissue: 80 % methanol + 0.1 % formic acid). For insect OS metabolite analysis, 20 µL of OS was mixed with 80 µL of extraction solvent and extraction was carried out as per Itkin et al. [32]. The LC-QTOF-MS analysis was performed on Agilent 6530 Q-TOF (Agilent, USA) mass spectrometer connected to HPLC Prime Infinity II 1260 system (800 bar) according to Vasav et al. [33]. The MS/MS fragmentation data was acquired at 10, 20 and 40 eV collision energy. In the case of targeted metabolite analysis, the peak area of metabolites was determined using Agilent Mass Hunter Qualitative Navigator B.08.00, and Qualitative Workflow B.08.00 using the customized database created using Agilent PCDL. The fold change of each metabolite was calculated by dividing mass feature peak area of sample values against control values. Individual metabolites were confirmed by comparing fragments generated by MS/MS data with standard compound or *in silico* fragments generated by CFM-ID software (http://cfmid.wishartlab.com/) [34] and also according to reported experimental fragmentation pattern [35].

2.5. Insect feeding assay with chlorogenic acid

Chlorogenic acid (CGA) (Sigma-Aldrich, USA) was mixed with the artificial diet at 50, 250, and 500-ppm concentrations and used to feed 1st instar *H. armigera* larvae. Each larva was pre-weighed, and those with equal mass were selected for the feeding (n = 30). The larvae were kept at 28 °C, and the diet was changed once after two days. Larvae mass was taken at two days of interval, and final observation was done on the 6th day of feeding.

2.6. Statistical analysis

Statistical analysis was carried out with the help of Duncan multiple range test (DMRT) and one-way ANOVA in the SPSS 20.0 software (http://www.spss.co.in). qRT- PCR results were presented as mean ± SE of three independent biological replicates and respective three technical replicates. Similar letters in the graphs indicate non-significant differences, while those with different letters indicate significant differences at $P < 0.05$. Venn diagram was drawn using http://bioinformatics.psb.ugent.be/webtools/Venn. Heatmap was generated with help of Clustvis web server [36].

3. Results

3.1. Differential accumulation of metabolites in H. armigera larval OS fed on different diets

Metabolites from insect OS have been reported. However, equivalent data comparing the impact of different plant-based diet on insect OS is limited. *H. armigera* larvae prefer tomato plants (*Solanum lycopersicum* L.; host) as their diet compared to the capsicum plants (*Capsicum annum* L.; non-host). This was also evident when *H. armigera* larvae fed on capsicum plants, they showed delayed growth and development (Supp. Fig. 1). To understand the *H. armigera* diet preferences and impact of different diets on the OS composition, oral secretion of larvae fed either on tomato (OS_H) or capsicum (OS_{NH}) leaves, or an artificial diet (OS_{AD}) were analyzed using Liquid Chromatography - Quadrupole Time of Flight- Mass Spectrometry (LC-QTOF-MS). Non-targeted metabolic analysis identified total 1296 mass peaks in OS_{AD}, OS_H, and OS_{NH}. Principal component analysis showed clear separation of all identified mass peaks from OS_{AD}, OS_H, and OS_{NH} (Supp. Fig. 2) contributed by PC1 (48.6 %) and PC2 (41.4 %). Among identified mass peaks, 599, 634 and 505 were from OS_{AD}, OS_H, and OS_{NH}, respectively. From all three OS metabolites, 75 were common, 165 (75 + 90) were shared by OS_{AD} and OS_H, 112 (75 + 37) were common between OS_{AD} and OS_{NH}, and 240 (75 + 165) were shared between OS_H, and OS_{NH}. Interestingly, 397, 304, 228 distinct mass peaks were identified from OS_{AD}, OS_H, and OS_{NH}, respectively (Fig. 1A). Moreover, radar plot analysis of classes of identified mass peaks showed that phospholipids, alkaloids and terpenoids were higher in OS_{AD}, OS_H, and OS_{NH}, respectively (Fig. 1B). From identified mass peaks on the basis of peak area, 43 significantly ($P < 0.05$) identified metabolites were confirmed by using standard parameters and MS/MS fragmentation patterns (Supp. Table 2; Supp. File 1). The analysis of these metabolites showed differential occurrence across the OS_{AD}, OS_H, and OS_{NH} (Fig. 1C). Out of the identified and confirmed metabolites, α-linolenic acid, samandarine, phytosphingosine-1-phosphate, 13-hydroxy-9-methoxy-10-oxo-11-octadecenoic acid, metanephrine, gentiatibetine were abundant in OS_H, while bolegrevilol, resolvin E1, oryzarol, etorphine, corchoroside A, gossyrubilone, O-geranylvanillin and stenostrol were prominent in OS_{NH}. The α-linolenic acid was also detected at moderate level in OS_{AD} and OS_{NH}. On the other hand, tocotrienol, sterebin D, dihydroxyacidissiminol, kanzonol K, chlorogenic acid metabolites were found in OS_H and OS_{NH} (Fig. 1C). The comparative analyses have identified differentially and diet-specific presence of metabolites in the *H. armigera* larval OS.

3.2. Induced defense responses are differentially regulated in tomato leaves treated with OS_{AD}, OS_H and OS_{NH}

In the plant-insect interactions, a rapid hormonal perturbation forms a signaling network that controls the induced defense responses. A significant transcriptome reprogramming has been observed in plants upon insect attack. The chewing herbivore insects induce plant defense mainly by physical damage followed by molecular signals from the insect OS. Here, we mimicked herbivory feeding by wounding leaves of tomato plant with a pattern wheel and applied *H. armigera* larval OS

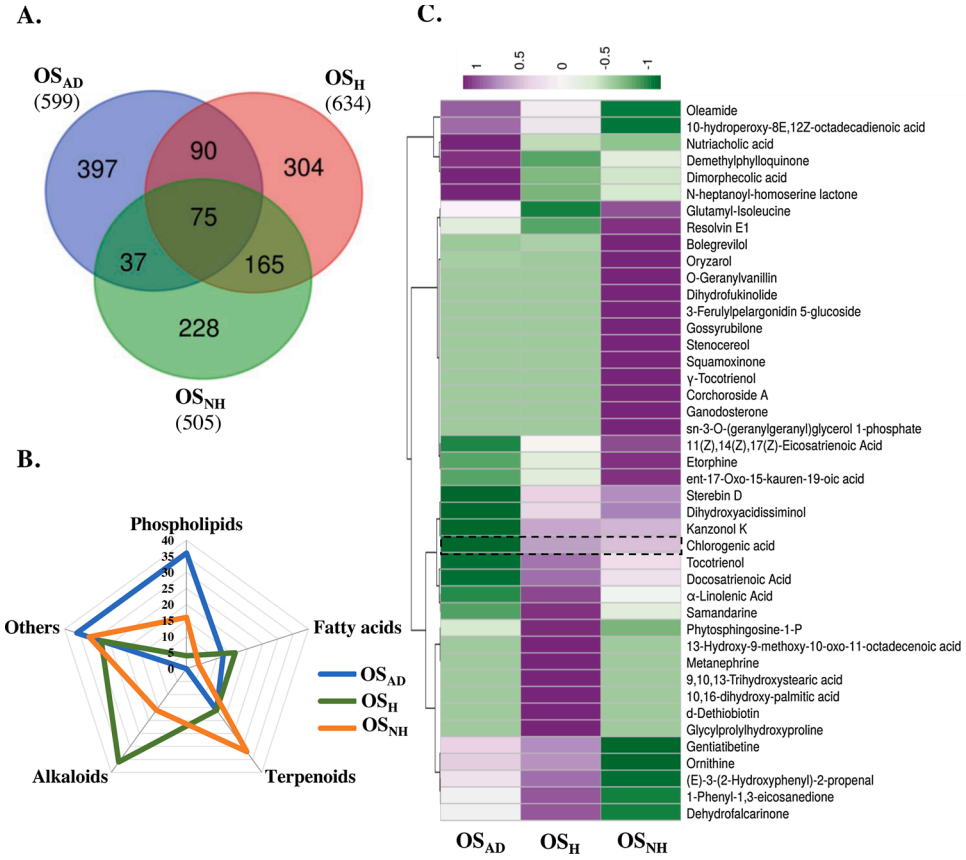

Fig. 1. Metabolites identified from *Helicoverpa armigera* oral secretion (OS) fed on different diets. A. Venn diagram of overall metabolite identified from *H. armigera* OS fed on different diets - artificial diet (OS_{AD}), host plant tomato leaves (OS_H), non-host plant capsicum leaves (OS_{NH}) using http://bioinformatics.psb.ugent.be/webtools/Venn. B. Radar plot depicts the classes of metabolites unique in OS_{AD}, OS_H, and OS_{NH} in terms of percentage. C. Heatmap of differentially identified metabolites in OS of *H. armigera* (OS_{AD}, OS_H and OS_{NH}). Total area under peak of respective metabolite was considered and heatmap was generated with help of Clustvis web server, which is showed in terms of scale bar from +1 to -1 [36].

(OS_H, OS_{NH}, or OS_{AD}, separately). To examine the modulation in phytohormonal signaling and their involvement in induced defense response, expression pattern of SA and JA biosynthetic and responsive genes were assessed. The expression of the *PHENYLALANINE AMMONIA-LYASE* (*SlPAL*), a SA biosynthesis gene and also key enzyme of phenylpropanoid pathway, was significantly upregulated (>2-fold) after 4 h upon W + OS_H and W + OS_{NH} treatments compared to wounded leaves of tomato plants. Further, the expression of *SlPAL* was continued to be significantly higher (>6-fold) till 24 h in response to the W + OS_H treatment (Fig. 2A). In contrast, the expression of another initial gene involved in SA synthesis through iso-chorismate pathway, the *ISOCHORISMATE SYNTHASE* (*ICS*) was upregulated after 4 h after wounding, however its expression was significantly reduced upon W + OS_H and W + OS_{NH} treatments (Fig. 2B). However, the expression of ICS was remained to be induced after 24 h of W + OS_{AD} treatment and mechanical wounding (Fig. 2B). Additionally, the significant upregulation of SA responsive marker gene *PATHOGENESIS-RELATED PROTEIN 1*(*SlPR1*)(>6-fold) after 4 h of treatment with W + OS_{AD}, W + OS_H and W + OS_{NH} compared to wounded leaves of tomato plants (Fig. 2C). Although after 24 h the *SlPR1* expression was reduced in all treatments compared to the 4 h treatment, *SlPR1* expression have remained higher (>5-fold) after W + OS_{NH} treatment. The upregulation of another SA responsive gene, *ENHANCED DISEASE SUSCEPTIBILITY*(*SlEDS*), was also higher (~10-fold) after 24 h of W + OS_{NH} treatment (Fig. 2D) compared to other treatments (W, W + OS_{AD}, and W + OS_H). Thus, it is evident that SA biosynthetic and signaling pathway more active in W + OS_H and W + OS_{NH} treatments compared to wounded tomato plants through PAL pathway (Fig. 2E).

Besides, we have also compared the impact of insect OS on the jasmonic acid (JA) pathway (Fig. 3E), one of the phytohormones involved in induction of plant defense against herbivores. JA biosynthetic genes, *LIPOXYGENASE* (*SlLOX*), and *ALLENE OXIDE SYNTHASE* (*SlAOS*) were upregulated after 4 h of the wounding and W + OS_H (Fig. 3A and B). The expression of *SlLOX* was found to be upregulated from 2 h to till 24 h in response to W + OS_{AD} treatment (Fig. 3A). Further, the *SlAOS* was upregulated only after 2 h of W + OS_{AD} treatment, however at subsequent timepoint the expression of *SlAOS* was reduced against W + OS_{AD} as compared to wounding and W + OS_H treatment (Fig. 3B). The

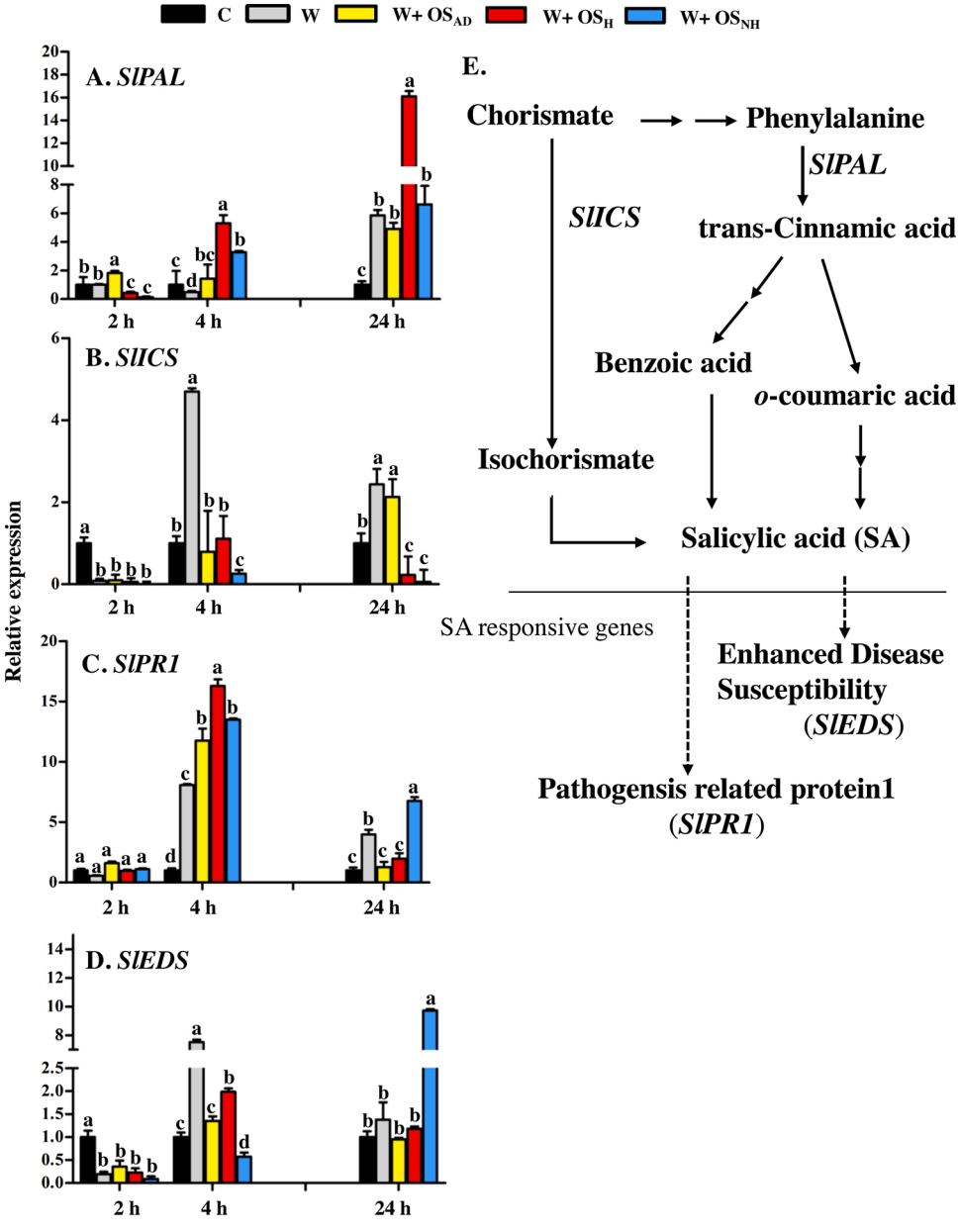

Fig. 2. Expression profiles of salicylic acid biosynthetic pathway and their responsive genes in tomato. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). A. *SlPAL* (*PHENYLALANINE AMMONIA-LYASE*), B. *SlICS* (*ISOCHORISMATE SYNTHASE*), C. *SlPR1* (*PATHOGENESIS-RELATED PROTEIN 1*), D. *SlEDS* (*ENHANCED DISEASE SUSCEPTIBILITY*), E. Schematic SA biosynthetic pathways; C- unwounded, W- wounded, W + OS_{AD} - wounded and OS of *H. armigera* fed on artificial diet, W + OS_H - wounded and OS of *H. armigera* fed on host (tomato), W + OS_{NH} - wounded and OS of *H. armigera* fed on non-host (capsicum) plant leaves.

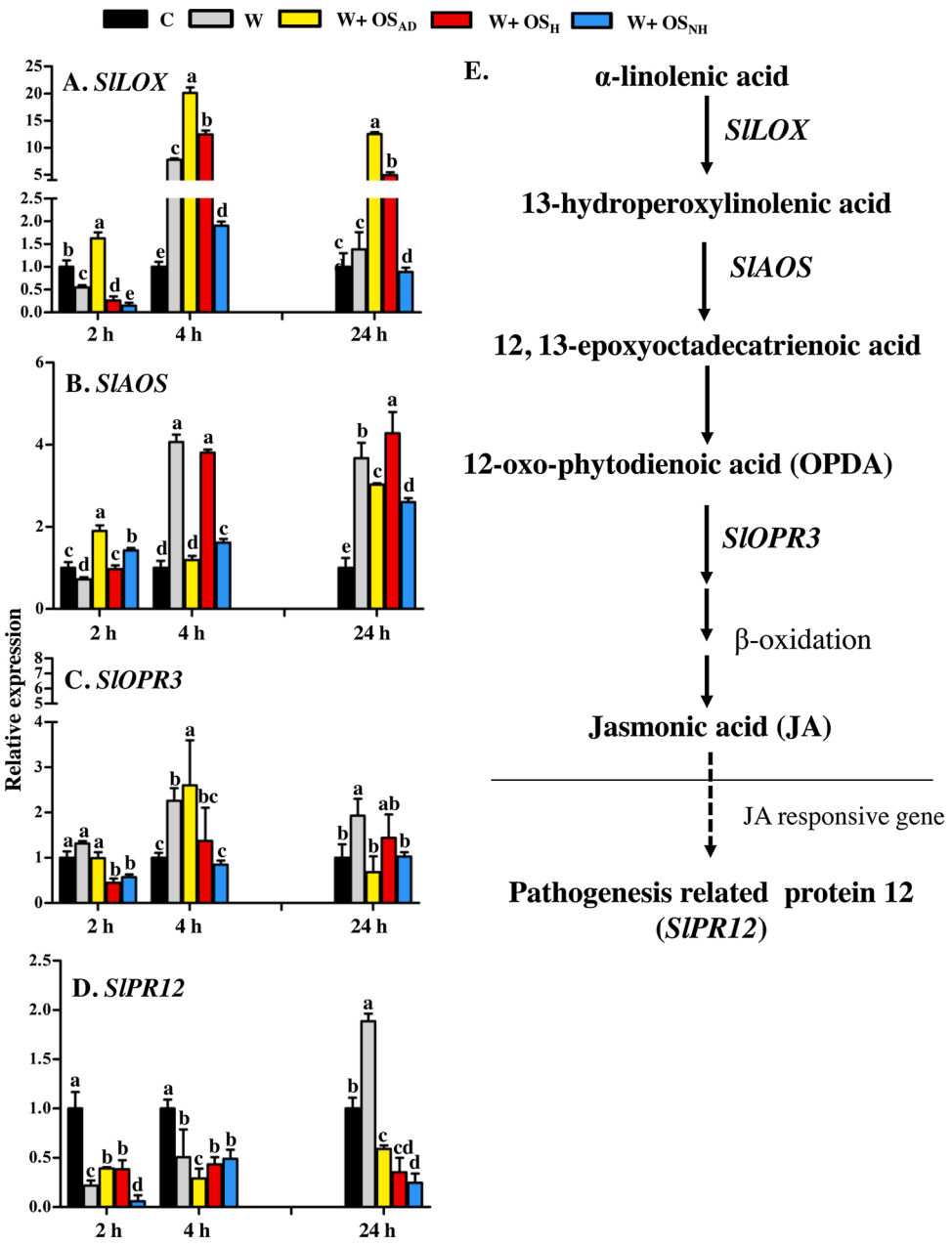

Fig. 3. Expression profiles of jasmonic acid biosynthetic pathway and their responsive genes in tomato. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). A. *SlLOX* (*LIPOXYGENASE*), B. *SlAOS* (*ALLENE OXIDE SYNTHASE*), C. *SlOPR3* (*12-OXOPHYTODIENOATE REDUCTASE 3*), D. *SlPR12* (*PATHOGENESIS-RELATED PROTEIN 12*), E. Schematic JA biosynthetic pathway; C- unwounded, W- wounded, W + OS_{AD} - wounded and OS of *H. armigera* fed on artificial diet, W + OS_{H} - wounded and OS of *H. armigera* fed on host (tomato), W + OS_{NH} - wounded and OS of *H. armigera* fed on non-host (capsicum) plant leaves.

expressions of *12-OXOPHYTODIENOATE REDUCTASE 3* (*SlOPR3*) gene, key enzyme of JA biosynthetic pathway was induced in response to mechanical wounding and W + OS_{AD} after 4 h of treatment (Fig. 3C). However, after 24 h the W + $OS_{AD,}$ W + OS_{H} and W + OS_{NH} treatment exhibited lower expression of *SlOPR3* treatment as compared to only wounded leaves (Fig. 3C). Also, decreased expression of JA responsive gene, *PATHOGENESIS-RELATED PROTEIN* 12 (*SlPR12*) with all treatments and time points except at 24 h in wounded plants (Fig. 3D). Overall, transcripts of JA biosynthetic and signaling pathway genes were higher in wounded leaves than the W + OS_{NH} treated tomato plants (Fig. 3A–D). These findings highlight the critical role of the insect OS components in the interface of plant-insect interactions. Further, this altered transcriptional response might stimulate accumulation of the specialized metabolites and defense mechanism.

3.3. *H. armigera* OS modulate specialized metabolites accumulation in tomato leaves

We used targeted and non-targeted LC-QTOF-MS to analyze differentially accumulated metabolites in tomato leaves. Different OS of *H. armigera* larvae (OS_{H}, OS_{NH}, and OS_{AD}) were applied to the wounded leaves to examine a direct contribution of different OS in plant metabolites accumulation. Metabolic profiling of the treated tomato leaves identified 657 mass peaks, out of which 63 were highly abundant based on peak area. PCA of all identified mass peaks showed clear and separate clusters contributed by PC1 (41.7 %) and PC2 (25.4 %) at different time points and metabolites from OS_{H} and OS_{NH} treated plants were distinct and appeared together (Fig. 4A). Among them 20 metabolites were confirmed by LC—MS/MS analysis (Supp. Table 3; Supp. File 2) and those metabolites were selected if their induction by wounding and OS treatment (either OS_{H}, OS_{NH}, or OS_{AD}) was significantly changed (≥1.5-fold change; $P < 0.05$) compared to unwounded tomato leaves. Selected metabolites were differentially accumulated throughout all treated

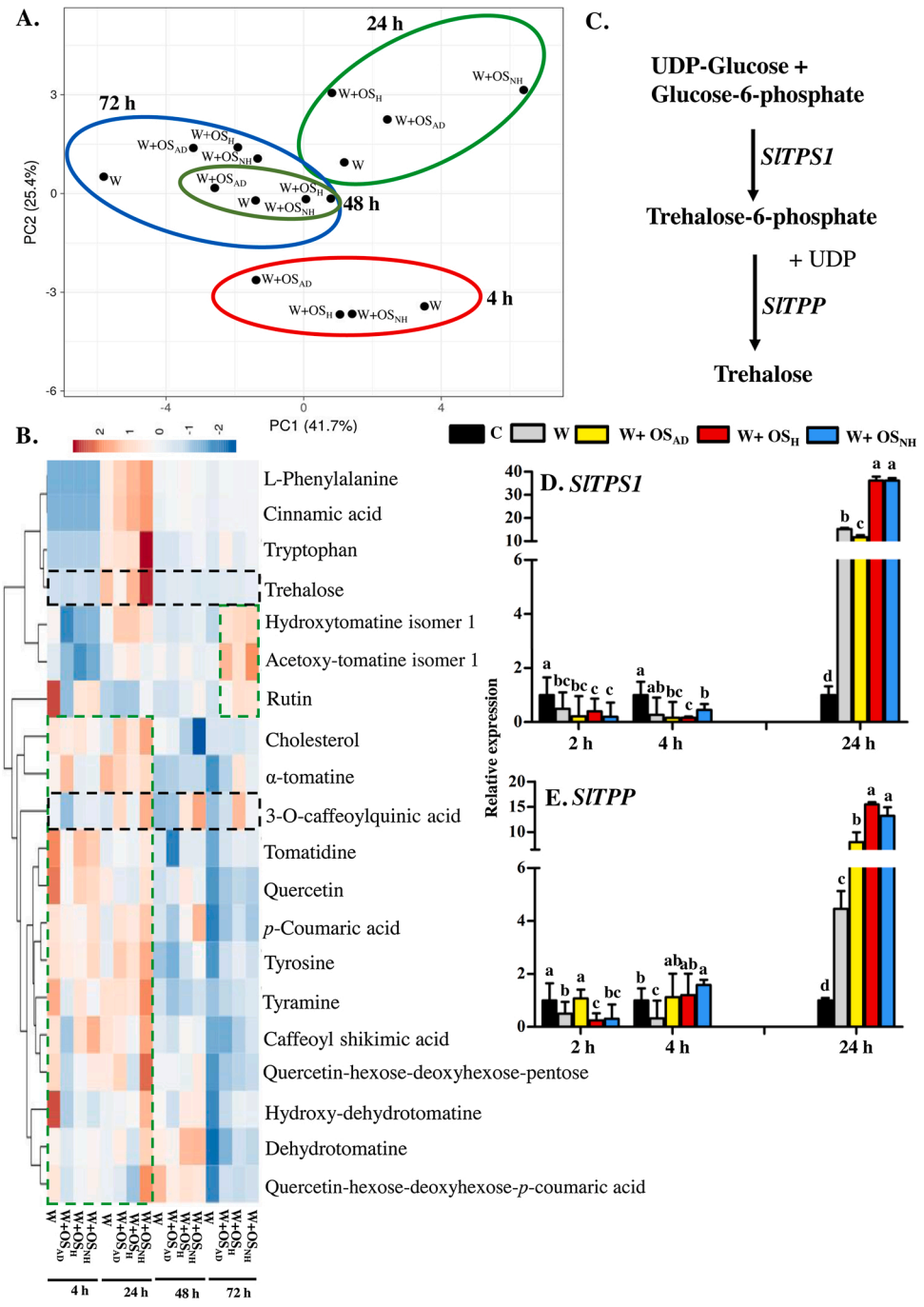

Fig. 4. Identified metabolites and expression analysis of trehalose biosynthetic pathway from tomato upon application of oral secretions on wounded leaves A. Principal component analysis of identified mass peaks from tomato leaves after wound and different *H. armigera* OS treatments with ESI (+) mode. PCA was generated with help of Clustvis web server [36]. Different clusters of mass peaks from tomato leaves after various treatments are shown at 4 (red circle), 24 (green circle), 48 (orange circle) and 72 h (blue circle). B. Heatmap of identified metabolites in tomato leaves after wound and different *H. armigera* OS treatments. Fold change was calculated by dividing mass peak area of treated samples against unwounded samples. Heatmap of W, W + OS_{AD}, W + OS_{H}, and W + OS_{NH} treatments at 4, 24, 48, and 72 h was generated with help of Clustvis web server [36]. Fold change values showed in scale bar from +2 to -2. C. Schematic trehalose biosynthetic pathway. Expression profiles of trehalose biosynthetic pathway genes in tomato. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control and the values represent means ± SE of three biological replicates each with three technical replicates. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). D. *SlTPS1* (*TREHALOSE-6-PHOSPHATE SYNTHASE*), E. *SlTPP* (*TREHALOSE 6-PHOSPHATE PHOSPHATASE*); C- unwounded, W- wounded, W + OS_{AD} - wounded and OS of *H. armigera* fed on artificial diet, W + OS_{H} - wounded and OS of *H. armigera* fed on host (tomato) plant leaves, W + OS_{NH} - wounded and OS of *H. armigera* fed on non-host (capsicum) plant leaves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

tomato leaves and belong to major groups like amino acids, alkaloids, and phenolics. Further analysis showed the accumulation of most of metabolite after 4 and 24 h of wounding as well as different OS treatments (OS_{AD}, OS_{H}, and OS_{NH}) compared to unwounded leaves (Fig. 4B; Supp. Table 4).

After 4 h, hydroxyl-tomatine isomer 1 (1.7-fold; $P < 0.05$), *p*-coumaric acid (1.6-fold; $P < 0.001$), rutin (1.7-fold; $P < 0.05$), quercetin-dihexose-deoxyhexose-p-coumaric acid (1.6-fold; $P < 0.001$), quercetin-dihexose-deoxyhexose-pentose (1.9-fold; $P < 0.001$), tyrosine (1.5-fold; $P < 0.001$), and tyramine (2.2-fold; $P < 0.001$) showed induced accumulation in wounded plants, while p-coumaric acid (1.7-fold; $P < 0.001$), caffeoyl shikimic acid (1.5-fold; $P < 0.001$), quercetin-dihexose-deoxyhexose-pentose (1.6-fold; $P < 0.001$), tyrosine (1.6-fold; $P < 0.001$) were higher in W + OS_{NH} (Fig. 4B; Supp. Table 4). Moreover, cholesterol (>1.5-fold; $P < 0.001$) and hydroxyl-tomatine (>1.5-fold; $P < 0.05$) were significantly upregulated after 24 h of wounding and different OS treatments. However, these metabolites decreased at subsequent time points. Further, hydroxyl-tomatine isomer 1 (2.2-fold; $P < 0.001$) and acetoxy-tomatine isomer (1.7-fold; $P < 0.01$) showed significantly higher accumulation in different OS treatments after 72 h (Fig. 4B; Supp. Table 4). Among the amino acids; L-phenylalanine (2.2-fold; $P < 0.05$), tryptophan (4.8-fold; $P < 0.001$), tyrosine (1.8-fold; $P < 0.01$) and tyramine (2.4-fold; $P < 0.01$) were significantly higher upon W + OS_{AD}, W + OS_{H}, and W + OS_{NH} treated tomato leaves at 24 h (Fig. 4B; Supp. Table 4). Significantly higher accumulation of a sugar, trehalose was observed in wounded (4.4-fold; $P < 0.01$) as well as upon OS treated leaves (W + OS_{H}: 4.7-fold; $P < 0.01$, and W + OS_{NH}: 7.2-fold; $P < 0.001$) (Fig. 4B; Supp. Table 4). Furthermore, several metabolites of

phenylpropanoid pathway were significantly upregulated after 24 h upon W + OS_{NH} treatment like cinnamic acid (2.4-fold; $P < 0.05$), *p*-coumaric acid (1.8-fold; $P < 0.01$), caffeoyl shikimic acid (1.6-fold; $P < 0.001$), 3-O-caffeoylquinic acid (chlorogenic acid (CGA); 1.6-fold; $P < 0.05$), quercetin (1.5-fold; $P < 0.01$), and quercetin-dihexose-deoxyhexose-*p*-coumaric acid (2.2-fold; $P < 0.001$) (Fig. 4B; Supp. Table 4). Furthermore, quercetin-dihexose-deoxyhexose-pentose (2.5-fold; $P < 0.001$) at 24 h was significantly elevated in the W + OS_{NH} treatment, while rutin (1.5-fold; $P < 0.05$) at 72 h was highly accumulated by the W + OS_H and W + OS_{NH} treatment. This overall modulation in metabolites accumulation by different OS treatments could be the reason for induction of plant defense against herbivore.

3.4. Induced expression of trehalose biosynthetic pathway genes in tomato upon application of *H. armigera* OS

Significantly higher accumulation of trehalose upon OS treated tomato leaves was noted compared to unwounded plants after 24 h (Fig. 4A and B). This observation indicated that trehalose might have crucial role in wounding and OS based plant defense induction. The significant increase in the expression of two important trehalose biosynthetic pathway genes (Fig. 4C), *TREHALOSE-6-PHOSPHATE SYNTHASE* 1 (*SlTPS1*) and *TREHALOSE 6-PHOSPHATE PHOSPHATASE* (*SlTPP*), in tomato leaves across wounding and different OS treatments was noted. Consistent with metabolite data, there was upregulation of *SlTPS1* (Fig. 4D) and *SlTPP* (Fig. 4E) transcripts level in all the treatments compared to the wounded tomato leaves. Interestingly, increased

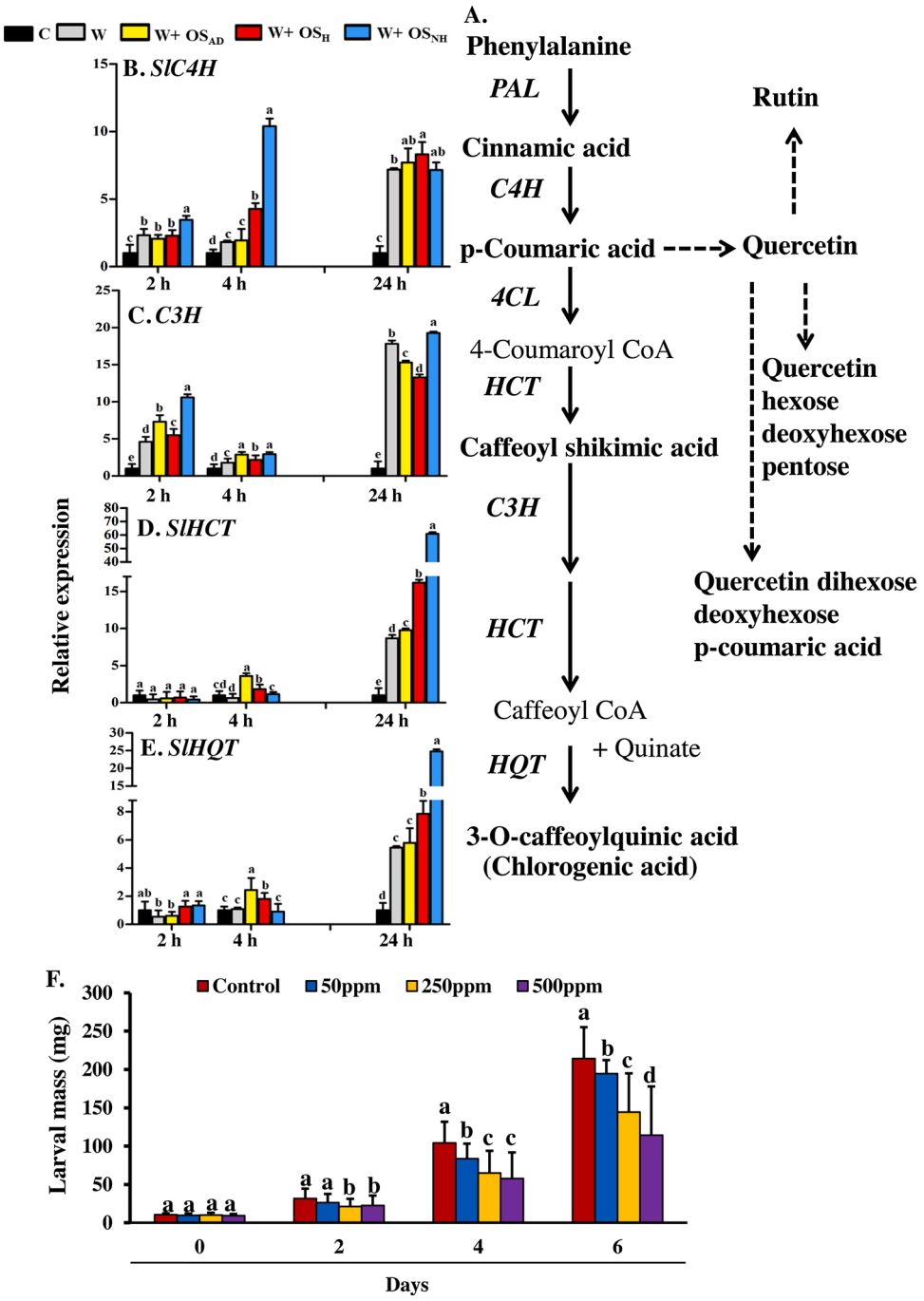

Fig. 5. Influence of chlorogenic acid (CGA) biosynthetic pathway in tomato defense and on *H. armigera* A. CGA biosynthetic pathway. qRT-PCR based relative expression of these genes were performed after 2, 4, and 24 h of treatments using *SlActin* as internal control. Fold change was calculated by dividing sample values against control values. Different letters (a, b, c, d) indicate significant difference ($P < 0.05$). B. C4H (*CINNAMATE-4-HYDROXYLASE*), C. *SlC3H* (*P-COUMARATE 3'-HYDROXYLASE*), D. *HCT* (*HYDROXYCINNAMOYL-COA:SHIKIMATE HYDROXYCINNAMOYLTRANSFERASE*), E. *HQT* (*HYDROXYCINNAMOYL COA QUINATE TRANSFERASE*). C- unwounded, W- wounded, W + OS_{AD} - wounded and OS of *H. armigera* fed on artificial diet, W + OS_H - wounded and OS of *H. armigera* fed on host (tomato) plant leaves, W + OS_{NH} - wounded and OS of *H. armigera* fed on non-host (capsicum) plant leaves. F. Dose-dependent effect of CGA on *H. armigera* larval growth. Each *H. armigera* 1st instar larva was pre-weighed, and those with equal mass were selected for the feeding (n = 30). Larval mass was recorded after feeding on control diet (artificial diet without CGA) and CGA added diet (50, 250, and 500 ppm- part per million) at various time intervals. Data shown are mean +/- SD. Bars represent the standard deviation of the means. Different letters (a, b, c, d) indicate significant ($P < 0.05$) difference.

expression of *SlTPS1* (>30-fold) and *SlTPP* (>10-fold) after 24 h of W + OS_H and W + OS_{NH} treatment than wounded plants was apparent (Fig. 4D and E). Elevated upregulation of trehalose biosynthetic genes and induced accumulation of trehalose after OS_H and OS_{NH} treatments underlines the significance of its role in plant defense responses.

3.5. Modulation of chlorogenic acid biosynthetic pathway in tomato upon application of H. armigera OS

Among phenylpropanoids, chlorogenic acid (CGA) synthesis and accumulation is crucially important during plant-herbivore interaction [37]. Precursor as well as the intermediates of CGA biosynthesis pathway (Fig. 5A) were significantly accumulated in the wounded tomato plants treated either with specifically OS_H or OS_{NH} and OS_{AD} compared to unwounded plants at 24 h (Fig. 4B). To evaluate the role of CGA biosynthetic genes in the accumulation of CGA and its intermediates, qRT-PCR of these genes was carried out. *CINNAMATE 4-HYDROXYLASE* (*SlC4H*), *HYDROXYCINNAMOYL-COA SHIKIMATE/-QUINATE HYDROXYCINNAMOYL TRANSFERASE* (*SlHCT*), P-COUMARATE 3'-HYDROXYLASE (*SlC3H*), and *HYDROXYCINNAMOYL COA QUINATE HYDROXYCINNAMOYL TRANSFERASE* (*SlHQT*) were significantly induced after 24 h of across all the treatments (Fig. 5B–E). The expression of *SlC4H* was significantly elevated at 4 h of W + OS_{NH} (>10-fold) and W + OS_H (>4-fold) treated plants than wounded tomato plants (Fig. 5B). Further, *SlC3H* was significantly up-regulated after 2 h (10-fold) in the W + OS_{NH} treated plants compared to wounded tomato plants (Fig. 5C). However, amongst the four genes, *SlHCT* and *SlHQT* are the major genes that encode for enzymes involved in final step of CGA biosynthesis. It was observed that after 24 h, the transcript level of both *SlHCT* (>15-fold) and *SlHQT* (>8-fold) were higher in response to W + OS_H and W + OS_{NH} treatment compared to just wounding or W + OS_{AD} (Fig. 5D and E). Higher accumulation of CGA and the expression of its biosynthesis genes in OS treated leaves compared to the wounded leaves, suggests a crucial involvement of CGA in the tomato plant defense against *H. armigera*.

3.6. Chlorogenic acid hinders the H. armigera larval growth

Metabolite and transcript profiling have revealed CGA as one of the significantly induced specialized defense metabolites in tomato. This led us to hypothesize that CGA could have an antibiosis effect against the generalist *H. armigera*. To examine the effect of CGA on larval growth, 1st instar *H. armigera* larvae were fed with different concentrations of CGA (50, 250, and 500 ppm) along with the artificial diet (control, without CGA). A significant reduction (about 30–40%) in the mass of larvae fed on artificial diet with CGA (250 and 500 ppm) was recorded on 4th and 6th day (Fig. 5F) compared to artificial diet without CGA. Also, dose-dependent growth inhibition of the larvae by CGA was noted from 2nd day and continued its effect till 6th day that could be one of the reasons for the retarded growth of *H. armigera* larvae.

4. Discussion

During the plant-herbivore interaction and co-evolution process, insects have preferred to feed on some plant species and avoided others [1]. Undoubtedly, there may be several reasons for their preferences, such as accessibility of food, plants' nutritional value, and plants defense responses [35]. During the insect feeding, OS enters in the host plant tissue, perceived by plants to activate specific defense to combat insect attack locally and systematically. It is increasingly accepted that herbivores could modulate host plants' natural defenses through OS components [5,11–13]. Numerous studies have identified different chemical components in the insect OS comprising enzymes, proteins, and metabolites [5,8,11,19,38–40]. However, most of these studies have been limited to induction of plant defense upon insect OS treatment when fed either on the host plant and/or artificial diet. Here, we have analyzed metabolite composition of *H. armigera* OS fed on different diets (artificial diet, leaves of host or non-host plant) and compared the responses of tomato plants upon the OS application on stimulation of known defense markers at metabolite and gene expression levels.

The nutritional quality of the plants positively modulates larval growth and development [41]. *H. armigera* larvae, when fed on capsicum and tomato leaves, a significant reduction in the larval growth was evident [42,43]. Interestingly, metabolite profiling of *H. armigera* larvae OS was found to be influenced by various diets artificial diet or leaves of tomato or capsicum. The metabolites from different classes like terpenoids, phenylpropanoids and alkaloids were identified when insects were fed on plant leaves compared to the artificial diet. It is also intriguing to see numerous phospholipids accumulation in the OS_{AD}, and many of them were diet-specific. Further, samandarine, one of the steroidal alkaloids known to have toxic effect on herbivores, was found in OS_H [44]. Another molecule, phytosphingosine-1-phosphate having role in plant signaling as well as in the stomatal closer during biotic and abiotic stress [45–47] was also detected in OS_H. Metanephrine, a phenolic metabolite derived from catecholamines identified from OS_H was found to be involved in alteration of insects' muscular contraction [48]. Moreover, a lipid peroxidation inhibitory diterpenoid, bolegrevilol, found in the OS_{NH} might be affecting the lipid metabolism of insect after feeding [49]. However, sterebin, a melanogenesis inhibitor and chlorogenic acid, insect growth inhibitor (by reducing the availability of amino acids) both were found in OS_H and OS_{NH} [50,51]. Corchoroside A found in OS_{NH} is one of the cardenolide glycosides. The cardenolide glycosides are known to have cytotoxic activity [52]. Resolvine, an active metabolite of polyunsaturated fatty acids having role in inflammation was detected in OS_{NH} [53]. Further, the terpenoids like gossyrubilone, O-geranylvanillin, and stenostrol a class of cholesterol and derivatives, are also identified only in OS_{NH}. The O-geranylvanillin was identified in *Chromolaena odorata* phenolic extract, which has shown antioxidant activity [54]. The tocotrienol, a member of vitamin E family identified in OS_H and OS_{NH}. The tocotrienol lowers cholesterol level by inhibiting hydroxy-methyl-glutaryl-coenzyme A (HMG-COA) reductase. Based on the analysis of highly accumulated metabolites in OS, it can be hypothesized that most of the metabolites are abundant in OS_{NH} and OS_H as compared to OS_{AD}. However, the metabolites in the insect OS can intimate the current status of the insect's metabolism after continuously feeding on a particular plant or its tissues. The differential accumulation and release of bioactive molecules from OS might alter the plant defense responses and needs further detail investigations.

Considering the limited available resources, plants prioritize many metabolic pathways that will help them to defend against insect attacks. Signaling of plant defense related pathways described to date are regulated by phytohormones [55]. Based on several studies, it has also been suggested that generalist herbivores, such as *S.exigua* and *S. littoralis*, may enhance their fitness by activating the SA pathway to weaken JA-mediated resistance [18,55]. Insect-specific elicitors from the insect OS or oviposition fluids are often responsible for modulating the plant defense responses. The previous report suggests that feeding by tobacco hornworm (*Manduca sexta*) larvae elicits prominent ethylene (ET) and JA bursts [56–58]. As reported, JA biosynthetic genes, *SlLOX* and *SlAOS*, were significantly upregulated at 4 h after wounding and W + OS_H treatment to the tomato leaves. In comparison, *SlLOX* and *SlAOS* upregulation was lower when wounded leaves were treated with OS_{NH}. LOX is involved in the oxidation of linolenic acid, which leads to activation of defense through JA biosynthesis. Induced lipoxygenase activity has been found in tomato plants treated with *S.exigua* OS [59]. Recently, Chen [5] have shown that OS of *H.armigera* differentially modulates the transcript level of *LOX* in *Arabidopsis* plants. Similarly, we also observed the altered transcript level of *LOX* in all treatments. The expression of JA responsive gene Pathogenesis related protein 12 was found to be attenuated in OS_H and OS_{NH} treated tomato plants. Salivary components of *H. zea* prevent nicotine induction in *Nicotiana tabacum* by

directly inhibiting the wound signaling molecule JA and/or antagonizing its interaction with other (i.e. SA) signaling pathways [11]. The suppression of JA responsive or signaling gene was also evident in SA upregulated *Arabidopsis* plant [60], suggesting induced synthesis and signaling of SA could act as an antagonist to JA signaling. It is known that SA plays a central role in defense against biotrophic pathogen as well as herbivore *Eurydema oleracea* and acts as an antagonist of JA-mediated defense responses [61,62]. Interestingly, SA biosynthetic pathway (*SlPAL*) and responsive (*SlPR1* and *EDS*) genes expression was upregulated in OS_H and OS_{NH} compared to the wounding alone. Whereas *SlICS* gene, involved in SA biosynthesis through another route, was observed to be upregulated in wounded tomato leaves, suggesting the SA biosynthesis through *SlPAL* might be more important than through *SlICS* during *H. armigera*-tomato interaction. The *S.exigua* OS elevates the accumulation and signaling of SA in Solanaceous plants [18,63]. OS components are known to suppress as well as induce SA and JA signaling pathways [5,12,64–66]. The uncharacterized small molecules (<3 kDa) from oral secretion of *S. littoralis* and *Pieris brassicae* were found to suppress wound-induced gene expression in *Arabidopsis* [67]. Recently Chen [5] have shown that the secretory protein HARP1 (*Helicoverpa armigera* R-like protein) from *H. armigera* oral secretion stabilizes JAZ degradation leading to suppressed JA signaling in *Arabidopsis*. Thus, it could be interesting to find OS components that suppress JA and activate SA to manipulate the plant defense.

The amino acids serve as primary metabolites in plants for growth and defense. An altered level of the amino acids like tryptophan, glutamine and glutamate was evident in response to herbivore attack [25,68]. The application of *H. armigera* OS on tomato plant resulted in a higher accumulation of primary metabolites like phenylalanine, tryptophan, tyrosine, and tyrosine could serve as precursor for the synthesis of specialized defensive metabolites. The steroidal glycoalkaloids and phenylpropanoid pathway metabolites involved in defense are mostly accumulated in Solanaceae plants after insect attack [69,70]. The OS treatment on wounded tomato plants resulted in induced accumulation of phenolics and their precursors like cinnamic acid, p-coumaric acid, caffeoyl shikimic acid at an early time point (after 24 h), whereas the alkaloids like hydroxytomatine isomer 1 and acetoxy-tomatine isomer at late time point (after 72 h) in our study. Also, the induced transcript level of a gene like *PHENYLALANINE AMMONIA-LYASE* (*SlPAL*) involved in phenylpropanoid pathway was observed at early time point (after 4 h) in response to OS_H and OS_{NH}. This suggests that in tomato plants phenylpropanoid pathway is the first preferred defense pathway in response to OS treatments. The regulation of transcript levels of genes involved in monoterpene biosynthesis and induced emission of volatile from *S.exigua* OS treated tomato plant is evident [59]. Numerous studies have identified alkaloids and phenolics that affect insects growth and development [24,71]. Several phenolic compounds like caffeoyl putrescine and CGA in Solanaceous plants were broadly explored upon herbivore treatment [72]. In comparative metabolites analysis, several metabolites of the phenylpropanoid pathway including L-phenylalanine, chlorogenic acid, quercetin, and rutin were highly accumulated in tomato leaves in response to insects OS treatments. Remarkably, OS metabolite analysis also implied a higher accumulation of CGA in insects' OS when they fed on plant leaves. CGA was previously known to have anti-nutritive properties for the *S. frugiperda*, *S. exigua* [71], and *S. litura* [37]. Further, chlorogenoquinones formed by oxidation of CGA, that bind to amino acids and proteins in insects and limiting the availability of amino acids required for growth and development, leading to reduced larval growth [37,51]. Consistent with a higher accumulation of CGA, we also observed increased expression of CGA biosynthetic pathway genes in OS_H and OS_{NH} treated tomato leaves. Thus, CGA can be considered as a strong anti-nutritive, altering the herbivore feeding behavior, growth, and survival.

Along with phenylpropanoid pathway, the OS treatments lead to a higher accumulation of signaling sugars trehalose, which was evident with the induced expression of trehalose biosynthetic genes (*SlTPS1* and *SlTPP*). Trehalose after exogenous application on plants shown to possess elicitor and priming properties and improved protection against abiotic and biotic stresses [73–80]. Also, trehalose treatment plays an important role in gene expression regulation linked to the plant defense responses involving phytohormones and various phytoalexins synthesis [81,82]. Along with protection against insects, the role of trehalose in induced plant defense signaling has been reported earlier [83]. Thus, trehalose also could influence the tomato plant defense against *H. armigera*.

In conclusion, current study investigated the influence of different plant-based diets on OS composition of generalist insect, *H. armigera* and its impact on tomato defense responses. Metabolite profiling has revealed that composition of *H. armigera* OS depend on the dietary component. Further, the plant-based diet significantly showed the higher content of alkaloids in OS_H and terpenoids in OS_{NH}. As expected, application of OS on wounded tomato leaves modulated the expression of SA and JA biosynthesis and responsive genes as well as trehalose biosynthetic genes. Interestingly, plant metabolite analysis revealed enhanced accumulation of steroidal glycoalkaloids and phenolic metabolites in response to insect OS. Also, CGA, one of the key components of plant defense showed enhanced accumulation through phenylpropanoid pathway upon OS_H and OS_{NH} application. Additionally, dose-dependent feeding of CGA displayed retardation of the *H. armigera* larval growth. Overall, this study indicated that plant-based diet might have major role in altering the composition of herbivore insect oral secretion and their significant influence on modulating the plant defense in tomato.

Author contributions

GSK, BAS, and APG conceived and designed the experiments. GSK and BAS performed qRT-PCR experiments as well as metabolite sample preparation. GSK and VTB acquired and analysed the metabolite data. GSK performed all the other experiments and analyses. GSK and BAS analysed the data and prepared figures and tables. GSK, BAS, AK, VTB and APG wrote and finalized the manuscript.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

GSK is supported by the Department of Science and Technology, New Delhi, under the INSPIRE fellowship (IF150067). BAS is grateful to the University Grants Commission (UGC) for Dr. D.S. Kothari Post-Doctoral Fellowship (2017-18-BL/17-18/0429). The Council of Scientific and Industrial Research, Government of India, New Delhi supported this work by under Agriculture, Nutrition and Biotechnology theme (MLP101526) to CSIR-National Chemical Laboratory. AK is supported by Department of Biotechnology under Ramalingaswami Fellowship program (BT/HRD/35/02/2006). Authors are thankful to Dr. Kirtikumar Kondhare for his valuable inputs in the manuscript. Authors are also thankful to Miss Sonal Madhiya for helping during different experiments and Horticulture Section, CSIR-NCL, Pune for the providing pot experimental facility.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2021.111120.

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