“Understanding the molecular and chemical basis of plant-insect communication”

Under the guidance of

Dr. Sagar Pandit

Submitted to

Indian institute of science education and research, Pune

Submitted by

Namithasree M

Certificate

This is to certify that this dissertation entitled “Understanding the chemical and molecular basis of plant-insect communication” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Namithasree M at IISER, Pune under the supervision of Dr. Sagar Pandit, Assistant Professor, Biology during the academic year 2019-2020.

Namithasree M

Sagar Pandit

Declaration

I hereby declare that the matter embodied in the report entitled “Understanding the chemical and molecular basis of plant-insect communication” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sagar Pandit and the same has not been submitted elsewhere for any other degree.

Namithasree M

Sagar Pandit

Abstract

Plant defense signaling involves a complex network of interacting signals majorly regulated by phytohormones. A large number of studies have shown the critical role played by the phytohormones jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) in this However; all these studies have been majorly limited to the context of folivory (leaf eating herbivory) although there are many other interactions present in nature. One such interaction is frugivory (fruit herbivory) of eggplant: *Solanum melongena* (Solanaceae) by the specialist insect herbivore *Leucinodes orbonalis* Guenee [shoot and fruit borer (SFB), Lepidoptera: Pyrallidae]. Eggplant, being one of the most important vegetable crops in Asia faces major threat from SFB, with infestation potential to the extent of 70 to 93 percent. None of the pest management strategies employed in field offered resistance to SFB to an adequate level. The chemistry of plant-insect interaction in this case is poorly understood. In this study, we report the involvement of two phytohormones ABA and ET in eggplant fruit response towards SFB attack. Our results show the increased metabolite level of ABA and increased transcript level accumulation of identified eggplant putative ET biosynthetic genes in response to SFB feeding. Our findings report that two major classes of defense metabolites of Solanaceae family, steroidal alkaloids and phenolics abundantly present in eggplant fruit did not respond to frugivory. Future studies focusing on the role played by these phytohormones and their downstream targets can provide new insights about this crop-pest interaction that can be exploited in pest management strategies.

List of figures

List of tables

Acknowledgements

First and foremost, I am grateful to my supervisor Dr. Sagar Pandit for his constant guidance and invaluable suggestions throughout the project work. I would like to express my sincere thanks to Ms. Rituparna Ghosh for her guidance and helping me throughout the project work in any way possible.

I wish to extend my thanks to all SP lab members, PhD students, project students present in lab, for their constant inputs and guidance whenever needed. I wish to express my deepest gratitude to Mr. Ganesh Pawar for his invaluable help in maintaining plants and insects.

I am grateful to thank my family and friends without whom this would not have been possible.

Introduction

Over the 350 million years of co-evolution [Gatehouse et al.,2002], plants and insects have evolved different interactions between them. While interactions like pollination, seed dispersal are beneficial to the plant, plants are constantly exposed to threats from a wide array of enemies such as herbivorous insects and pathogens. Plants have evolved to employ a diverse array of defense strategies against them. The defense system in plants can be broadly classified into direct and indirect defenses. Direct defenses include plant traits that directly help the plant in defending the attacker while indirect plant defenses include releasing compounds that attract the natural enemy of the attacker [Thaler et al., 2002, Walling et al., 2000]. Plant defense includes both physical and chemical strategies. Physical strategies include the presence of structures like trichomes, thorns, spines, and thicker leaf whereas chemical strategies involve the local and systemic accumulation of toxic insect-repelling chemicals such as proteinase inhibitors, terpenoids, alkaloids, phenols and quinones [War et al., 2012, Skibbe et al.,2008]. Chemical defenses have been reported from all trophic levels and play a crucial role in deterring the enemy [Pasteels., 2006]. Chemical defenses may be constitutive and induced. Constitutive defenses are always present in the plant regardless of the attack while induced defenses are produced in the plant upon the perception of an attack. [Heil et al, 2001].

Plant defense signaling

Successful defense depends on the ability of plants to perceive the attack and elicitation of appropriate signaling cascades which will lead to the activation of downstream attacker specific defense responses. Plant defense signaling is well studied in leaf. The earliest events in plant defense signaling include a rapid perturbation of plasma membrane potential (Vm) leading to a significant increase in intracellular Ca^{2+} concentration which happens within few seconds to minutes following the first bite of the herbivore [Maffei et al., 2007]. Within the next few minutes to hours, a qualitative and quantitative change in the levels of phytohormones are seen [Wasternack and Hause, 2002, Checker et al., 2018, Pieterse et al., 2009]. Following signaling, gene activation and the accumulation of defense metabolites can take days [Maffei et al., 2007]

Role of phytohormones

Apart from their role in growth, development and other physiological processes, phytohormones play a crucial role in mounting the induced direct and indirect defenses. Jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA) are the major players involved in this. [Erb et al., 2012, Vos et al.,2013] This complex network of hormone signals can interact antagonistically or synergistically with each other, often referred as hormone crosstalk [Seilaniantz et al., 2011, Figure 1] Hormone crosstalk co-ordinate the plant defense and provide the plant with attacker specific defenses while helping to minimize fitness costs [Vos et al., 2015].

In general, several studies have shown that SA pathway is effective against biotrophic pathogens while JA pathway is primarily activated against necrotrophic pathogens and herbivorous insects [Walling, 2000, Glazebrook et al., 2005]. JA-SA antagonism and ET/JA synergism are two well-studied hormone cross talks. [Caarls et al.,2015, Seilaniantz et al., 2011, Spoel et al., 2003]. JA mutant plant showed higher resistance to cucumber mosaic virus in *Arabidopsis* by activation of SA mediated defenses [Takahashi et al., 2004]. Exogenously supplied SA inhibited JA induced defense gene expression in tobacco leaves [Nikki et al., 1998]. In *Nicotiana attenuata* JA induced accumulation of nicotine against *Manduca sexta* herbivory strongly reduced in ethylene insensitive plants [Onkokesung et al., 2010]. Furthermore to the role played by these classical defense hormones, recent studies have shown the decisive role of hormones such as abscisic acid (ABA), indole acetic acid (IAA), indole butyric acid (IBA), and gibberellins (GA) in this orchestrated plant defense response against herbivory. [Checker et al., 2018, Seilaniantz et al 2011] Their positive or negative cross talks with other hormone signal transduction pathways help the plant fine tune the defense responses.

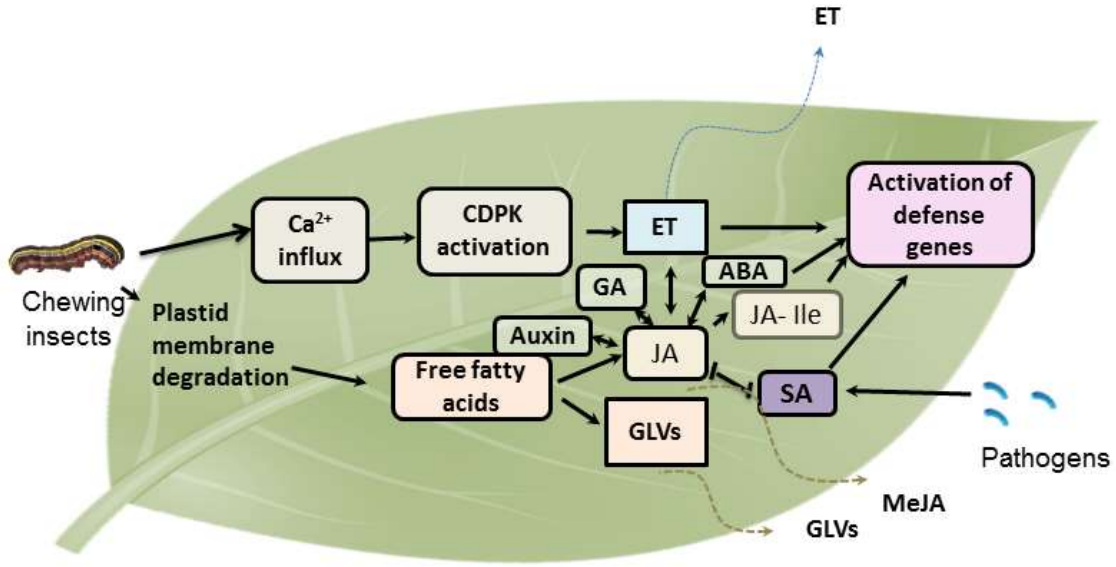

Figure 1: Defense signaling in leaf

A simplified schematic representation of phytohormone mediated plant defense signaling network, involving JA, SA, ET and ABA. Chewing insects activate JA, and ABA mediated signaling whereas pathogen attack generally activate SA mediated signaling.

Arrows and end blocked lines represent synergistic and antagonistic interactions respectively.

(CDPK- Ca2+ dependent protein kinases, ET- Ethylene, JA- JA-Jasmonic acid, JA-Ile- Jasmonic acid isoleucine, GLVs- Green leaf volatiles, SA- Salicylic acid, ABA- Abscisic acid, GA- Gibberellin)

The lipid derived signal, Jasmonates (JA together with biologically active derivatives of JA, and intermediates of JA biosynthesis pathway) are regarded as the master regulators of plant defense [Koo et al., 2018, Creelman et al., 1997]. Various studies have demonstrated the essential role of JA as a local and systemic wound signal orchestrating defense against chewing insects and necrotrophic pathogens [Yan et al., 2013.The herbivory and damage associated plastid membrane degradation results in the release of a free fatty acid product (linolenic acid), which is the substrate of JA biosynthesis [Wasternack and Hause, 2007]. This lead to wound induced accumulation of JA in the plant within one to two hours of attack resulting in transcriptional reprogramming of 10% or more of the genome including enhancement and repression of transcription of various genes [Devoto et al., 2005, Pauwels et al., 2008, Zhang et al., 2008, Staswick et al., 2008 Halitschke et al.,

2001, Baldwin et al., 1998]. Herbivory induced JA burst further induced carnivore attracting volatiles in lima bean and zea mays in response to the attack of spider mites and beet army worm respectively [Dicke et al., 1999, Schmelz et al., 2003]. Thus, Jasmonates mediate the efficient translation of the perception of primary wound or stress signal to appropriate defense response [Doan et al., 2004, Bruinsma et al., 2009].

Ethylene is a gaseous phytohormone, in many cases acting in parallel with JA in defense against necrotrophic pathogens and herbivorous insects [Xu et al., 1994, Lorenzo et al., 2003].Several studies suggest the role of ET as a modulator of different hormone pathways [Broekgaarden et al., 2015, Groen et al., 2014] .Biosynthesis of ethylene occurs through two important steps, conversion of S-adenosyl-L-methionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC-synthase (ACS), Conversion of ACC into ethylene by ACC-oxidase (ACO), the latter being the rate limiting step [Kato et al., 2000, Balaji et al., 2008]

ABA is another important phytohormone generally considered as a stress hormone against abiotic stresses such as drought and high salinity [Imai et al., 1995] .However there are increasing number of studies in the last few years describing the role of ABA in herbivore and pathogen associated defense responses [Mauch et al, 2005, Dinh et al., 2013]. A recent study suggests the role of ABA as a putative long distance signal in systemic plant defense [Erb et al., 2002]. However there are reports which talk about the multi faced role of ABA acting as a repressor of defense responses and increasing the susceptibility of the plant towards attack [Ton et al., 2009]

The role played by each phytohormone mediated signaling pathways depend on the plant, the plant part which is being attacked, the type of attacker and their specific way of entering the host and feeding. The time courses of accumulation of different phytohormones also vary according to these parameters in a plant-attacker specific manner.

All the current knowledge regarding plant defense signaling and defense responses are known primarily in the context of a plant- folivore interaction (a follivore is a leaf eating herbivore) although there are many other types of herbivorous interactions present in nature such as shoot borers, rhizovores (root herbivory), frugivores (fruit

herbivory), granivores (seed herbivory) etc. [Gurevitch et al., 2002]. The nature of the plant-insect interaction in these cases has been overlooked by scientists so far.

Here, we tried to understand the fruit defense responses. Studies on plant defense theory predicts a strong selection for the defense of fleshy fruits as they are directly linked to seeds, nutritionally rich, and pulp and seeds are exposed to various threats from predators during the long time course of development and post ripening period [Whitehead and Bowers et al., 2014].There has been studies which report the presence of toxic secondary metabolites in fruits in much abundant levels compared to leaves and other plant parts [Herrera et al., 1982, Cippoliniet al., 1997]. Other than their role in seed development and dispersal, very few studies tried to address their role in fruit defense. In *Piper* fruits, the major class of secondary metabolites, amides showed no effect on the seed predator *Sibaria englemani,* but showed strong negative effects on the growth rate of certain fungi present in the plant [Whitehead and Bowers et al., 2014].Attack from the pathogen *Colletotrichum gloeosporioides,* caused a localized production of reactive oxygen species in unripened mango fruits which then resulted in increased lignin accumulation and chitinase activity in the mango peel (epicarp) [Sinniah et al., 2013]. Wild parsnips produce parthenocarpic fruits as a decoy to divert frugivores away. Specialist frugivore, parsnip webworms showed less growth rate on those fruits [Zangerl et al., 1991].An evolutionary study suggests that fleshy pulp of fruits primarily evolved as a defense strategy against seed predators, therefore the fleshy pulp of fruits can play a significant role in defense[Mack et al.,2000].

This study aimed at understanding the interaction of a frugivore with its host plant. We have chosen to study the interaction between eggplant (*Solanum melongena*) and its specialist pest *Leucinodes orbonalis* commonly known as shoot and fruit borer (SFB).

Study system

Eggplant (*Solanum melongena*), is an agronomically important vegetable crop in South and South East Asia. It is a native of India commonly known as brinjal in the country. World-wide production of eggplant has reached 54 million tonnes in the year 2018 and Asia contributes to 93.1% of its production share (FAOSTAT, 2020).Eggplant fruit is well known for its very high content of antioxidant phenolic

compounds and their antitumoral and anticholestremic properties [Singh et al., 2008, Whitaker et al., 2003]. It is a remarkable source of vitamins, minerals, antioxidants and fibers. It is cultivated throughout the year and hence a major source of income for many rural farmers in India. Although eggplant faces attacks from many pests such as white flies, flea beetles, cutworms, armyworms, aphids etc., attack from the eggplant shoot and fruit borer (SFB), *Leucinodes orbonalis* (Lepidoptera: Pyrallidae) is the major challenge faced by farmers in terms of production and marketing. It is the most serious and destructive pest of brinjal causing more than 70% loss in the marketable yield [Saran et al., 2018, Latif et al., 2010]. It is a specialist pest on eggplant although some other Solanaceae plants are also reported to be occasional hosts of this pest [Fletcher et al., 1916, Hargreaves et al., 1937]. Larva causes serious damage to both vegetative and reproductive stage of eggplant. The adult moth lays around 100-250 eggs in its life time mostly on lower surface of leaves, tender shoots of the plant and when eggs hatch, freshly hatched larvae (neonates) bore inside the petioles, veins and midribs of leaves and start feeding on the internal tissue. This results in the wilting and drooping of shoot. Soon the larva bore into fruits and stays inside the fruit. A significant amount of its larval period, larva stays inside the fruit. It is this larval stage which is the most devastating stage of this insect attack. Larval feeding along with continuous deposition of its excreta inside the fruit make the fruits unfit for human consumption. One larva can damage 4-6 fruits [Jayaraj and Manisegaran, 2010]. Infested fruits can be identified by their boring holes.

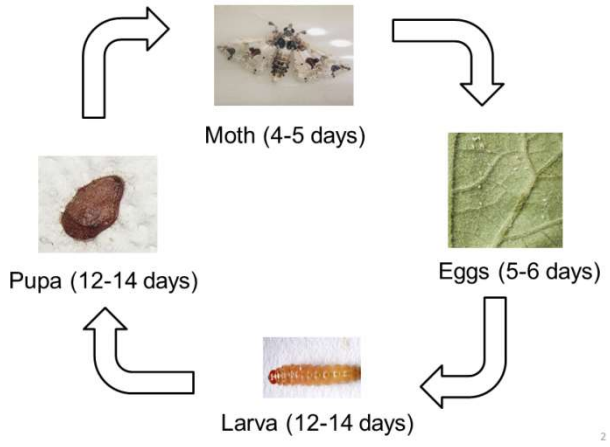

Figure 2: Life cycle of SFB

Female moth lays around 100-250 eggs in its life mostly on the lower surface of leaves and tender shoots during night time. Neonates (freshly hatched larvae) start boring tender shoots, leaf petioles, veins, midribs and soon into fruits. Larva feeds inside the fruit and form pupae outside. Moths emerge from pupae after two weeks and life cycle continues.

The unique nature of feeding of SFB by boring and staying inside the fruit makes the control of SFB through pesticides difficult. Intensive application of these pesticides have given rise to problems of pest resistance, secondary pest outbreak; increased production costs and environmental hazards [Chakraborti et al., 2011, Gaur and Chaudhary, 2009] .High frequency applications also cause adverse health effects to humans. Alternate non-chemical strategies such as screening of resistant varieties, sex pheromone trapping of male moths, destruction of infested shoots and fruits, and pupas at regular intervals, were employed in fields, but none of them offered control of SFB to an appreciable level. In this context, Bt eggplant was developed as the first genetically modified (GM) food crop in India by the Maharashtra Hybrid Seed Company Ltd (Mahyco), Pune, with lepidopteron specific cry1Ac gene from *Bacillus thuringiensis.*[Gaur and Chaudhary, 2009]. Although field trials from Bangladesh and Philippines show adequate resistance of Bt plants to SFB, further studies report that insects are evolving resistance to Bt crops in a faster rate [Shelton et al., 2019, Hautea et al., 2006, Sheikh et al., 2017].However, the controversies regarding the safety concerns of commercialisation of genetically modified food crops in India lead to the ban of Bt eggplant in the country [Shelton et al., 2010, Mathur et al., 2012].

While the two closely related Solanum crops of eggplant, potato (*solanum tuberosum*) and tomato (*Solanum lycopersicum*) are well studied in terms of their interactions with their pests, eggplant is much less recognized [Hirakawa et al., 2014]. As a pest that poses a great threat to the economic value of eggplant, the chemistry of interaction between SFB and eggplant is not well studied.

In this study, we tried to understand the plant innate responses towards frugivory. We found the involvement of two phytohormones, ABA and ET in fruit response towards larval feeding. Increased ABA metabolite levels and transcript level accumulation of putative ET biosynthetic genes was observed in response to larval feeding. Two Major classes of secondary metabolites of Solanaceae family did not respond to frugivory.

2. Materials and methods

2.1. Plants and growth conditions

Plants of *Solanum melongena* of the variety, Kashi Taru (KT) were used in this study. Plants of this variety are tall and erect, height 120-130cm and leaves and stem dark green. Fruits are long, purple with average length 31cm and average diameter 5cm (ICAR- Indian institute of vegetable research). Seeds were obtained from Indian institute of vegetable research (IIVR), Varanasi. Seeds were germinated in autoclaved mixture of cocopeat, black and red soil in 2:1:1 proportion respectively. Three weeks old seedlings were transferred to pots (diameter 10cm) filled with mixture of cocopeat, black and red soil in 1:1:1 proportion respectively. Plants were maintained inside a climatic chamber (25 ± 2°C, 65 ± 5% RH, 16h light and 8h dark photoperiod).Two months old plants were planted into the field at Indian Institute of Science Education and Research (IISER), Pune. A distance of 1m was maintained between plants.

2.2. Insects and rearing conditions

Leucinodes orbonalis larvae were collected from infested eggplants of the experimental field inside IISER, Pune. To initiate a laboratory population of ESFB, larvae collected from the field were maintained in aerated polypropylene containers [length (l) 30cm × breadth (b) 20cm × height (h) 10cm] incubated inside a climatic chamber (25 ± 2°C, 65 ± 5 %RH, 16h light and 8h dark photoperiod) and fed on fruits of KT variety. Pupae were maintained in separate containers in dark. For mating, male-female moth pairs were kept in a mating jar [20cm (l) × inner diameter (ID) 10cm] containing healthy twigs (6-8cm) of KT variety for oviposition. Moths were reared on a 10% (v/v) aqueous sucrose solution. After egg hatching, the same twig was offered to the neonates for the first few hours. First instar larvae were transferred to rearing containers and KT fruits were given to feed on.

2.3. Experimental set-up to examine the change in phytohormone and defense metabolite levels upon frugivory

Three months old KT plants in the field were caged (mesh size with i.d. 1.5mm) to avoid natural SFB infestation (Figure 2A). Cages were 2ft x 2ft x 4.5ft (length x breadth x height) in size with adequate aeration inside and plant growth was not

disturbed (Figure 2A). Fruits of the same maturation stage were selected for experiments (Days after pollination (DAP) 20). It was observed that an average sized 20DAP fruit have a length of ~6 inches .Mechanical damage (MD) (1cm depth x 4 mm diameter) was done to fruits with the help of an artificial borer to mimic the boring activity of the insect. Manual infestation of selected fruits was done with third-fourth instar SFB larvae. Insects were allowed to feed for 0, 1, 3, 6, 12, 24, 48 and 120 hours. Initial time points were chosen for analysing change in phytohormone levels whereas late time points were chosen to analyse change in levels of induced defense metabolites. Fruits were caged during the assay with a net bag (mesh size-1mm) in order to prevent larval escape (Figure 2B). As MD control, only mechanical boring was done to fruits. Healthy fruits (control) were covered with a net bag for tissue collection as no wound control. During collection of tissues, fruits were dissected into six parts which are epicarp, mesocarp, placenta, crown, seeds, and Pedicel (stalk of the fruit) (Figure 2C). Collected tissues were flash -frozen in liquid nitrogen and stored at -80°C.

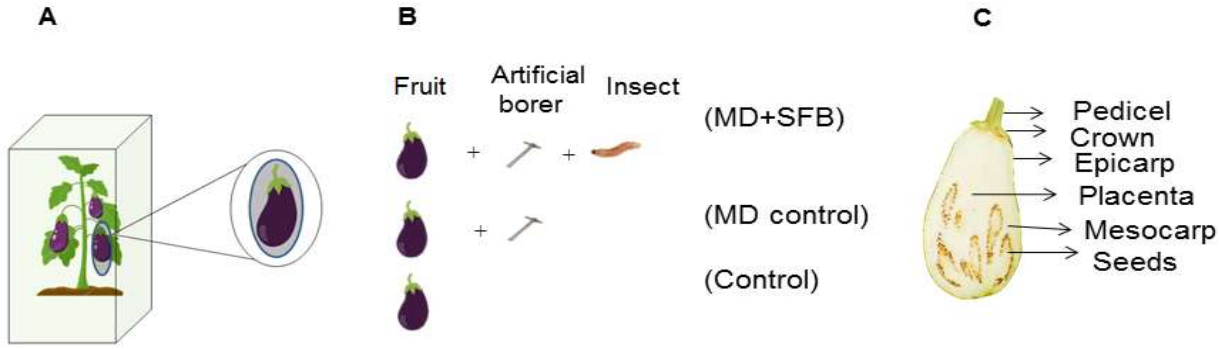

Figure 3: Experimental set-up to examine the change in phytohormone and defense metabolite levels upon frugivory

(A) - Schematic showing KT plants caged in the field to avoid natural SFB infestation, KT fruits (DAP 20) caged during the assay time. (B) - Three treatments given to fruits, MD+SFB- Fruits mechanically damaged with artificial borer to mimic the boring activity of the insect and third- fourth instar SFB larvae released inside. MD control- Mechanically damaged control tissues, Control- Healthy fruits bagged (C) - Schematic showing the tissue types collected

2.4. Assay of metabolites by UPLC/ESI/QTOF-MS

Harvested tissues were homogenized in liquid nitrogen. Approximately 300mg of homogenized tissues were weighed and placed in 2ml microcentrifuge tubes. One millilitre of extraction buffer (absolute methanol) spiked with 250ng/ml of adonitol and 500ng/ml of nicergoline as internal standards was added to each sample. Samples were vortexed and incubated at room temperature for ten minutes. The homogenate was centrifuged repeatedly at 12,000rpm and the supernatant was collected. High molecular weight lipids were precipitated by incubation at -80°C and removed by further centrifugation. 50µl of this clear solution was injected into a SCIEX X500R UPLC/ESI/QTOF-MS system. Analytes were separated on a C18 column (5µm, 50 X 4.6mm; Gemini) using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) . The gradient of 0 min 0% B, 3 min 10% B, 6 min 50% B, 7 min 70% B, 10 min 100% B, 12 min 100% B, with a flow rate of 0.5ml/min was used.

Phytohormones and phenolics were analysed by negative electrospray ionisation and steroidal alkaloids by positive electrospray ionisation.

Table 1: Metabolites analysed in this study, their molecular mass and respective mode of electrospray ionisation.

Analytes	Molecular formula	Mass (g/mol)	Mode of electrospray ionisation
Abscisic acid (ABA)	$C_{15}H_{20}O_4$	264.32	Negative
Gibberellic acid (GA)	$C_{19}H_{22}O_6$.	346.38	Negative
Indole-3-acetic acid (IAA)	$C_{10}H_9NO_2$	175.18	Negative
Indole-3-butyric acid (IBA)	$C_{12}H_{13}NO_2$	203.24	Negative
Jasmonic acid (JA)	$C_{12}H_{18}O_3$	210.27	Negative
Jasmonic acid isoleucine(JA-ILE)	$C_{18}H_{28}NO_4$	322.4	Negative
Salicylic acid (SA)	$C_7H_6O_3$	138.12	Negative
Chlorogenic acid (CGA)	$C_{16}H_{18}O_9$	354.31	Negative
Caffeic acid (CFA)	$C_9H_8O_4$	180.16	Negative

Quinic acid (QNA)	$C_7H_{12}O_6$	192.17	Negative
Gallic acid	$C_7H_6O_5$	170.12	Negative
Eugenol	$C_{10}H_{12}O_2$	164.20	Negative
Vanilin	$C_8H_8O_3$	152.15	Negative
Benzyl salicylate	$C_{14}H_{12}O_3$	228.24	Negative
Rutin	$C_{27}H_{36}O_{19}$	664.6	Negative
Solasodine	$C_{27}H_{43}NO_2$	413.65	Positive
Solasonine	$C_{45}H_{73}NO_{16}$	884.1	Positive
Solamargine	$C_{45}H_{73}NO_{15}$	868.1	Positive

2.5. Larval escape

Mechanical damage (MD) (1cm depth x 4mm diameter) was done to healthy, uninfested fruits in the field plants (KT) with the help of an artificial borer and third-fourth instar SFB larvae were released inside (n=12). Fruits were caged with a net bag (mesh size-1mm) and observed twice a day. This was done in order to monitor the amount of time larva stays inside a single fruit.

2.6. Standardization of ET detection through gas chromatography- mass spectrometry (GC-MS)

ET detection and quantitation method has been standardised using ET analytical standards. 20 ml airtight glass vials with third-fourth instar SFB larvae feeding on it and control glass vials with uninfested tissue were incubated for 0 hour (5 minutes), 1 hour, and 3 hours. After each incubation time point, the headspace was sampled using 1 mL gas-tight syringe and direct gas injection on the PLOT column (Thermo Scientific).

2.7 Gene expression analysis

Identification of putative eggplant *ACO* genes

ACO gene sequences for *Nicotiana tabacum* (GenBank ID: AB012857.1 , NCBI Reference Sequence: NM_001325309.1, NM_001325398.1, NM_001325967.1), *Capsicum chinense* (GenBank ID: AB434925.1, AJ879117.2), *Solanum tuberosum* (GenBank ID: AF384820.1, AF384821.1,NCBI Reference Sequence: NM_001288220.2), *Lycopersicon esculentum* (GenBank ID: AJ715790.1), *Nicotiana*

attenuata (GenBank ID: AY426756.1, EF123111.1,), *Nicotiana suaveolens* (GenBank ID: DQ984136.1), *Capsicum annuum* (GenBank ID: JX515597.1), *Solanum lycopersicum* (NCBI Reference Sequence: NM_001246938.2 NM_001247095.2, NM_001247108.1, NM_001329913.1), *Nicotiana glutinosa* (GenBank ID: U54565.1, U54566.1, U62764.1) These sequences were used in "Basic local alignment search tool (BLAST)" search against eggplant genome database (http://www.eggplantgenome.org/). Sequences with low e value were selected and further filtered based on 90% or more similarity. As a result, five putative eggplant *ACO* genes were identified.

Primer designing

Multiple sequence alignment was done using the MEGA (Molecular Evolutionary Genetics Analysis, ver 7.0.26) software. Pairwise alignment of sequences was done in order to design primers for qPCR. 3' UTR regions of these sequences were utilized to design primers for ACO genes as they were dissimilar from each other. Each primer was aligned to all the other sequences to ensure that there is no non-specific binding.

Primer specificity was verified by PCR followed by gel electrophoresis and qPCR standard curve obtained from serial dilutions of cDNA.

Table 2- Primers used in the real time quantitative PCR study

Gene ID	Gene name	Forward primer	Reverse primer	Amplicon Length (bp)
SMEL_002g156760.1	*SmACO2a*	GGGAATGGGAACAAAAGATTG	AACAAGAAAAAGCACCCCACT	95
SMEL_007g283770.1.01	*SmACO5*	AAGGGTTATTCTTTCCGAGTT	GCTCATACACATCAGCTTCTCA	102

		T		
SMEL_007g287810.1.01	*SmAC01a*	GCTTAGATTCCAATTCAATTGGAG	GATACAATATTAGGACCCTTTGA	138
SMEL_007g287840.1.01	*SmACO1b*	GAAAATGAATCTGGTCTTGGAA	CACAGCAGAAAAACACACCA	95
SMEL_010g339190.1.01	*SmACO2b*	ATTAGTACAAAACTAAGTGGCATAC	CAAACTTTAGTCCTCTTTACCAC	118

Real time quantitative PCR

Total RNA was extracted from approximately 150mg of frozen mesocarp tissue samples using the Trizol reagent (Himedia) followed by chloroform extraction. Concentrations of total RNA were determined by measuring the absorbance at 260nm and the ratio of the absorbance at 260/280nm was used to assess the RNA purity in a nanodrop spectrophotometer. RNA integrity was verified by electrophoresis using a 1% agarose gel.

First strand cDNA was synthesized from 500ng total RNA using Primescript TM first strand cDNA synthesis kit (Takara) according to the manufacturer's instructions.

Transcript abundance of ACO homologs was analysed by quantitative real-time reverse transcription polymerase chain reaction (qRT)-PCR. Cyclophilin a was used as an internal standard.

qPCR was performed in a total volume of 5µl (2.5µl of TB Green Premix Ex Taq II, 0.25µl of each gene specific primer, and 1µl of cDNA) using a Bio-Rad CFX96 Real-

Time System. Following cycler conditions were used: 95ºC for one minute, 39 cycles of 95ºC for 45 seconds, 55ºC 45 seconds, 72ºC for 45 seconds.

2.8. Statistical analysis

All the data were analysed by the one-way analysis of variance (ANOVA) followed by the least significant difference (Fisher's protected LSD) calculated by using StatView software (ver. 5.0). In all the statistical analyses, we considered not detected compounds to have value zero.

3. Results

3.1 Increased ABA levels after SFB feeding.

Out of seven phytohormones screened in this study, JA, JA ILE, and ABA were detected in mesocarp, placenta, pedicel tissues and JA, and ABA were detected in crown through UPLC/ESI/QTOF-MS .SFB feeding induced higher levels of ABA in mesocarp, placenta, and crown tissues after 120 hours of feeding compared to control tissues (Figure 4C).ABA levels increased approximately 6-7 folds from basal level.

Fruits subjected to 120 hours of SFB feeding were found to be rottened whereas mechanically damaged control fruits did not.

No phytohormones were detected in seeds.

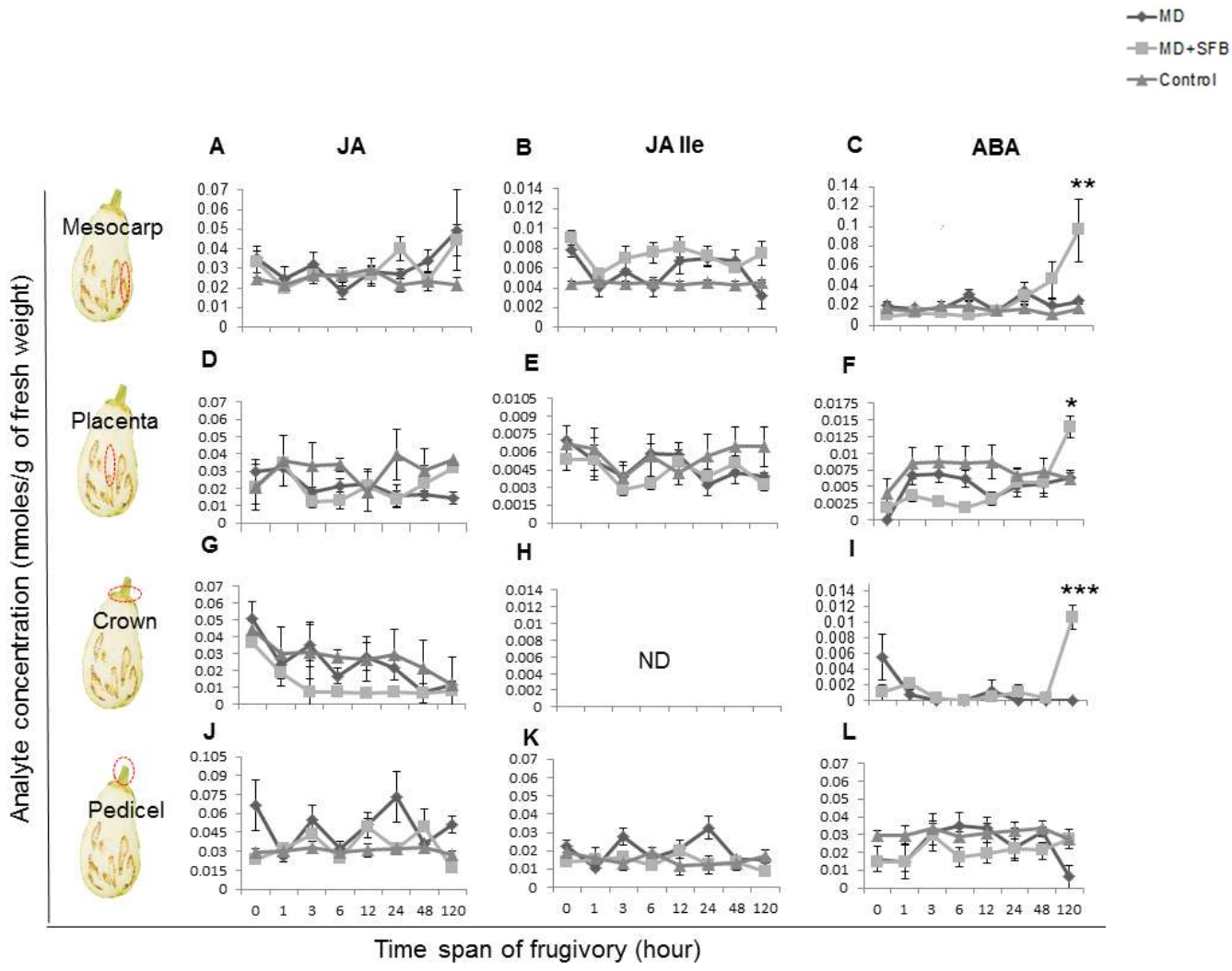

Figure 4: Profiling of phytohormones across tissue types

(Mean ±SE), n=5 biological replicates.

JA concentrations (nmoles/g of fresh weight) in mesocarp (A), placenta (D), crown (G), and pedicel (J) tissues of healthy fruits (control), fruits damaged by feeding of third-fourth instar SFB larvae (MD+SFB) ,and fruits damaged by mechanical damage mimicking the boring activity of insect (MD). No significant change in the levels of JA. JA-Ile concentrations (nmoles/g of fresh weight) in mesocarp (B), placenta (E) and pedicel tissues (K) of healthy fruits (control), fruits damaged by feeding of third-fourth instar SFB larvae (MD+SFB) ,and fruits damaged by mechanical damage mimicking the boring activity of insect (MD). JA Ile levels were not detectable in crown (H). No significant change in the levels of JA ILE. ABA concentrations (nmoles/g of fresh weight) in mesocarp (C), placenta (F), crown(I), and pedicel (L) tissues of healthy fruits (control), fruits damaged by feeding of third-fourth instar SFB larvae (MD+SFB) ,and fruits damaged by mechanical damage mimicking the boring activity of insect (MD).Fruits damaged by SFB feeding showed a significant induction of ABA in mesocarp, placenta, and crown after 120 hours of feeding, (approximately 6-7 folds increase compared to control and mechanically damaged fruits.) (ANOVA, * is used for $p<0.05$, ** is used for $p<0.01$, *** is used for $p<0.001$, ND-Not detected, JA-Jasmonic acid, JA Ile- Jasmonic acid isoleucine, ABA- Abscisic acid).

3.2. Survey of secondary metabolites as putative indicators of frugivory induced defense responses

3.2.1. Steroidal alkaloids

Solasodine (SD) and Solamargine (SM) were detected in mesocarp, placenta, and crown tissues. Mesocarp and placenta did not show any change in the levels of these metabolites. A small increase (two folds from the basal level) in the levels of SD is found in crown after 48 hours of SFB feeding (Figure 5). No steroidal alkaloids were detected in seeds.

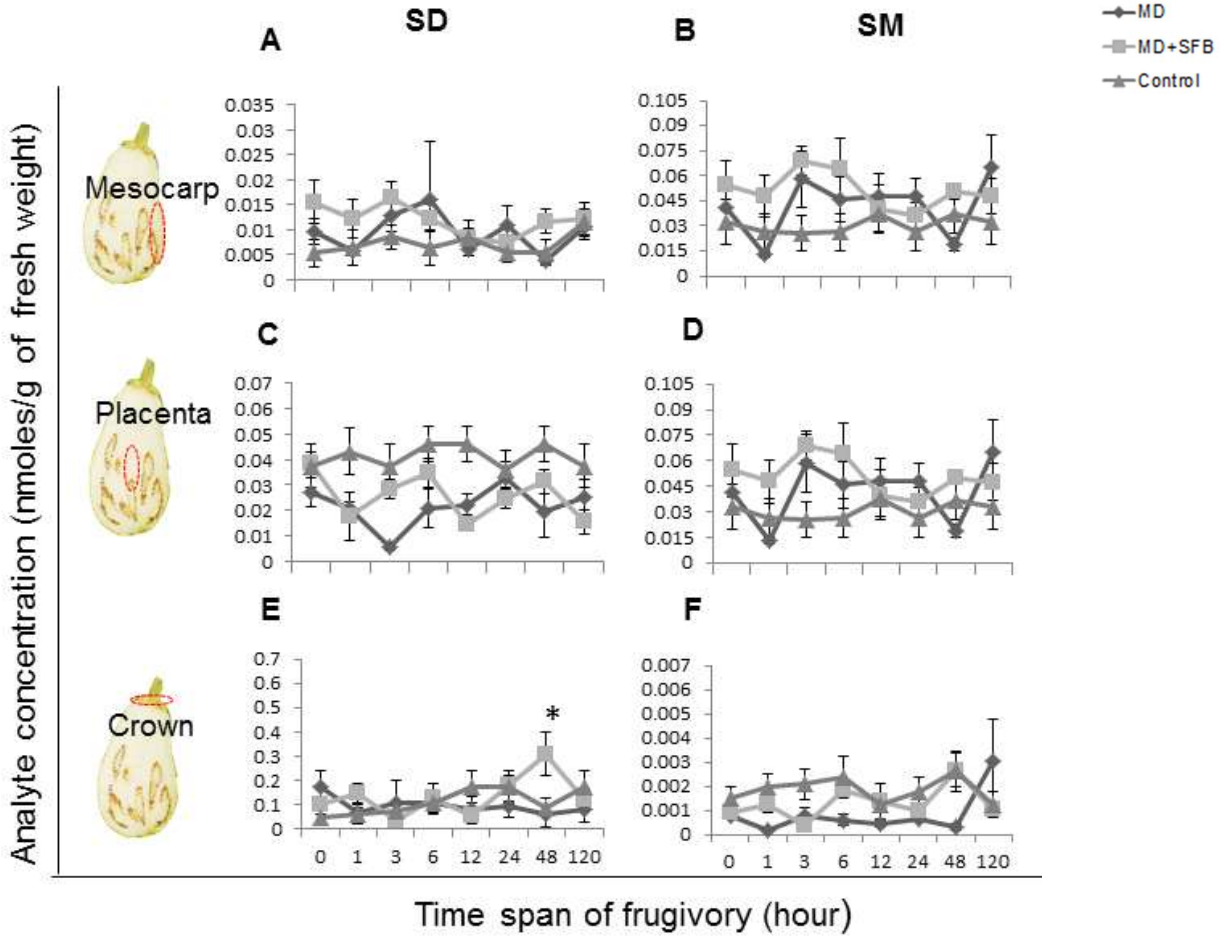

Figure 5: Profiling of steroidal alkaloids across tissues

(Mean ±SE), n=5 biological replicates.

SD concentrations (nmoles/g of fresh weight) in mesocarp (A), placenta (C), crown (E) tissues. Increase in the SD levels (approximately twice) in the crown after 48 hours of feeding by third-fourth instar SFB larvae (E). No significant change in the levels for mesocarp and placenta tissues after SFB feeding and mechanical damage. SM (nmoles/g of fresh weight) concentrations in mesocarp (B), placenta (D) and crown (F) tissues. No significant change in the levels of SM in mesocarp, placenta and crown tissues after SFB feeding and mechanical damage.

(ANOVA, * is used for $p<0.05$, SD- Solasodine, SM- Solamargine)

3.2. 2. Phenolics

Out of the eight phenolic compounds screened as putative indicators of frugivory induced defense response, only three of them (CGA, CFA, and QNA) were found in eggplant fruit. No significant change in the levels of these metabolites is observed in mesocarp, placenta, and crown tissues (Figure 6)

No phenolics were detected in seeds.

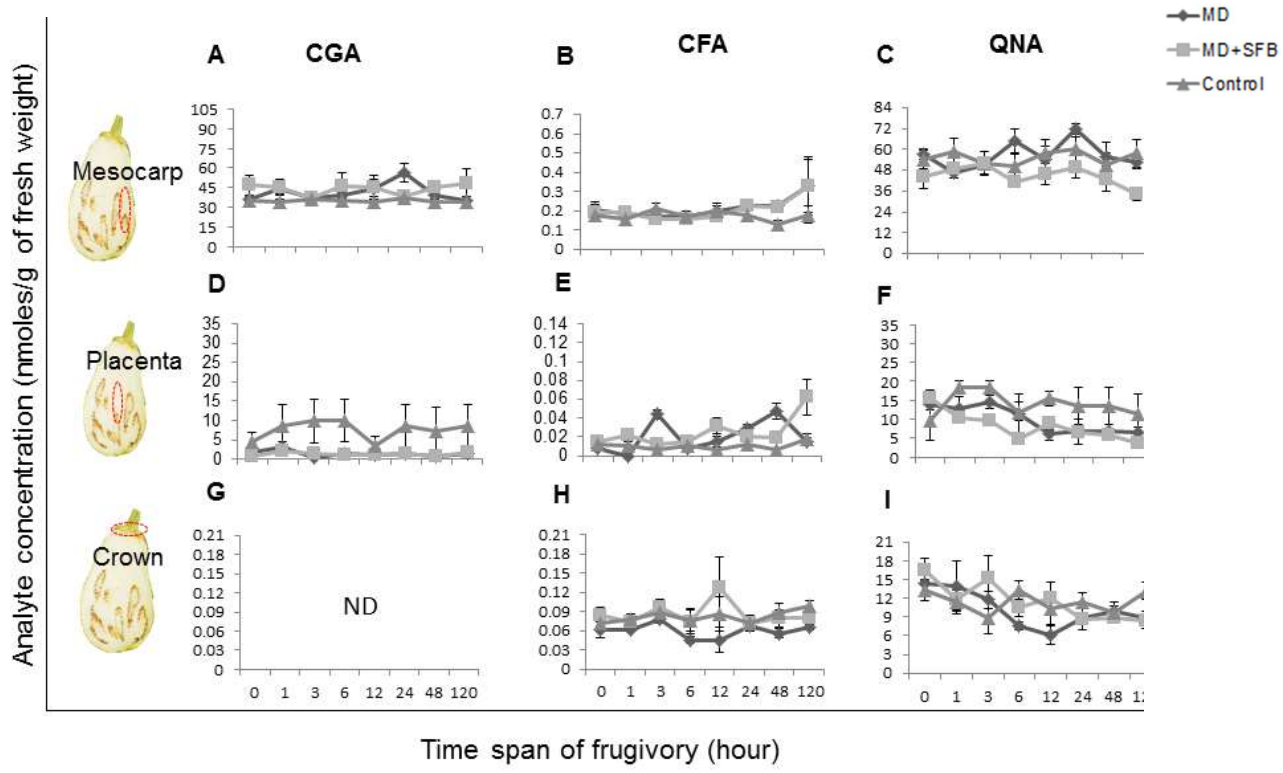

Figure 6: Profiling of phenolics across tissue types

(Mean ±SE), n=5 biological replicates.

Concentration of CGA (nmoles/g of fresh weight) in mesocarp(A) and placenta (D). CGA is not detected in crown (G). No significant change in the levels of CGA in mesocarp, placenta tissues after SFB feeding and mechanical damage.Concentrations of CFA (nmoles/g of fresh weight) in mesocarp (B), placenta(E) and crown (H) tissues. No significant change in the levels of CFA in mesocarp, placenta and crown tissues after SFB feeding and mechanical damage.Concentrations of QNA (nmoles/g of fresh weight) in mesocarp (C), placenta(F) and crown (I) tissues. No significant change in the levels of QNA in mesocarp, placenta and crown tissues after SFB feeding and mechanical damage**.** (ND- Not detected, CGA- Chlorogenic acid, CFA- Caffeic acid, QNA- Quinic acid)

3.3 Increased ABA levels and larval escape by third day

Larval escape was observed on third day. Out of twelve fruits, eight fruits were found with the appearance of a second hole indicating larval attempt to leave the fruit. Mesocarp and placenta tissues collected from the same fruits showed increased ABA level on third day (Figure 8)

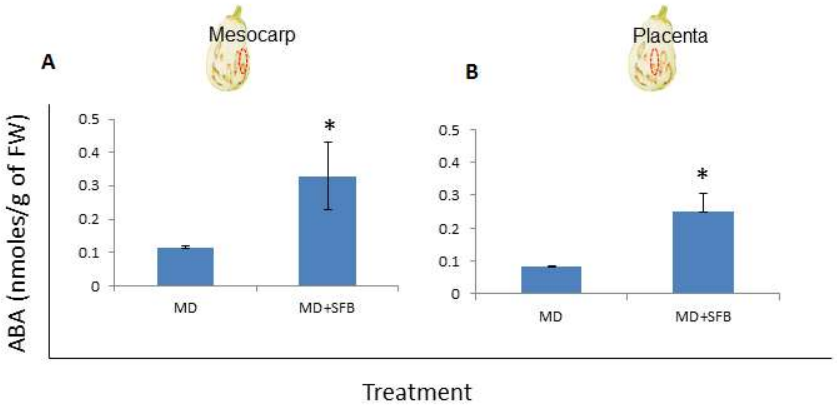

Figure 7: Increased ABA levels by third day

Increased ABA levels found in Mesocarp (A), placenta (B) tissues collected from the fruits larvae tried to escape on third day of continuous feeding. (ANOVA, * is used for $p<0.05$, ** is used for $p<0.01$)

3.4. Ethylene

3.4.1 Standardisation of ethylene detection through gas chromatography-mass spectrometry.

Ethylene analytical standard was detected in GC-QQQ (Figure 8).

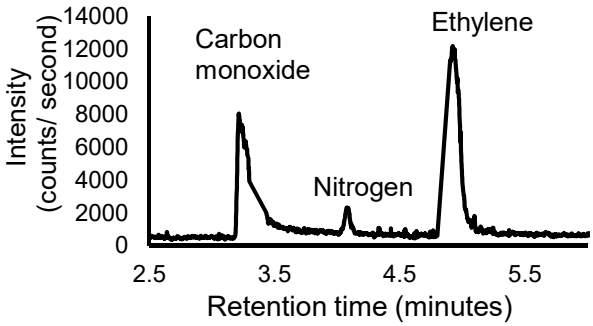

Figure 8: ET analytical standard detection

ET analytical standard was detected in GC-QQQ**.** Carbon monoxide (Mol wt-28.01 g/mol), Nitrogen (Mol wt- 28.01 g/mol), and Ethylene (Mol wt- 28.05 g/mol) peaks were clearly separated using the PLOT column (Thermo Fisher Scientific).

3.4.2 Identification of putative eggplant *ACO* genes

We identified five putative eggplant *ACO* genes through BLAST study. A phylogenetic tree constructed based on ACO nucleotide sequences from Solanaceae family members demonstrates their homology. The identified putative eggplant *ACO* genes were named *Solanum melongena ACO1a* (SMEL_007g287810), *Solanum melongena ACO1b* (SMEL_007g287840.1.01), *Solanum melongena ACO2a* (SMEL_002g156760.1), *Solanum melongena ACO2b* (SMEL_010g339190.1.01), *Solanum melongena ACO5* (SMEL_007g283770.1.01) based on their phylogenetic similarity to other *ACO* genes of Solanaceae family members (Figure 9).

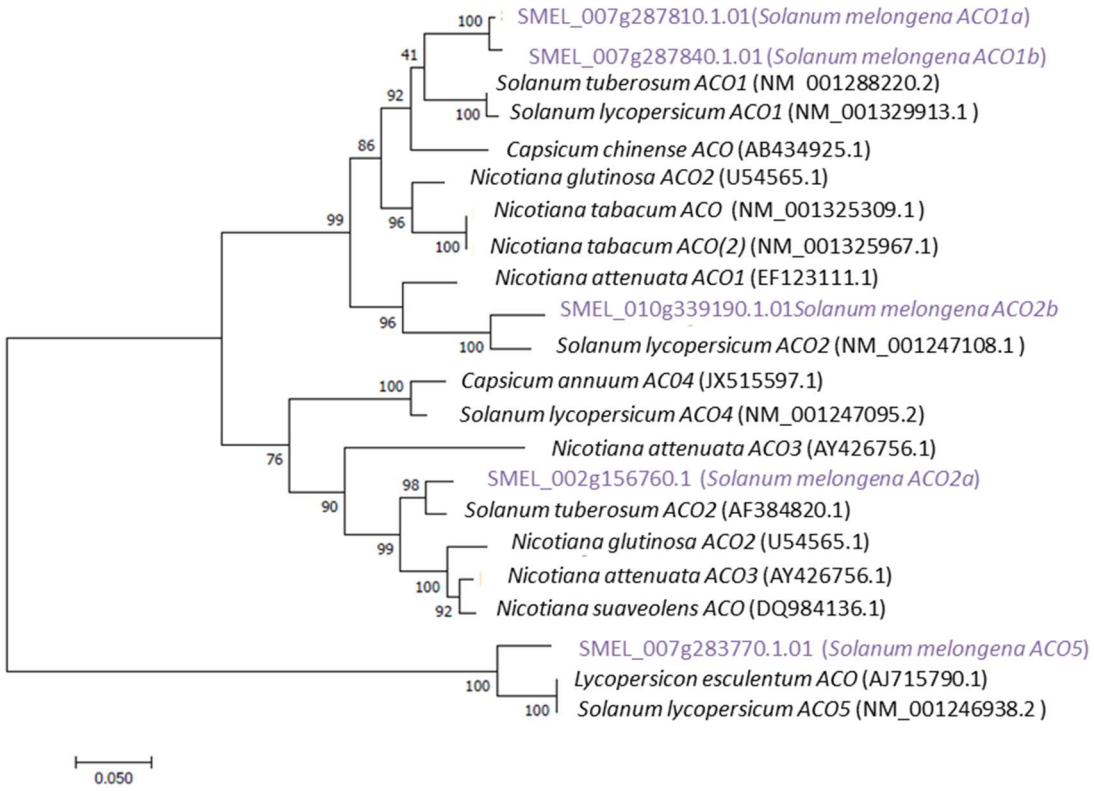

Figure 9: *ACO* phylogenetic tree

A phylogenetic tree is constructed from multiple alignment of ACO full length nucleotide sequences from different Solanaceae family members obtained from NCBI database.The analysis was done using the MEGA (Molecular Evolutionary Genetics Analysis, ver 7.0.26) software. Five identified putative eggplant *ACO* genes are named *Solanum melongena*

ACO1a, *Solanum melongena ACO1b, Solanum melongena ACO2a*, *Solanum melongena ACO2b, Solanum melongena ACO5* based on their phylogenetic similarity to other ACO genes of Solanaceae family members

3.4.3 Elevated transcript levels of putative eggplant *ACO* genes.

We analyzed the transcript level expressions of identified putative eggplant ACO genes (*SmACO1a, SmACO1b, SmACO2a, SmACO2b*, and *SmACO5*). *SmACO1a,* and *SmACO5* transcripts responded to SFB feeding. Transcript levels of *SmACO2b* were undetectable. Transcript levels of control fruits were hardly detectable in all cases. Transcript levels of *SmACO1a* accumulated at 48 hours of larval feeding (Figure 10A). Transcript levels of *SmACO5* accumulated at 3 hours of larval feeding and later declined. Transcipt levels of *SmACO5* responded to both larval feeding and mechanical damage after 0 hour (5 minutes). (Figure 10D). *SmACO1b and SmACO2a* transcript levels did not respond to larval feeding (Figure 10B, 10C).

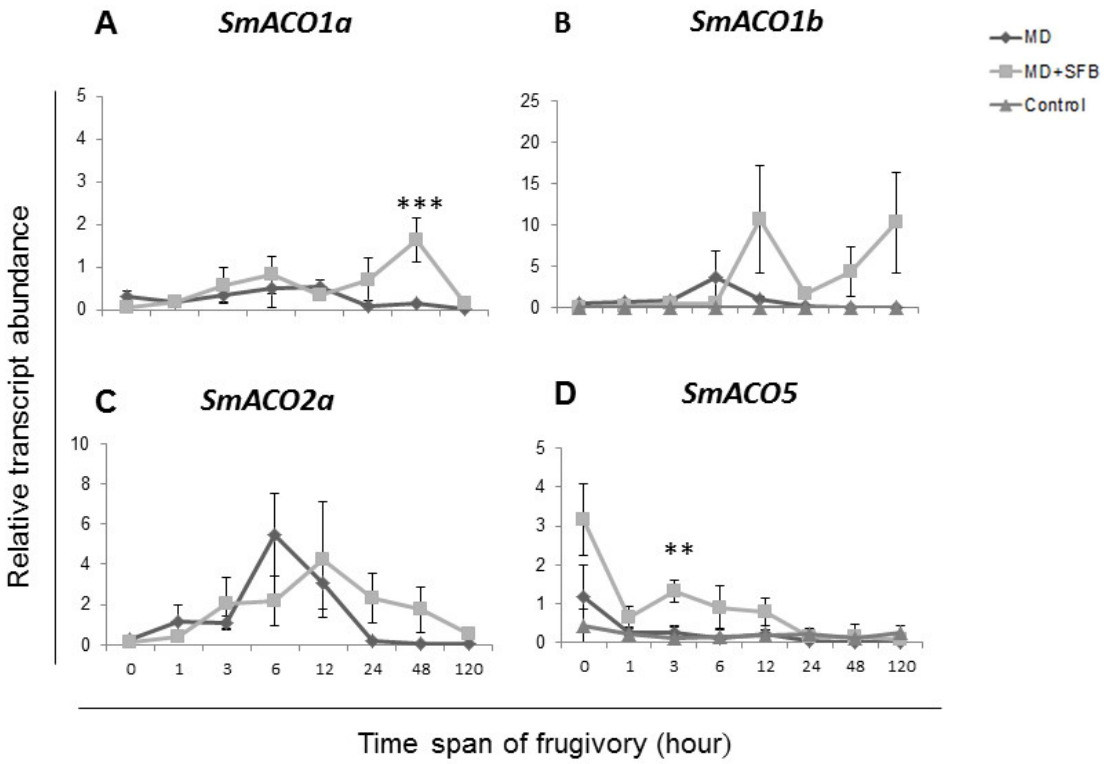

Figure 10: Gene expression analysis of putative eggplant *ACO* genes during frugivory.

Relative transcript abundance ±SE of *SmACO1a*, *SmACO1b, SmACO2a*, and *SmACO5* as analysed by RT-PCR, n=5 biological replicates .Transcript levels of the individual genes were normalized to the transcript abundance of an internal control gene (Cyclophilin a). Transcript levels were undetectable for *SmACO2b*.

(B) Transcript level of *SmACO1b* did not respond to larval feeding.
(C) Transcript level of *SmACO2a* did not respond to larval feeding. Transcript levels of control tissues were undetectable.
(D) Transcript level accumulation of *SmACO5* in mesocarp tissues after 3 hours of feeding by third-fourth instar SFB larvae.Transcipt levels responded to both larval feeding and mechanical damage after 0 hour (5 minutes).
(ANOVA, * is used for $p<0.05$, ** is used for $p<0.01$)

4. Discussion

Plant defense signaling involves a complex network of interacting signal transduction pathways, majorly regulated by phytohormones. Phytohormones play a crucial role in fine tuning plant's defense responses by further activating appropriate downstream defense responses. The role of classical defense hormones, JA, SA and ET in this orchestrated plant defense response is widely studied. Along with that, recent studies have shown the critical role played by phytohormones such as abscisic acid (ABA), auxins (IAA) and gibberellins (GA) against biotic stress [Checker et al., 2018, Seilaniantz et al 2011].

While all the current knowledge regarding plant defense responses are mainly limited to the context of folivory, this study aimed to understand the defense signaling in eggplant fruit upon the attack of the shoot and fruit borer. Fruits protect seeds and help the plant in disbursing the seeds. It forms a barrier between outer environment and seeds during the course of seed development. As a major sink organ of the plant, whether fruits are capable of eliciting defense signals when being attacked is a question we tried to address here. We conducted a frugivory assay in our experimental field where we subjected fruits to the feeding by third-fourth instar SFB larvae for 1, 3, 6,12,24,48,and 120 hours and analysed the change in levels of phytohormones across mesocarp, placenta, crown, seeds, pedicel and epicarp tissues. Feeding for 120 hours elicited 6-7 folds higher levels of the phytohormone ABA in the fruit (mesocarp, placenta and crown tissues) and fruit were found to be rottened. Our later experiments point to the fact that one larva does not feed for 120 hours (5 days) inside a fruit in natural conditions. We monitored the number of days larva stays inside a single fruit and found that larva leaves a fruit by three days of feeding .Elevated ABA levels was observed in the mesocarp, placenta tissues of this day fruit .Although ABA has long been understood as a stress signal against abiotic

stresses, recent studies show the role of ABA signaling in defense against herbivorous insects [Bodenhausen et al., 2007,Thaler et al., 2004].These studies demonstrate the synergistic action of JA and ABA against necrotrophic pathogens leading to reduced herbivory. In *Arabidopsis*, Priming of systemic defense responses against specialist herbivore *Pieris rapae* is activated by ABA by co-regulating the MYC branch of defense together with JA [Vos et al., 2013]. Tomato and *Arabidopsis* plants with reduced ABA content are shown to be more susceptible to the generalist herbivorous insects *Spodoptera littoralis* and *Spodoptera exigua* [Bodenhausen and Reymond, 2007, Thaler et al., 2004].

ET is a gaseous plant hormone regulating fruit ripening in non-climacteric fruits. However several studies have shown the role of ET as an important signaling molecule regulating plant defense responses against herbivores. We identified five putative eggplant, ET biosynthetic gene, *ACO* through BLAST study and analysed their transcript level expressions (*SmACO1a, SmACO1b, SmACO2a, SmACO2b*, and *SmACO5*). Among them *SmACO1a* and *SmACO5* transcripts responded to larval feeding, transcript level accumulation in infested mesocarp tissues was observed at 48 and 3 hours of feeding by SFB respectively. Elevated transcript levels of putative ET biosynthetic genes suggest a possible role of ET in the eggplant fruit response to frugivory. ET induced defense responses include accumulation of phenolic compounds, increase in proteinase inhibitor activity and release of volatile organic compounds (VOCs) [Dahl et al.,2007, Kahl et al., 2000].

We investigated the change in levels of any secondary metabolites of fruit upon frugivory as an indication of defense signaling. Phenolics and alkaloids are two major classes of secondary metabolites present in the Solanaceae family. Production of steroidal alkaloids is a characteristic of Solanaceae family. These metabolites possess interesting insecticidal properties. It is generally considered that many alkaloids are induced upon herbivory whereas phenolics are generally not [Adler et al., 2006].However, recent studies have shown the increase in anti-herbivore phenolic content of plants in response to herbivory, especially the increased accumulation of chlorogenic acid (CGA), against attacks from different types of insects which include stem borers, both sucking and chewing insects. *Tricobaris mucorea* stem borer larvae induced a 1000 fold accumulation of CGA in the pith tissues of *Nicotiana attenuata*. Attack from *Spodoptera litura* larvae induced

significant levels of both CGA and CFA in tomato leaves after 24 hours of feeding [Kundu et al., 2018, Lee et al., 2007]. Eggplant fruit is known for its very high content of phenolics, especially the hydroxycinnamic acid conjugates, CGA, CFA and QNA. Here, we see no change in the levels of phenolic content of the fruit upon the attack. We observed a small increase (approximately twice from the basal level) in the levels of the steroidal alkaloid, solasodine (SD) in the crown tissue in response to SFB feeding for 48 hours.

This study aimed to understand the response of eggplant fruit towards the attack of shoot and fruit borer. Among phytohormones, jasmonates and salicylates are regarded as the global signals for defense gene expression in plants [Raymond and farmer, 1998]. Attack from lepidopteron insects are in general followed by an induction of JA (10-15 folds) within first one to two hours of attack typically in cases of folivory[Shivaji et al., 2010, Diezel et al., 2009, Halitschke et al., 2001, Baldwin et al., 1998, Schulze et al., 2006]. Although less extensive and qualitatively different, pith and root infestations have also shown an increased JA level response towards herbivory [Diezel et al., 2011, Fragoso et al., 2014, Tytgat et al., 2014. When jasmonate mediated defense responses primarily mediate leaf defense responses against lepidopteron attack, our study report no involvement of jasmonates in fruit upon the attack of *Leucinodes orbonalis* (Lepidoptera: Pyrallidae). This study shows the involvement of the phytohormones ABA and ET in fruit response towards insect attack. We found no change in the levels of important secondary metabolites of Solanaceae. Future studies focusing on the role played by these phytohormones and their downstream targets can provide new insights about this crop-pest interaction.

References

1. Adler and Lynn, “Leaf herbivory and nutrients increase nectar alkaloids." Ecology letters 9.8 (2006).
2. Baldwin, Kessler, et al. "Volatile signaling in plant–plant–herbivore interactions: what is real?" Current opinion in plant biology 5.4 (2002)
3. Broekgaarden, Colette, et al. "Ethylene: traffic controller on hormonal crossroads to defense." Plant physiology 169.4 (2015)
4. Bruinsma, Maaike, et al. "Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores." Journal of experimental botany 60.9 (2009)
5. Caarls, Lotte, et al. "How salicylic acid takes transcriptional control over jasmonic acid signaling." Frontiers in plant science 6.1 (2015)
6. Carriere, Yves, et al. "Evolutionary ecology of insect adaptation to Bt crops." Evolutionary Applications 3.5 (2010)
7. Checker, Gulyani, et al. "Role of phytohormones in plant defense: signaling and cross talk." Molecular aspects of plant-pathogen interaction 6.3 (2015)
8. Cipollini, Martin et al. "Secondary metabolites of fleshy vertebrate-dispersed fruits: adaptive hypotheses and implications for seed dispersal." The american naturalist 150.3 (1997)
9. Creelman, Robert, et al. "Biosynthesis and action of jasmonates in plants." Annual review of plant biology 48.1 (1997)
10. Devoto, Alessandra, et al. "Expression profiling reveals COI1 to be a key regulator of genes involved in wound-and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions." Plant molecular biology 58.4 (2005)
11. Diezel, Celia et al. "Pithy protection: *Nicotiana attenuata*’s jasmonic acid-mediated defenses are required to resist stem-boring weevil larvae." Plant physiology 155.4 (2011)
12. Dinh, Truong et al. "The HERBIVORE ELICITOR-REGULATED1 gene enhances abscisic acid levels and defenses against herbivores in *Nicotiana attenuata* plants." Plant Physiology 162.4 (2013)

13. Doan, Ervin, et al. "Temporal effects on jasmonate induction of anti-herbivore defense in *Physalis angulata*: seasonal and ontogenetic gradients." Biochemical systematics and ecology 32.2 (2004).
14. Erb, Matthias, et al. "Belowground ABA boosts aboveground production of DIMBOA and primes induction of chlorogenic acid in maize." Plant signaling & behavior 4.7 (2009)
15. Erb, Matthias, et al. "Belowground ABA boosts aboveground production of DIMBOA and primes induction of chlorogenic acid in maize." Plant signaling & behavior 4.7 (2009): 639-641.
16. Erb, Matthias et al. "Role of phytohormones in insect-specific plant reactions." Trends in plant science 17.5 (2012)
17. Fragoso, Variluska, et al. "Root jasmonic acid synthesis and perception regulate folivore-induced shoot metabolites and increase *Nicotiana attenuata* resistance." New phytologist 202 (2014).
18. Gatehouse and John "Plant resistance towards insect herbivores: a dynamic interaction." New phytologist 156.2 (2002).
19. Glazebrook, “Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens.” Annual review of phytopathology (2005)
20. Gaur and Chaudhary “The development and regulation of Bt brinjal in India (Eggplant/Aubergine).” International service for the acquisition of agri-biotech applications (2009)
21. Groen, Simon et al. "The evolution of ethylene signaling in plant chemical ecology." Journal of chemical ecology 40.7 (2014)
22. Hautea, Desiree, et al. "Field performance of Bt eggplants (*Solanum melongena*) in the Philippines: Cry1Ac expression and control of the eggplant fruit and shoot borer (*Leucinodes orbonalis* Guenée)." Public library of science 11.6 (2016).
23. Heil, Koch, et al.,”Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius*, is an induced, indirect, defensive response elicited by jasmonic acid.” Proceedings of the national academy of sciences of the United States of America. 98.3 (2001)
24. Hirakawa, Hideki, et al. "Draft genome sequence of eggplant (Solanum melongena) the representative solanum species indigenous to the old world." DNA research 21.6 (2014)

25. Imai, Ryozo, et al. "Expression of an ABA-induced gene of tomato in transgenic tobacco during periods of water deficit." Journal of experimental botany 46.9 (1995)
26. Jayaraj and Manisegaran. “Management of fruit and shoot borer in brinjal.” .The Hindu Sci-Tech. (2010)
27. Kahl, Siemens et al. “Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. Planta” 210 (2010).
28. Kato, Masaya, et al. "Wound-induced ethylene synthesis and expression and formation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, phenylalanine ammonia-lyase, and peroxidase in wounded mesocarp tissue of *Cucurbita maxima*." Plant and cell physiology 41.4 (2000)
29. Koo and Abraham "Metabolism of the plant hormone jasmonate: a sentinel for tissue damage and master regulator of stress response." Phytochemistry reviews 17.1 (2018)
30. Kundu, Anish, et al. "*Spodoptera litura*-mediated chemical defense is differentially modulated in older and younger systemic leaves of *Solanum lycopersicum*." Planta 248.4 (2018)
31. Latif, Rahmanet al. "Efficacy of nine insecticides against shoot and fruit borer, *Leucinodes orbonalis* Guenee (Lepidoptera: Pyralidae) in eggplant." Journal of pest science 83.4 (2010)
32. Lee, Gisuk, et al. "What happens in the pith stays in the pith: tissue-localized defense responses facilitate chemical niche differentiation between two spatially separated herbivores." The plant journal 92.3 (2017).
33. Lorenzo, Oscar, et al. "ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense." The Plant Cell 15.1 (2003)
34. Mack and Andrew “Did fleshy fruit pulp evolve as a defence against seed loss rather than as a dispersal mechanism." Journal of biosciences 25.1 (2000)
35. Maffei, Massimo, et al. "Before gene expression: early events in plant–insect interaction." Trends in plant science (2007).
36. Mauch-Mani, Brigitte, et al “The role of abscisic acid in plant–pathogen interactions." Current opinion in plant biology 8.4 (2005)

37. Bodenhausen and Reymond ‘Signaling pathways controlling induced resistance to insect herbivores in Arabidopsis ‘Molecular plant-microbe interactions 20 (2007)
38. Pasteels and Jacques "Chemical defence, offence and alliance in ants–aphids–ladybirds relationships." Population ecology 49.1 (2007)
39. Pauwels, Laurens, et al. "Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells." Proceedings of the national academy of sciences 105.4 (2008)
40. Pieterse, Corné et al. "Networking by small-molecule hormones in plant immunity." Nature chemical biology 5.5 (2009)
41. Seilaniantz, Alexandre, et al. "Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism." Annual review of phytopathology 49 (2011)
42. Sheikh, Ahmad, et al. "An overview on resistance of insect pests against Bt crops." Journal of entomology and zoology studies 5 (2017)
43. Shelton, Anthony, et al. "Bt Brinjal in Bangladesh: The First Genetically Engineered Food Crop in a Developing Country." cold spring harbor perspectives in biology 11.10 (2019)
44. Shivaji, and Renuka, "Plants on constant alert: elevated levels of jasmonic acid and jasmonate-induced transcripts in caterpillar-resistant maize." Journal of chemical ecology 36.2 (2010)
45. Singh, Luthria, et al. “Polyphenols content and antioxidant capacity of eggplant pulp.” Food Chemistry. 114,(2009)
46. Skibbe, Melanie, et al. "Induced plant defenses in the natural environment: Nicotiana attenuata WRKY3 and WRKY6 coordinate responses to herbivory." The Plant Cell 20.7 (2008)
47. Spoel, Steven ,et al. "NPR1 modulates cross-talk between salicylate-and jasmonate-dependent defense pathways through a novel function in the cytosol." The plant cell 15.3 (2003)
48. Staswick and Paul "JAZing up jasmonate signaling." Trends in plant science 13.2 (2008)

49. Thaler, Jennifer et al. "Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects." Ecology 85.1 (2004)
50. Thaler, Jennifer, et al. "Jasmonate-deficient plants have reduced direct and indirect defenses against herbivores." Ecology letters 5.6 (2002)
51. Ton, Jurriaan, et al. "The multifaceted role of ABA in disease resistance." Trends in plant science 14.6 (2009)
52. Turlings, Ted, et al. "Timing of induced volatile emissions in maize seedlings." Planta 207.1 (1998)
53. Dahl, Caroline, et al. "Deciphering the role of ethylene in plant–herbivore interactions." Journal of plant growth regulation 26.2 (2007)
54. Vos, Irene, et al. "Costs and benefits of hormone-regulated plant defences." Plant pathology 62 (2013)
55. Vos, Irene, et al. "Impact of hormonal crosstalk on plant resistance and fitness under multi-attacker conditions." Frontiers in plant science 6 (2015)
56. Walling and Linda “The myriad plant responses to herbivores." Journal of plant growth regulation 19.2 (2000)
57. War, Rashid, et al. "Mechanisms of plant defense against insect herbivores." Plant signaling & behavior 7.10 (2012)
58. Wasternack, Claus, et al. "Jasmonates and octadecanoids: signals in plant stress responses and development." Progress in nucleic acid research and molecular biology. 72 (2002)
59. Xu,Chang, et al. "Plant defense genes are synergistically induced by ethylene and methyl jasmonate." The plant cell 6.8 (1994)
60. Yan, Liuhua, et al. "Role of tomato lipoxygenase D in wound-induced jasmonate biosynthesis and plant immunity to insect herbivores." Public library of science1 2 (2013)
61. Zhang and. Turner. "Wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis." Public library of science one 3.11 (2008).

.