Understanding chemical and molecular basis of eggplant- insect herbivore interaction

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

Umesh KP

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

8th April, 2020

Supervisor: Dr. Sagar Pandit

Umesh KP

Certificate

This is to certify that this dissertation entitled Understanding chemical and molecular basis of eggplant- insect herbivore interaction towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Umesh KP at Indian Institute of Science Education and Research under the supervision of Dr. Sagar Pandit, Assistant Professor, Department of Biology, during the academic year 2019-2020.

Umesh KP

Dr. Sagar Pandit

Committee: IISER Pune MS projects committee (Department of Biology)

Name of your Guide- Dr. Sagar Pandit

Name of Your TAC- Dr. Stuart Campbell

This thesis is dedicated to my mom and dad for their endless love, support and encouragement.

Declaration

I hereby declare that the matter embodied in the report entitled Understanding chemical and molecular basis of eggplant- insect herbivore interaction 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

Umesh KP

26/03/2020

Dr. Sagar Pandit

Index

Acknowledgments

First and foremost I would like to thank my thesis advisor Dr. Sagar Pandit for his constant support and guidance.

I would like to thank IISER Pune for providing me the facilities to work.

I would like to thank my external examiner Dr. Stuart Campbell for his support and encouragement.

I would like to thank Ms. Prashasti Pandey for the constant support, encouragement and help she gave me.

I would like to thank Mr. Manish Kumar, Mr. Girish Sananse and Mr. Animesh Anand for the support and help they gave me.

I would like to thank Ms. Namithasree M and all SP lab members for the support they have given me.

I would like to thank Mr. Ganesh Pawar and other staffs in IISER Pune for helping me with the facilities in IISER Pune.

Above all, I would like to thank my family and friends for the constant support and encouragement I received from them.

General Introduction

In natural ecosystem plants and insects interact with each other in the language of chemical compounds [1]. Plants being sessile in nature, have to encounter abiotic stress (temperature, light, humidity) and biotic stress (parasites, pathogens, insect herbivores and grazing animals) [3-4]. The most recurrent biotic stress is insect herbivory which appears as major threat to overall growth and development of the plant [3, 11, 12]. Insects, to counter-adapt plant's defense strategies have also developed several mechanisms to reduce or overcome the defense established by plants against insect herbivores [3]. Plants and insects have co-evolved and developed strategies to sustain this dynamic interaction. In modern world, where the food production is struggling to meet food requirement, insect herbivores creates a huge crop loss in agricultural fields, and thereby reduces the food production. Hence to produce novel technologies to reduce crop loss, it is important to understand the interaction between plants and insects.

Eggplant or brinjal (*Solanum melongena*; Solanaceae) is an agronomically important crop in South Asia and the tropical world [7]. *Spodoptera litura*, a lepidopteran generalist herbivore which devours more than 87 plants of economic importance is also a pest of eggplant [8]. It causes huge crop loss in agricultural fields as it voraciously feeds on the leaves of crop plants. In this study I am trying to study the chemical and molecular basis of eggplant-*S. litura* interaction. Eggplant produces a variety of secondary metabolites in which steroidal alkaloids and phenolics are two major class of secondary metabolites which are abundant in eggplant. The thesis is divided into two chapters, in which the first chapter deals with role of steroidal alkaloids in eggplant-*S. litura* interaction, whereas the second chapter deals with the role of phenolics in eggplant-*S. litura* interaction.

Chapter 1

Plant derived chlorogenic acid: an alternative to synthetic pesticides against generalist herbivore *Spodoptera litura*

Abstract

Phenolics are a class of plant metabolites having role in both plant development and defence response, which are present in a wide range of plant families. Chlorogenic acid (CGA) is a secondary metabolite which is known for its deterrent nature against insect herbivores. Here we report the role of CGA in eggplant- *S. litura* interaction. In a randomized field of different eggplant varieties, a differential occurrence of *S. litura* larvae was observed on different eggplant varieties, and the *S. litura* occurrence negatively correlated with the abundance of CGA. Feeding *S. litura* on artificial diets spiked with CGA showed high neonate mortality, reduced larval mass gain, increased larval lifecycle etc., which shows the negative effect of CGA on larval performance and fitness. Larvae was shown to be metabolising CGA into caffeic acid and quinic acid, by an ester hydrolysis reaction. Two genes COE4 and COE15 were shown to be induced upon the ingestion of CGA in the larval midgut. Exogenous application of CGA and ds RNA of COE4 and COE15 were carried out, which opens a new horizon in the field of agricultural science to reduce crop loss.

Introduction

In natural ecosystem plants and insects interact with each other in the language of chemical compounds. The most recurrent biotic stress: insect herbivory, is a major threat to the overall growth and development of the plant. Hence, it is not astonishing that the plants have developed an elaborate defense mechanism like physical and chemical barriers to deter the herbivory. Plant secondary metabolite: phytotoxins are powerful chemical weapons which play a crucial role in plant-herbivore interactions [35]. These toxins target the insect's metabolism and alter their physical and physiological conditions.

Solanum melongena (Solanaceae) also known as eggplant, is a widely consumed vegetable crop by people of countries like Central, South and Southeast Asia, some

parts of Africa and Central America [36]. The crop is native to India and is grown in all seasons [37]. It is also known as poor man's crop because of its high productivity and low cost of cultivation. Tropical armyworm or *Spodoptera litura* (Lepidoptera: Noctuidae), is a generalist herbivore that cause significant crop loss mainly on plants belonging to *Solanaceae* family [26]. It is a pest on many agronomically important crops including tomato and eggplant. There is a dearth of information about ways to alleviate *S. litura*'s feeding on the crops. Being one of the major destructors, *S. litura* is exposed to various kinds of insecticides which have led to the accumulation of abominable chemicals and eventually the development of resistance. *S.litura* is the one of the first agronomically important insect herbivore to gain resistance against insecticides [27]. So it is important to study naturally existing plant metabolites, which can be used as a potential alternative to synthetic pesticides.

Phenolics are plant compounds, having important role in plant development [30]. Phenolics also plays an important role in the defense system of the plant against the insect herbivores, at the same time it acts as attractant towards symbiotic organisms of the plant [29]. In most of the time phenolics are stored in the plant as a defensive metabolite, and it serves as an alternative to chemical control of pathogens in crop fields [30]. Oxidized plant phenolics conjugates with dietary proteins and hinder the absorption or digestion of the proteins [25]. The covalent interaction between oxidized plant phenolics and dietary proteins are deleterious to insect herbivores [24]. The oxidized form of chlorogenic acid (CGA) significantly reduces the protein quality in *Spodoptera exigua*[3]. Plant phenolics are reported to have both beneficial effects as well as harmful effects on humans [28].

In a randomized field of different eggplant varieties, I found a differential occurrence of *S. litura* larvae on different eggplant varieties. The differential occurrence of *S. litura* negatively correlated with the abundance of CGA in the eggplant leaf. In this study I tried to decipher the role of CGA in eggplant-*S.litura* interaction and its possible applications to reduce crop loss. I investigated the effects of CGA on the performance of *S. litura*, the metabolism of CGA by *S. litura*, and the genes involved in the metabolism of CGA. Our study shows the negative effects of CGA on larval mass gain and neonate mortality. *S. litura* metabolizes CGA into caffeic acid (CFA) and quinic

acid (QNA) in the larval midgut. Two carboxylesterase genes were identified to be responsible for the ester-hydrolysis of CGA into CFA and QNA.

Freshly hatched neonates were reared on artificial diets spiked with physiological concentration of CGA, CFA and QNA till pupation stage. The pupation rate were calculated on larvae fed on each metabolite diet. The emergence of moths from the pupae were also calculated on larvae fed on each diets.

Results

Differential occurrence of *S. litura* on different eggplant varieties

In a randomized field of different eggplant varieties (Figure 1-A), a differential occurrence of 3rd and 4th instar larvae of *S. litura* was observed [work done by Dr. Sagar Pandit]. To validate this, *S. litura* larvae were reared on leaves of different eggplant varieties. Feeding rate of 3rd and 4th instar larvae on leaves of different eggplant varieties corroborates with the field observation of differential occurrence of 3rd and 4th instar larvae on different eggplant varieties. The larval mass gain of *S. litura* was also calculated on different eggplant varieties, which showed a similar trend as that of the differential occurrence in the randomized field (Figure 1-C, D). In addition to this, 100% neonate mortality was observed and high larval mortality of early instar larvae (1st instar) was observed when they were reared on different eggplant varieties in laboratory condition. Early instar *S. litura* larvae were seen on *Ricinus communis* (castor plants) around the eggplant field, at the same time the late instar larvae were not seen on castor plants, instead on different eggplant varieties.

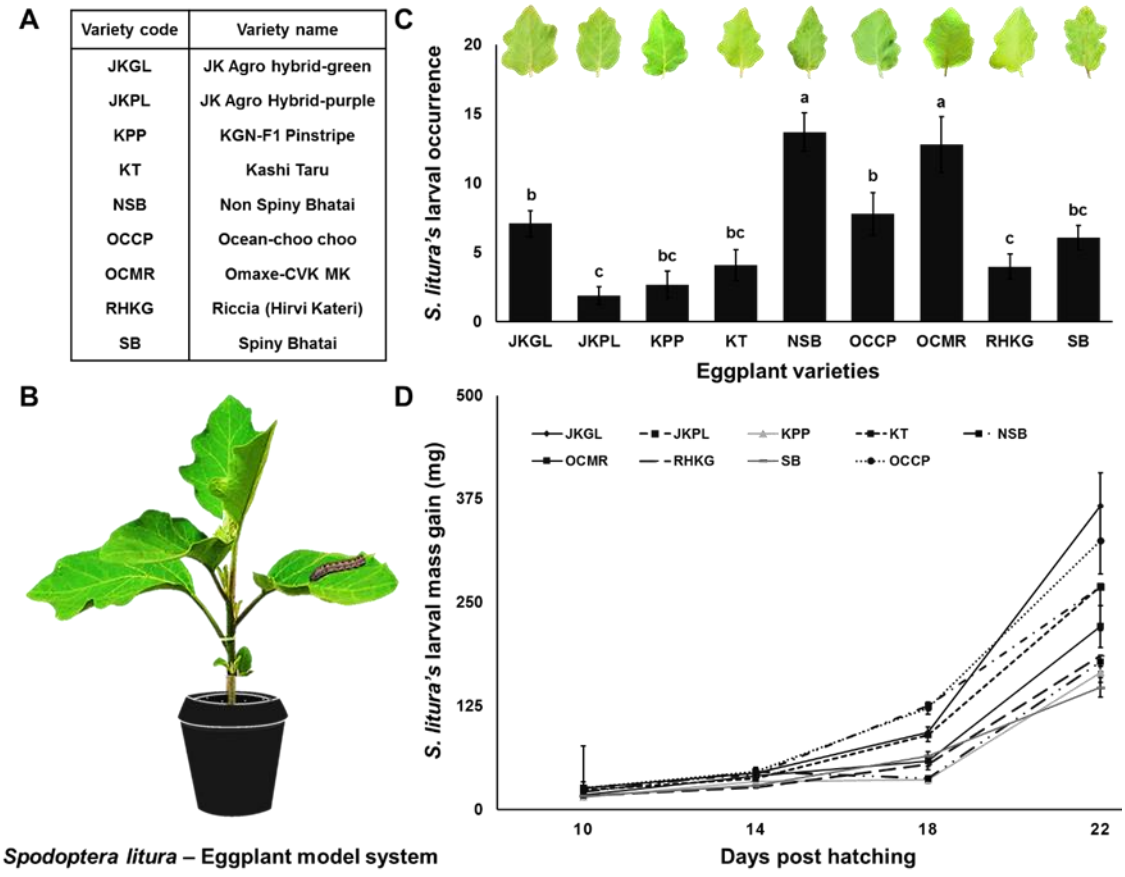


Figure 1 | *S. litura* larvae shows differential occurrence on different eggplant varieties. (A) Codes of different eggplant varieties with their names used for study. **(B)** *Spodoptera litura* – eggplant model system showing *S. litura* feeding on eggplant leaves. **(C)** *S. litura's* larval occurrence on different eggplant varieties in a randomized field in IISER, Pune shows maximum larval occurrence on NSB and OCMR variety ($f_{8,81}$ = 11.949, significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 10 for each variety). **(D)** *S. litura's* larval mass gain on different eggplant varieties measured on 10 dph (days post hatching), 14 dph,18 dph and 22 dph; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 10 for each variety. ANOVA, analysis of variance.

Differential occurrence of *S. litura* larvae negatively correlates with abundance of CGA in eggplant leaves

To determine the putative cause of differential occurrence of *S. litura*, U(H)PLC-ESI-QTOF based metabolite profiling of leaves of 9 eggplant varieties from randomized field was done. It showed an abundance of CGA which varied between eggplant varieties (Figure 2-A). Average physiological concentration of CGA in leaves of different eggplant varieties was calculated as 650 µg/g of fresh leaf tissue. Abundance

of CGA showed a negative correlation with *S. litura* larval occurrence ($p \leq 0.05$, R value = -0.8459) (Figure 2-B).

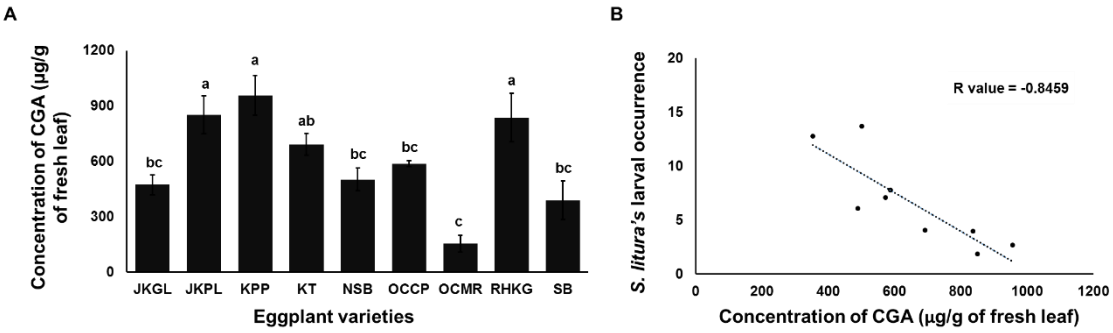
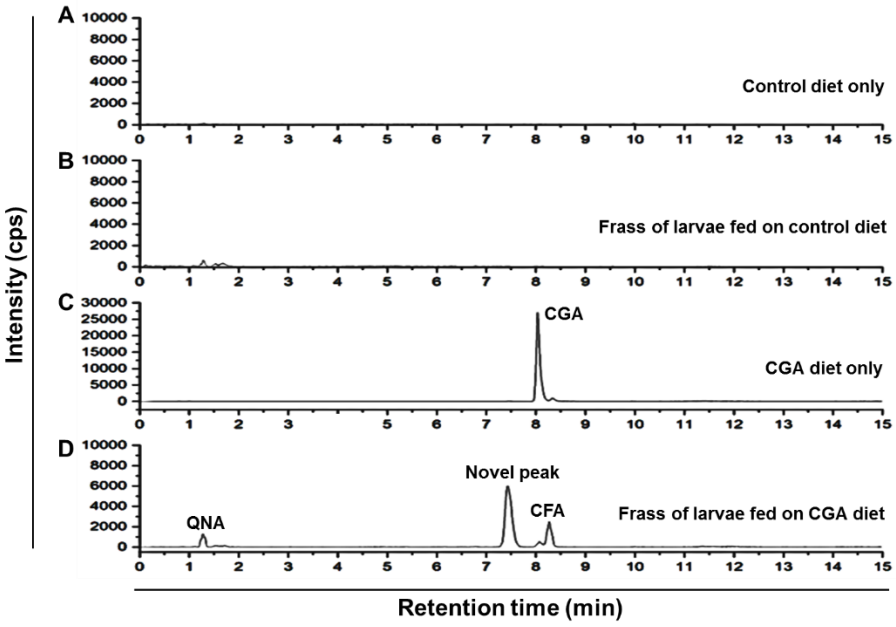


Figure 2 | UPLC-ESI-QTOF based metabolite profiling of leaves of different eggplant varieties shows abundance of CGA in eggplant leaves and their negative correlation with *S. litura's* larval occurrence. (A) Concentration of CGA in microgram per gram of fresh leaves of different eggplant varieties shows KGN-F1 pinstripe has highest concentration among all the varieties; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 6 for each variety. **(B)** Correlation of concentration of CGA microgram per gram of fresh leaves of different eggplant varieties with *S. litura's* larval occurrence shows a negative correlation; significance value $p \leq 0.05$, f = 3.214. ANOVA, analysis of variance.

CGA is metabolized by *S. litura* into CFA and QNA

Despite CGA showing negative effect on *S litura,* it feeds on eggplant leaves. To understand the mechanism by which *S. litura* is able to do so, U(H)PLC-ESI-QTOF based analysis of frass of *S. litura* larvae fed on artificial diets spiked with physiological concentrations of CGA was done. It was observed that CFA and QNA was present in the frass of *S. litura* (Figure 3-D). No CGA or metabolized products of CGA were detected in the frass of *S. litura* fed on control diets. These results indicate that CGA is metabolized into CFA and QNA by the larvae.



E

Metabolite	RT (min)	[M-H]⁻ (Da)
CGA	8.03	353.088
CFA	8.35	179.034
QNA	1.45	191.095

Figure 3 | UPLC-ESI-QTOF based metabolite analysis of artificial diet spiked with different metabolites and frass of *S. litura* larvae. Extracted ion chromatogram (XIC) of CGA, CFA and QNA from total ion chromatogram (TIC) of **(A)** artificial diet **(B)** frass of *S. litura* larvae fed on artificial diet spiked with CGA **(C)** artificial diet spiked with CGA **(D)** frass of *S. litura* larvae fed on artificial diet spiked with CGA; analysis of CGA-spiked diet showed presence of CGA but not CFA and QNA; however, when fed on CGA-spiked diet, larval frass showed the presence of CFA, QNA and a novel peak with mass of CGA. **(E)** List of metabolites with their retention time (RT) and their mass in $[M-H]^-$ (Da).

Neonate mortality is high on diets containing CGA

To validate the toxic effect of CGA on *S. litura* larvae, neonates (freshly hatched larvae) were reared on artificial diet spiked with average physiological concentration in 9 different eggplant verities of CGA (650 µg/g of artificial diet). A high neonate mortality was observed within 24 hrs of feeding on diets containing CGA (Figure 4-A). Similarly, neonate mortality was also observed on artificial diets spiked with varying concentrations of CGA (100 µg/g, 200 µg/g, 400 µg/g, 650 µg/g, 800 µg/g and 1 mg/g of artificial diet). Neonate mortality increased with increase in concentration of CGA (Figure 4-B).

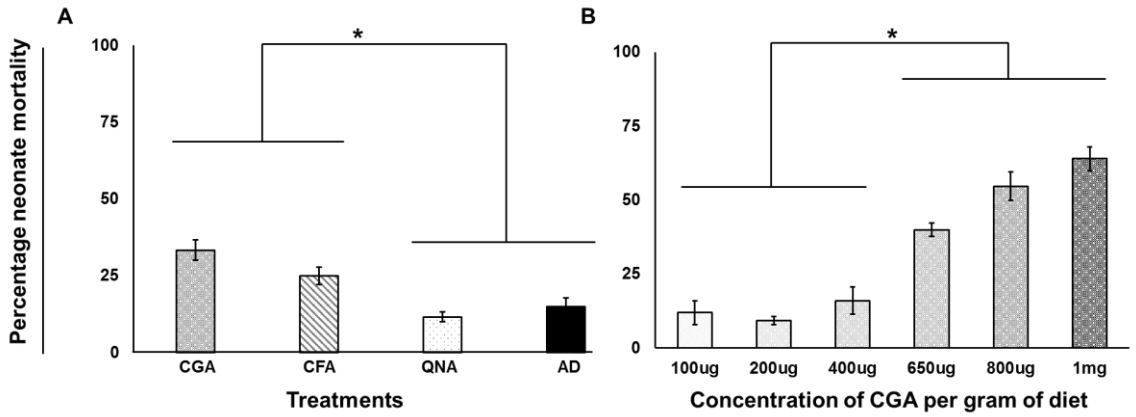


Figure 4 | Percentage neonate mortality of *S. litura* larvae within 24 hrs after feeding artificial diet spiked with different metabolites. (A) Percentage neonate mortality was calculated for neonates reared on artificial diets spiked with physiological concentrations of CGA (650 µg/g of AD), CFA (10 µg/g of AD) and QNA (650 µg/g of AD) respectively for 24 hrs; high neonate mortality was observed for neonate reared on artificial diets spiked with CGA and CFA; significant differences ($p \leq 0.05$) ($f_{3,23} = 43.21$) between means (± s.e.) determined by one-way ANOVA; n = 10 for each variety. **(B)** Percentage neonate mortality was calculated for neonates reared on artificial diets spiked with varied concentrations of CGA for 24 hrs; increase in neonate mortality was seen with increase in concentration of CGA ($f_{5,35} = 79.223$) spiked in artificial diet; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 6 for each variety. ANOVA, analysis of variance.

Larval mass gain is reduced upon ingestion of CGA

To study the negative effect of CGA on *S. litura* larvae's mass gain, larvae were reared on diets spiked with CGA, CFA and QNA from neonatal stage. Larval mass gain measured at an interval of 4 days showed a reduction upon the ingestion of artificial diet spiked with CGA. This was also observed upon the ingestion of diet spiked with the physiological concentration of CFA, but at a lower extent (Figure 4-A).Larval mass gain was also calculated for larvae fed on diets spiked with different concentration of CGA (100 µg/g of diet, 200 µg/g of diet, 400 µg/g of diet, 650 µg/g of diet, 800 µg/g of diet and 1 mg/g of diet) at an interval of 4 days. The larval mass gain decreased with increase in the concentration of CGA (Figure 4-B). This infers that *S. litura* larvae grow less on diets containing CGA.

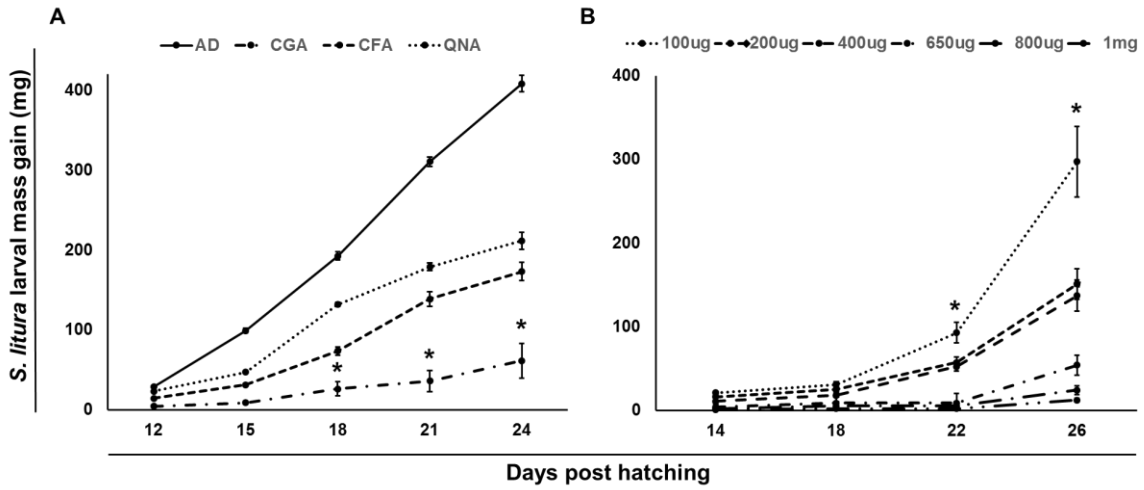


Figure 5 | Reduced larval mass gain *of S. litura* after feeding on artificial diets spiked with metabolites. (A) Larval mass was measured on 12 dph (days post hatching), 15 dph, 18 dph, 21 dph and 24 dph after feeding *S. litura* larvae of 1 dph on artificial diet (AD) spiked with physiological concentration of CGA (650 µg/g of AD), CFA(10 µg/g of AD) and QNA (650 µg/g of AD) with water spiked diets as control; CGA showed a significant reduced larval mass gain ($f_{3,119} = 86.21$, $f_{3,119} = 90.21$) as compared to other treatments; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 20 for each treatment. **(B)** Larval mass was measured on 14 dph (days post hatching), 18 dph, 22 dph and 26 dph after feeding *S. litura* larvae of 1 dph on artificial diet (AD) spiked varying concentration of CGA (100 µg, 200 µg, 400 µg, 650 µg, 800 µg and 1 mg per g of AD), CFA and QNA with water spiked diets as control; larval mass gain was seen reducing with increase in concentration of CGA; significant differences ($p \leq 0.05$) ($f_{5,119} = 10.321$, $f_{5,119} = 19.291$) between means (± s.e.) determined by one-way ANOVA; n = 20 for each treatment. ANOVA, analysis of variance.

No significant difference in pupation and moth emergence rate on *S. litura* larvae fed on different diets

To study larval performance, it is essential to study larvae's ability to pupate and emerge as a moth. Percentage of pupation was observed on *S. litura* larvae reared on different diets. Larvae were reared on artificial diets spiked with physiological concentration of CGA, CFA and QNA respectively from neonate stage till pupation. There was no significant difference observed between the percentage pupation of *S. litura* larvae reared on different diets (Figure 6-A). Following this, moths were allowed to emerge from the pupae which were formed after feeding on diets spiked with CGA, CFA and QNA. There was no significant difference between percentage moth emergence between larvae fed on different metabolite diets (Figure 6-B).

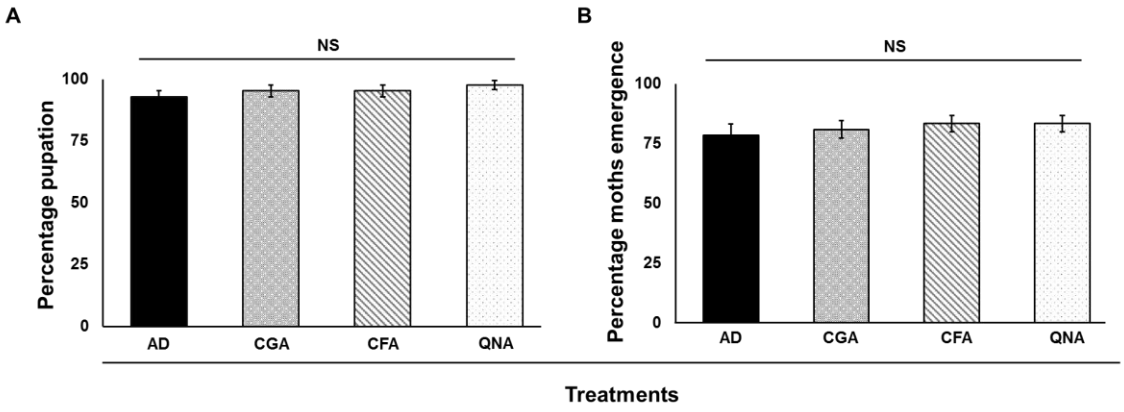


Figure 6 | Pupation and moth emergence of *S. litura* larvae fed on artificial diets spiked with metabolites. (A) Percentage pupation was calculated on *S. litura* larvae of 1 dph reared on artificial diet (AD) spiked with physiological concentration of CGA (650 µg/g of AD), CFA (10 µg/g of AD) and QNA (650 µg/g of AD) with water spiked diets as control; no significant difference was observed between the treatments; n=20 for each treatment. **(B)** Percentage moth emergence was calculated on *S. litura* larvae of 1 dph reared on artificial diet (AD) spiked with physiological concentration of CGA (650 µg/g of AD), CFA (10 µg/g of AD) and QNA (650 µg/g of AD) with water spiked diets as control; no significant difference was observed between the treatments; n=20 for each treatment. NS, not significant.

Excretion efficiency determination assay

To understand the difference between the concentrations of metabolite ingested with the concentration of metabolite excreted, excretion efficiency determination assay was performed with *S. litura* larvae. In this assay, the larvae were fed on a measured quantity of artificial diet spiked with physiological concentration of CGA, CFA and QNA respectively, for 24 hrs After 24 hrs the frass was collected, and U(H)PLC-ESI-QTOF based analysis of metabolites in the frass and diet was carried out. The quantity of CFA and QNA ingested by the larvae were equal to the quantity of CFA and QNA excreted by the larvae (Figure 7-C, D). In case of CGA spiked diet, CGA was metabolized into CFA and QNA. Quantity of CGA in larvae fed on CGA spiked diet decreased and on the other hand, quantity of CFA and QNA increased in frass, which was not present in the diet (Figure 7-B).

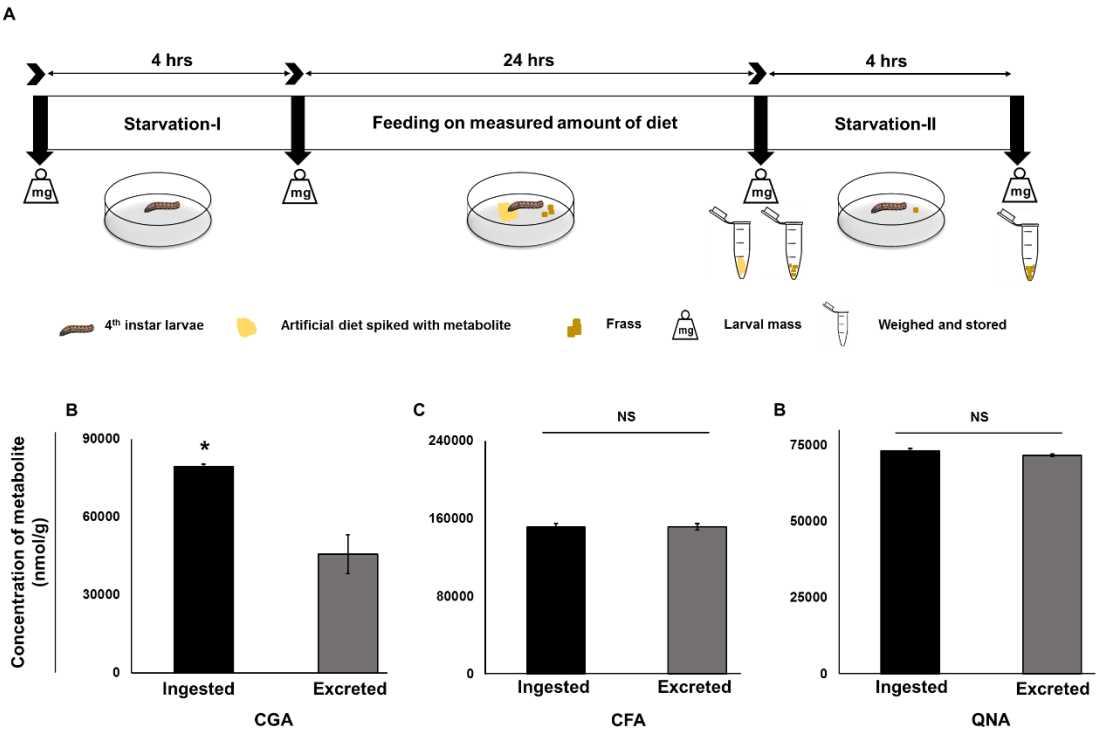


Figure 7 | Excretion efficiency determination assay shows CGA is excreted without any metabolism. (A) Schematic showing timeline of excretion efficiency determination assay used to quantify ingested and excreted CGA, CFA and QNA; 4th instar larvae were used for all the assays; artificial diets (AD) were spiked with physiological concentration of CGA (650 µg/g of AD), CFA(10 µg/g of AD) and QNA (650 µg/g of AD); diet and pooled frass (after and before starvation-II) were measured and extracted using 70% methanol and analyzed by U(H)PLC-ESI-QTOF. Concentration (nmol/g) of ingested and excreted **(B)** CGA **(C)** CFA and **(D)** QNA per gram of AD spiked with respective metabolites by *S. litura* showed a significant difference in AD spiked with CGA ($f_{1,11}$ = 100.36) and no significant difference in AD spiked with CFA and QNA; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 6 for each treatment. ANOVA, analysis of variance; NS, not significant.

Carboxlyesterase genes are expressed in the midgut of *S. litura*

From the metabolite profiling of frass of *S. litura* fed on diet spiked with CGA, its metabolism into CFA and QNA is observed. Metabolism of CGA into CFA and QNA is an ester hydrolysis reaction. Insect *carboxlyesterases* are involved in a broad range of functions, which includes both metabolism of essential molecules and also in the detoxification of harmful exogenous compounds [21]. Hence I shortlisted the *carboxlyesterase* genes expressed in the midgut of *S. litura* from NCBI (Table 1).

Sequence id.	Sequence name	Gene code used for experiment
DQ445461.2	Spodoptera litura clone est2 carboxylesterase mRNA, complete cds	COE1
EU783914.1	Spodoptera litura carboxylesterase mRNA, complete cds	COE2
KU375439.1	Spodoptera litura putative carboxylesterase CXE23 mRNA, complete cds	COE4
KU375442.1	Spodoptera litura putative carboxylesterase CXE23 mRNA, complete cds	COE5
KU375441.1	Spodoptera litura putative carboxylesterase CXE28 mRNA, complete cds	COE6
KU375438.1	Spodoptera litura putative carboxylesterase CXE22 mRNA, complete cds	COE7
KU375425.1	Spodoptera litura putative carboxylesterase CXE1 mRNA, complete	COE14
KY304476.1	Spodoptera litura carboxyl/choline esterase 016a (CCE016a) mRNA	COE15
KY130419.1	Spodoptera litura acetylcholinesterase 2 mRNA, complete cds	COE16

Table 1

Some COE genes are upregulated in expression upon ingestion of CGA spiked diets

In order to investigate the midgut expression pattern of the COE genes shortlisted in Table 1, qRT PCR was performed on the midgut transcriptome of larvae fed on CGA, CFA, QNA and control diets. qRT PCR was performed on COE genes using ACTB (beta-actin) and UCCR (ubiquinol-cytochrome c reductase) as internal controls (IC). The expression level of ACTB and UCCR changed the least with starvation and insecticidal activity in *S. litura.* At the same time, COE4 and COE15 showed a significant induction in the midgut of the larvae upon feeding them with diets spiked with CGA (Figure 8).

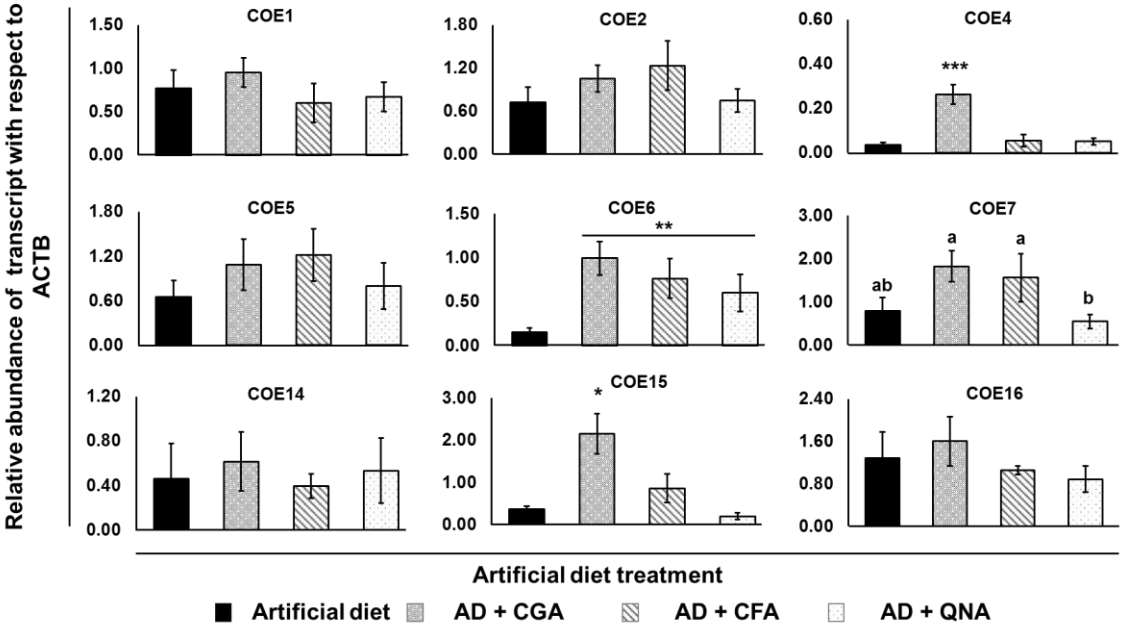


Figure 8 | Upregulation of *COE* genes in *S. litura* larval midgut. Larvae were fed on artificial diets spiked with CGA, CFA and QNA respectively for 24 hrs; midgut was dissected out and qRT-PCR was performed on larval midgut transcriptome for genes COE1, COE2, COE4, COE5, COE6, COE7, COE14, COE15 and COE16; significant increase in expression level of COE4 ($f_{3,59} = 8.0922$) and COE15 ($f_{3,59} = 7.04995$) was observed upon ingestion of artificial diet spiked with CGA; ACTB gene of *S. litura* was used as internal control for qRT-PCR; similar trend of expression was observed when UCCR was used as internal control for; significant differences (* for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$) between means (± s.e.) determined by one-way ANOVA. ANOVA, analysis of variance.

Exogenous application of CGA on eggplant leaves

To study the role of COE gene(s) in metabolizing CGA into CFA and QNA and helping *S.litura* in counter-adaptation against plant's defensive mechanism, plant mediated RNA interference (PMRi) method was taken into consideration. To induce the COE genes, high concentration of CGA needs to be applied on eggplant leaf. Exogenous application of CGA was done to confirm whether high concentration of CGA causes any effect on the leaves. No deleterious morphological phenotype was observed on eggplant leaves on pasting CGA.

DISCUSSION

Preliminary observations in a randomized field of different eggplant varieties revealed a differential occurrence of *S. litura* was observed. To decipher the chemical basis of this differential occurrence, a metabolite profiling of all the eggplant varieties in the field was carried out. Results of this showed a high CGA content in all eggplant

varieties, and a negative correlation between the CGA content and larval occurrence. CGA is a well-known defense molecule in plants which has been reported to work against a broad range of insect herbivores [33]. Therefore, the study of CGA is important, as it can be used as an alternative to synthetic pesticides to control populations of the generalist crop pest *S. litura* which is notorious for causing huge crop loss owing to its voracious feeding [26].

CGA is a defensive metabolite found across a wide range of plant groups, including *S. litura*'s most preferred host [34]. Therefore insects have been exposed to CGA for decades during their evolution, and are possibly endowed with counter-adaptation strategies that enable them to overcome its toxic effect. U(H)PLC-ESI-QTOF based analysis of larval frass of *S. litura* fed on artificial diets spiked with CGA showed that larvae metabolize CGA into CFA and QNA by an ester hydrolysis reaction.

CGA interacts with dietary proteins of larvae and interferes in their digestion and absorption in the larval gut [23], possibly due to which I observed a negative effect of this metabolite on larval mass gain. On diets spiked with CGA *S. litura* larvae showed the least larval mass gain. Over the period of feeding, larval mass gain reduced as the concentration of CGA increased in the diet. I observed that a low larval mass gain is associated with a lengthier larval life cycle. Larvae grown on CGA diets for generations were seen to be lagging behind the larvae grown on artificial diet for the same time period. The employment of CGA can therefore be used as a good strategy in agricultural fields to combat the voracious feeding of *S. litura* larvae.

Spiking CGA in the artificial diet also significantly increased neonate mortality in *S. litura*, showing increased mortality with increase in concentration of spiked CGA. At the same time, CGA was shown to have no effect on pupation and moth emergence of the larvae, when larvae are fed on diets spiked with CGA since neonate stage.

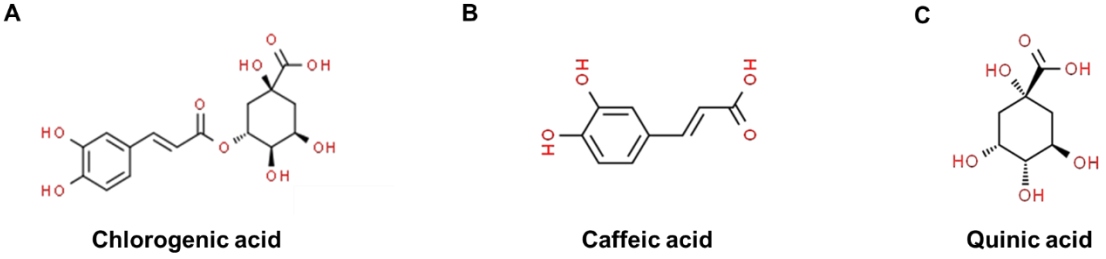


Figure 9 | Structures of the secondary metabolites in eggplant. (A) Chlorogenic acid, **(B)** Caffeic acid, **(C)** Quinic acid. (chemspider.com)

In plants, CGA is synthesised by combining caffyl CoA with quinic acid through an ester bond [25]. Our results suggest that ingested CGA is metabolized in larval midgut to form CFA and QNA through an ester hydrolysis reaction. U(H)PLC-ESI-QTOF based analysis showed CGA to be stable in room temperature in artificial diet and also in the alkaline pH of the larval gut. This indicated that the hydrolysis of CGA in the gut environment is enzymatic. Since, metabolism of CGA into CFA and QNA is an ester hydrolysis reaction, *carboxylesterase* gene could be putative candidate for this reaction. *Carboxylesterase*s are known for their role in the detoxification of harmful exogenous molecules, apart from their role in plant development [1]. 9 COE genes known to be expressed in the midgut of *S. litura* were shortlisted. Of these, COE4 and COE15 showed a significant induction in the midgut upon the ingestion of diets spiked with CGA. For functional characterization of COE4 and COE15 genes, virus induced gene silencing (VIGS) was performed on eggplant.

The excretion efficiency determination assay showed that CGA is metabolized into CFA and QNA by *S. litura*. From the structure of CGA, CFA and QNA (Figure 7), it can be observed that 1 molecule of CGA dissociates to form 1 molecule of CFA and 1 molecule of QNA. The quantity of CGA significantly decreased in the frass of the larvae fed on diets spiked with CGA, on the other hand the concentration of CFA and QNA increased in the frass, despite their absence in the ingested diet. Excretion efficiency determination assay also showed that the quantity of CFA and QNA ingested was equal to the quantity of CFA and QNA excreted by the larvae, which shows that CFA and QNA are excreted by the larvae without any metabolism (Figure 7-C, D).

Simultaneously along with VIGS, exogenous application of dsRNA of COE4 and COE15 along with CGA on eggplant leaf was carried out. Leaves of many plants including *Nicotiana attenuatum* got corroded upon the pasting of high concentration of CGA, but eggplant leaves did not show any such phenotype, hence this novel idea of pasting of defensive metabolite along with dsRNA of its metabolising gene in the insect was performed. It has already been reported about the exogenous application of dsRNA to silence genes in lepidopterans [32]. But using dsRNA to silence the detoxification gene along with a higher concentration of the defensive metabolite is a

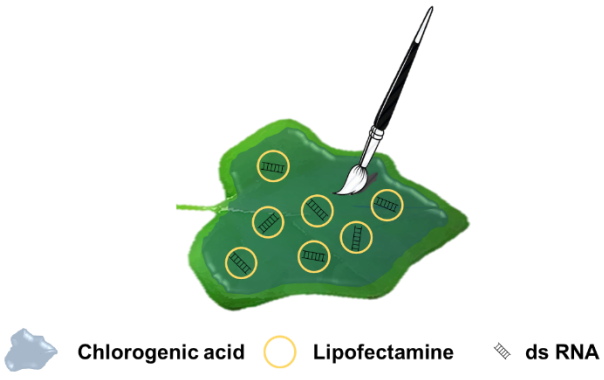
novel strategy, which can be used as an efficient strategy to reduce the crop loss in agricultural field. Because of the anti-oxidant nature of CGA [33], it is beneficial for human health, and unlike VIGS, dsRNA pasting focuses on the exogenous application of nucleic acids, which can open a new horizon in the field of agricultural sciences.

FUTURE WORK

Virus induced gene silencing (VIGS) of COE4 and COE15 genes have been performed so far, but the larval performance assays with those VIGS plants are yet to be carried out. Effect of silencing these genes in *S. litura,* should be studied in the context of its effect on the performance of the larvae in terms of larval mass gain, effect on pupation, moth emergence, larval mortality, neonate mortality etc.

To study the significance of COE gene(s) in metabolizing CGA into CFA and QNA and helping *S.litura* in counter-adaptation against plant's defensive mechanism, plant mediated RNA interference (PMRi) method will be performed. dsRNA (double stranded RNA) of COE4 and COE15 genes will interfere *carboxylesterase* activity when ingested and prevent the larvae from detoxifying it. Exogenous application of dsRNA to silence a metabolite-detoxification gene, with the simultaneous application of a higher concentration of the defensive metabolite might potentially be an effective technique to reduce the crop loss in agricultural fields.

A



B

Treatment number	Treatments
1	WT leaf
2	WT+ Lipofectamine
3	WT+ CGA
4	WT+ Lipofectamine+ CGA
5	WT+ COE4/15
6	WT+ Lipofectamine+ COE4/15
7	WT+ COE4/15+ CGA
8	WT+ Lipofectamine+ COE4/15+ CGA

Table 2

Figure 10| Complementation of COE4 and COE15 dsRNA with CGA. (A) Pasting of dsRNA coated with lipofectamine and CGA on wild type (WT) eggplant leaf as various combinations as mentioned in **(B)** table 2.

Unlike VIGS, dsRNA pasting focuses on the exogenous application of nucleic acids and without really interfering with the physiology of plants, can open a new horizon in the field of agricultural sciences.

The significance of COE genes is also need to be understood in the context of the next trophic level, which is the predators of *S. litura.*

Materials and methods

Plants

Seeds of 9 different varieties (3 pure-line breed and 6 hybrid) of *Solanum melongena* (eggplant) were germinated in red soil, vermiculite, perlite and cocopeat soil mixture in 3:1:1:1 ratio in controlled conditions (Relative humidity: 70-80%; Photoperiod: 16 hr light-8 hr dark; Temperature: 22 ± 2°C). Plantlets were transferred to pots after 15 days and were grown under natural conditions for 10 days followed by plantation in a randomized way in an open field at IISER, Pune. Plants were provided with macroelements and microelements (HiMedia) regularly for their better growth and development.

Insects

Spodoptera litura larvae were collected from various crop fields in and around Pune, India. Larvae were reared on the leaves of *Ricinus communis* for maintaining the larval culture, and on artificial diet (minimal nutrition required for insect survival) for experiments[34], under controlled conditions (Relative humidity: 65%; Photoperiod: 16 hr light-8 hr dark; Temperature: 25 ± 2°C). Moths were provided with 10% sucrose solution and were reared in dark conditions. Eggs and early instar larvae from culture were transferred to artificial diet spiked with metabolites for all the experiments in this study. Fresh diet was provided to the larvae every alternate day until pupation.

Larval mass gain assay

For larval performance assay, freshly hatched neonates were fed on artificial diets spiked with physiological concentration of CGA (650ug/g of diet), CFA (10ug/g of diet) and QNA (650ug/g of diet) respectively. Freshly spiked diets were provided every alternate day to rear larvae till pupation. Larval mass was recorded for four times at interval of every 4 days. Frass and haemolymph were collected for UPLC/ESI/QTOF based analysis.

Larval performance assay was also performed on larvae fed on artificial diets spiked with different concentrations of CGA i.e., 100 µg/g of diet, 200 µg/g of diet, 400 µg/g of diet, 800 µg /g of diet and 1mg/g of diet. Freshly spiked diets were provided every alternate day to rear larvae till pupation. Larval mass was recorded for four times at interval of every 4 days.

Impact of metabolites on pupation and moth emergence

Freshly hatched neonates were reared on artificial diets spiked with physiological concentration of CGA, CFA and QNA till pupation stage. The pupation rate were calculated on larvae fed on each metabolite diet. The emergence of moths from the pupae were also calculated on larvae fed on each diets.

Extraction of metabolites and UPLC/ESI/QTOF (LCMS) based analysis

For metabolite analysis, 200 mg of leaf tissues were fine crushed and extracted in 1 mL of 70% methanol. Cell debris was removed by centrifugation (12000 x g, 10 min). Supernatant was collected and incubated at -80˚C overnight for protein precipitation. Samples were centrifuged again(12000 x g, 20 min, 4˚C). Frass and hemolymph samples were also extracted in a similar manner, using 70% methanol in the ratio of 100 mg of frass in 1 ml of extraction buffer and 10ul of haemolymph in 100ul of extraction buffer. Metabolite extracts from all tissues were analysed on AbSCIEX-X500R-QTOF (UPLC/ESI-QTOF-MS). Samples were separated on a Phenomenex Gemini® C18 column (50x4.6 mm, 5µm, 118Å), using Millipore water with 0.1% formic acid and methanol with 0.1% formic acid as mobile phase. A 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 0.5 ml/min was used. Compounds were analysed in positive ion mode (capillary voltage, 4,500 V; capillary exit, 130 V; dry gas temperature, 200 °C).

Neonate mortality assay

Neonates (freshly hatched larvae) were fed artificial diet spiked with physiological concentration of CGA, CFA and QNA. Neonate mortality was observed after 24 hours of feeding on these diets.

Similarly neonate mortality was also observed on neonate fed with artificial diets spiked with different concentration of CGA. Neonates were fed on artificial diets spiked

with the following concentrations of CGA, 100 µg/g of diet, 200ug/g of diet, 400 µg/g of diet, 800 µg/g of diet and 1mg/g of diet.

Stability of CGA in artificial diet and alkaline pH

Artificial diet spiked with physiological concentration of CGA was kept at 25°C for 24 hours. Similarly diet spiked with CGA was also kept in alkaline medium for 24 hrs at 25°C. Diets were processed for U(H)PLC/ESI-QTOF-MS based analysis

Excretion efficiency determination assay (Waldbauer assay)

Ingestion of metabolite spiked diet and excretion by *S. litura* larvae were budgeted using an excretion efficiency determination assay, following standardization [26]. Freshly molted larvae into 4th instar were first starved for 4 hrs, and then fed on measured amount of artificial diets spiked with physiological concentration of CGA, CFA and QNA for 24 hrs. After 24 hrs of feeding, larvae were again starved for 4 hours. Mass of larvae and frass was measured before and after every starvation. Frass of larvae before starvation and after starvation was pooled, and then were analyzed by UPLC-ESI-QTOF.

Screening for carboxylesterase genes

CDS sequences of carboxylesterase genes expressed in the midgut of *S. litura*, was screened from NCBI. Four internal control genes were also screened whose expression changes the least upon starvation and insecticide presence. qPCR primers were designed for all the screened carboxylesterase gene with annealing temperature as 60°C using Primer3 software version 4.0.

RNA isolation from insect midgut and cDNA synthesis

S. litura larvae reared on castor leaves till 4th instar were transferred to artificial diets spiked with physiological concentration of CGA, CFA and QNA for 24 hours. The larval midgut were dissected out at 4°C and washed in TE buffer before storing at -20°C. Total midgut RNA was isolated using manufacturer's protocol (RNA XPress reagent, Himedia). cDNA synthesis was performed with the isolated RNA, using manufacturer's protocol (PrimeScript 1st strand cDNA Synthesis Kit, TAKARA).

q-RT PCR was performed on the larval midgut transcriptome with ACTB and UCCR as the internal controls.

In vitro carboxylesterase activity assay

Larvae were fed on artificial diets spiked with physiological concentration of CGA, CFA, QNA respectively. Larval midguts were dissected out at 4°C and homogenized. Esterase activity was measured using α-naphthol or β-naphthol standard curves as described by Van Asperen et.al [27]. Protein contents in different larval midgut were determined by Bradford method, using bovine serum albumin as the standard.

Cloning of COE4 and COE15 in *Agrobacterium tumifaciens*

A 354bp sequence from COE4 and 377bp sequence from COE15 genes were sequence amplified using gene specific primers from the larval midgut transcriptome. The PCR products were further amplified with gene specific primers flanking with restriction enzyme sequence of Kpn1 and BamH1 in forward and reverse primers respectively to clone them in pTRV2 binary vector. COE4 and COE15 were cloned independently in respective pTRV2 binary vectors and were used to transform *E. coli.* The recombinant plasmids were further isolated from *E. coli* and were used to transform *Agrobacterium tumifaciens.*

Virus induced gene silencing

pTRV2 and pTRV1 VIGS vectors were used in this experiment. COE4, COE15 fragments from the larvae and the HQT gene fragments from the eggplant were cloned in respective pTRV2 VIGS vectors. Eggplant were grown in sterile conditions at 22°C in plant growth chamber for VIGS infiltration. Plants in 3 leaved stage were used for VIGS infiltration. The experiment consisted of seven treatments with 25 plants for each treatment.

Treatment no.	VIGS lines	Genes used for VIGS infiltration
1	WT	Nil
2	EV	Empty vector of pTRV2
3	COE4i	COE4
4	COE15i	COE15
5	COE4HQTi	COE4 and HQT
6	COE15HQTi	COE15 and HQT
7	HQTi	HQT
8	PDSi	PDS

Table 3

A primary culture of *A. tumifaciens* pTRV1, pTRV2 and pTRV2 with respective constructs were grown at 27°C for 16hrs in LB medium containing three antibiotics (25ug/mL rifampicin and 50 µg/mL kanamycin). A secondary culture of 500uL of each *Agrobacterium* culture was inoculated into a 100mL LB medium containing antibiotics, 10 mM MES and 20 µM acetosyringone and grown overnight in a 27 °C shaker at the speed of 200rpm. All the *A. tumifaciens* cultures were grown until the O.D600 reached 2. These *A. tumifaciens* cultures were centrifuged and the cells were harvested and resuspended in an infiltration buffer (10 mM $MgCl_2$, 10 mM MES (2-(4-Morpholino)-Ethane Sulfonic Acid), 200 µM acetosyringone (39,59-Dimethoxy-49-hydroxy-acetophenone), pH 5.6). The infiltration buffers for all the 8 treatments were mixed according to the ratio mentioned in the Table 3, and were incubated in a roller mixer for 3hrs. The *A. tumifaciens* cultures containing infiltration buffers were infiltrated in the lower surface of the leaves using a syringe. The syringe method was followed as per [22].

Complementation of dsRNA and CGA on eggplant leaves

CGA was applied on eggplant leaves in different concentration: 650 µg, 1 mg, 2 mg and 5 mg per mL. Leaves were checked every day for 3 days for any possible signs of corrosion.

Statistical analysis

All quantitative data in this study (metabolites in leaf tissue, larval tissues, and larval frass and haemolymph) were analysed by one-way ANOVA and the statistical

significance ($p \leq 0.05$) was determined by Fisher's least significant difference or Games Howell post hoc test. Significance ($p \leq 0.05$) of the results of all larval performance, mortality and survivorship assays was evaluated using one-way ANOVA and the statistical significance ($p \leq 0.05$).

Chapter 2

Effects of eggplant steroidal alkaloids on *Spodoptera litura*

Abstract

Steroidal alkaloids (SAs) are the secondary metabolites in *Solanum melongena* (Solanaceae) which are known to play an important role in plant defense mechanisms. Here I report the chemical basis of interaction between an agronomically important crop *Solanum melongena* and its generalist herbivore *Spodoptera litura*. A differential occurrence of *S. litura* was observed on different eggplant varieties in a randomized field. Upon metabolite profiling of eggplant leaves, a negative correlation between larval occurrence and concentration of SAs: solasodine (SD), solasonine (SN) and solamargine (SM) was observed. It was interesting to see that *S. litura* was unable to metabolize SAs and excreted them without any modification. Concentration-mortality relationship of SAs was studied on neonates and second instar larvae of *S. litura.* A reduced larval mass gain was observed upon feeding them with artificial diets spiked with SAs as compared to control diet. Larval mass gain showed a negative correlation with increase in the concentration of respective SAs.

Introduction

In natural ecosystem plants and insects interact with each other in the language of chemical compounds. The most recurrent biotic stress: insect herbivory, is a major threat to the overall growth and development of the plant. Hence, it is not astonishing that plants have developed an elaborate defense mechanism like physical and chemical barriers to deter the herbivory. Plant secondary metabolite: phytotoxins are powerful chemical weapons which play a crucial role in plant-herbivore interactions [1]. These toxins target the insect's metabolism and alter their physical and physiological conditions. Interestingly, phytotoxins are in the form of glucosylated pro-toxins which avert their toxic effect on the plant itself [2].

Solanum melongena (Solanaceae) also known as eggplant, is a widely consumed vegetable crop by people of countries like Central, South and Southeast Asia, some parts of Africa and Central America [6]. The crop is native to India and is grown in all seasons [7]. It is also known as poor man's crop because of its high productivity and low cost of cultivation. Tropical armyworm or *Spodoptera litura* (Lepidoptera: Noctuidae), is a generalist herbivore on Solanaceae plants. It is a pest on many agronomically important crops including tomato and eggplant. There is a dearth of information about ways to alleviate *S. litura*'s feeding on the crops. Being one of the major destructors, *S. litura* is exposed to various kinds of insecticides which have led to the accumulation of abominable chemicals and eventually the development of resistance. It has been observed that despite the presence of SGAs in eggplant, *S. litura* voraciously feeds on it.

In the class of plant secondary metabolites, steroidal glycoalkaloids (SGAs) are particularly compelling to study due to their significance in diverse aspects. SGAs are peculiar secondary metabolites of Solanaceae family, present in various parts of plant viz. flowers, fruits, leaves, roots, and tubers [2]. Pharmaceutically important values of SGAs like treating fungal diseases and tumor-inhibiting activities are reported. Antimicrobial and insecticidal properties of glycoalkaloids are well known for long. [10]. Being a major chemical defense metabolite, SGA's role in the plant defense system is indisputable.

Nevertheless, SGAs showing beneficial use, when consumed in pertinent amounts, they may show toxic effects on bacteria, fungi, viruses, insects, animals, and humans as well [3]. Certain unavoidable drawbacks go parallel with research in the field of steroidal alkaloids. For instance, the detrimental effect of SGAs varies with the complexity of an organism and its developmental stage [9]. Scarcely anything is known about ways in which insects withstand the ingestion of steroidal glycoalkaloids. An interesting concern regarding the study of glycoalkaloids is its property to show synergism [11].

In this report, I tried to determine the effect of steroidal alkaloids present in eggplant on *S. litura*. In a randomized field, differential occurrence of *S. litura* on eggplant varieties were observed. *S. litura* being a folivore, metabolite profiling of different

varieties of eggplant leaves was carried out. SGAs were found in varying concentrations in different eggplant variety and showed a negative correlation with *S. litura*'s occurrence. Out of all SGAs, solasodine, solasonine, and solamargine were found to be most abundant in eggplant. Both solasonine (solatriose-based) and solamargine (chacotriose-based) are composed of aglycone: solasodine and their respective sugar chain [4]. To better understand the effect of SGAs on *S. litura,* I determined the correlation of metabolite concentration with larval mass gain as well as with mortality rate of neonates and second instar larvae on diets spiked with solasodine, solasonine and solamargine. Results from excretion efficiency determination assay confirmed that *S. litura* is unable to metabolize the steroidal alkaloids, and are excreted through frass without any modification.

RESULTS

S. litura larvae occur differentially on different eggplant varieties

In a randomized field of different eggplant varieties (Figure 1-A), a differential occurrence of 3rd and 4th instar larvae of *S. litura* was observed [work done by Dr. Sagar Pandit]. To validate this, *S. litura* larvae were reared on leaves of different eggplant varieties. Feeding rate of 3rd and 4th instar larvae on leaves of different eggplant varieties corroborates with the field observation of differential occurrence of 3rd and 4th instar larvae on different eggplant varieties. The larval mass gain of *S. litura* was also calculated on different eggplant varieties, which showed a similar trend as that of the differential occurrence in the randomized field (Figure 1-C, D). In addition to this, 100% neonate mortality was observed and high larval mortality of early instar larvae (1st instar) was observed when they were reared on different eggplant varieties in laboratory condition. Early instar *S. litura* larvae were seen on *Ricinus communis* (castor plants) around the eggplant field, at the same time the late instar larvae were not seen on castor plants, instead on different eggplant varieties.

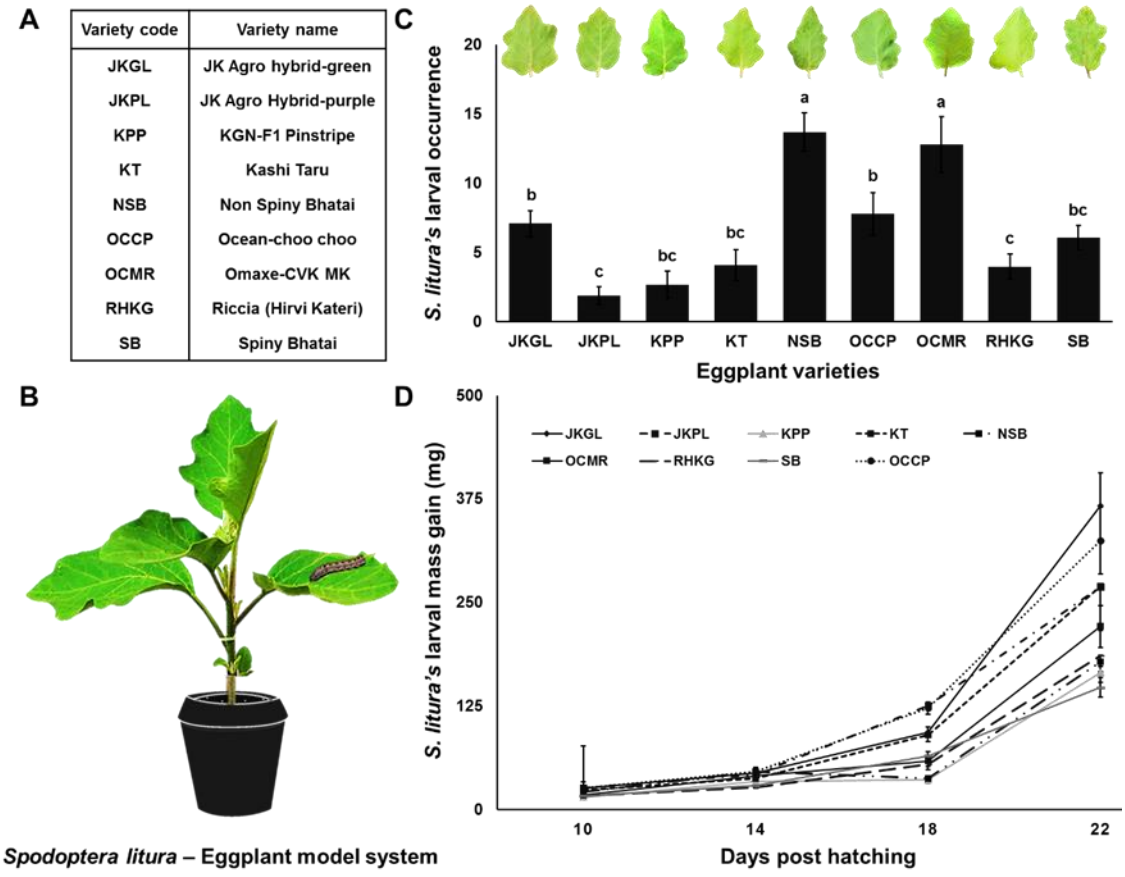


Figure 1 | *S. litura* larvae shows differential occurrence on different eggplant varieties. (A) Codes of different eggplant varieties with their names used for study. **(B)** *Spodoptera litura* – eggplant model system showing *S. litura* feeding on eggplant leaves. **(C)** *S. litura's* larval occurrence on different eggplant varieties in a randomized field in IISER, Pune shows maximum larval occurrence on NSB and OCMR variety ($f_{8,81}$ = 11.949, significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 10 for each variety). **(D)** *S. litura's* larval mass gain on different eggplant varieties measured on 10 dph (days post hatching), 14 dph,18 dph and 22 dph; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 10 for each variety. ANOVA, analysis of variance.

Differential occurrence of *S. litura* larvae negatively correlates with abundance of steroidal alkaloids in eggplant leaves

U(H)PLC-ESI-QTOF based metabolite profiling of leaves of 9 eggplant varieties from randomized field showed an abundance of steroidal alkaloids solasodine (SD), solasonine (SN) and solamargine (SM) varies between eggplant varieties. Average physiological concentration of SAs in leaves of different eggplant varieties was observed as SD (0.5 ng/g of fresh mass of leaf), SN (10 ng/g of fresh mass of leaf) and SM (10 ng/g of fresh mass of leaf). SD showed a negative correlation to *S. litura*

larval occurrence ($p < 0.05$) whereas SN and SM also showed a negative correlation which was not significant (Figure 2).

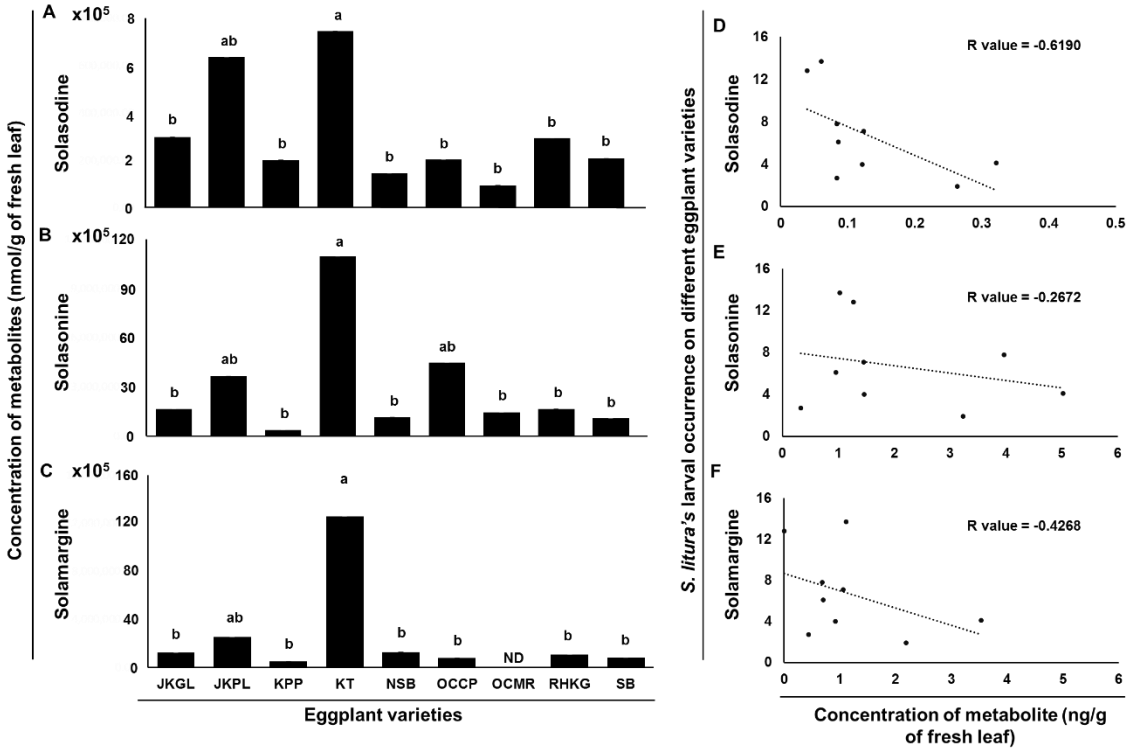


Figure 2 | UPLC-ESI-QTOF based metabolite profiling of leaves of different eggplant varieties shows presence of steroidal alkaloids and their negative correlation with *S. litura's* larval occurrence. Concentration of **(A)** solasodine **(B)** solasonine and **(C)** solamargine in nmol per gram of fresh leaves of different eggplant varieties shows Kashi Taru has highest concentration of steroidal alkaloids among all the varieties; n = 6 for each variety. Correlation of concentration of **(D)** solasodine **(E)** solasonine and **(F)** solamargine in nmol per gram of fresh leaves of different eggplant varieties with *S. litura's* larval occurrence shows a negative correlation (significance value $p \leq 0.05$, $f_{8,45} = 3.404$ $f_{8,45} = 2.89$, $f_{8,45} = 3.112$). ANOVA, analysis of variance; ND, not detected.

SD, SN and SM are excreted without metabolism

U(H)PLC-ESI-QTOF based analysis of frass of *S. litura* larvae fed on artificial diets spiked with physiological concentrations of SD, SN and SM respectively showed no metabolism of SAs (Figure 3C). Similarly, neither these metabolites nor their metabolized products were detected in the haemolymph (Figure 3C) indicating that these SAs are not sequestered by the larvae in these tissues.

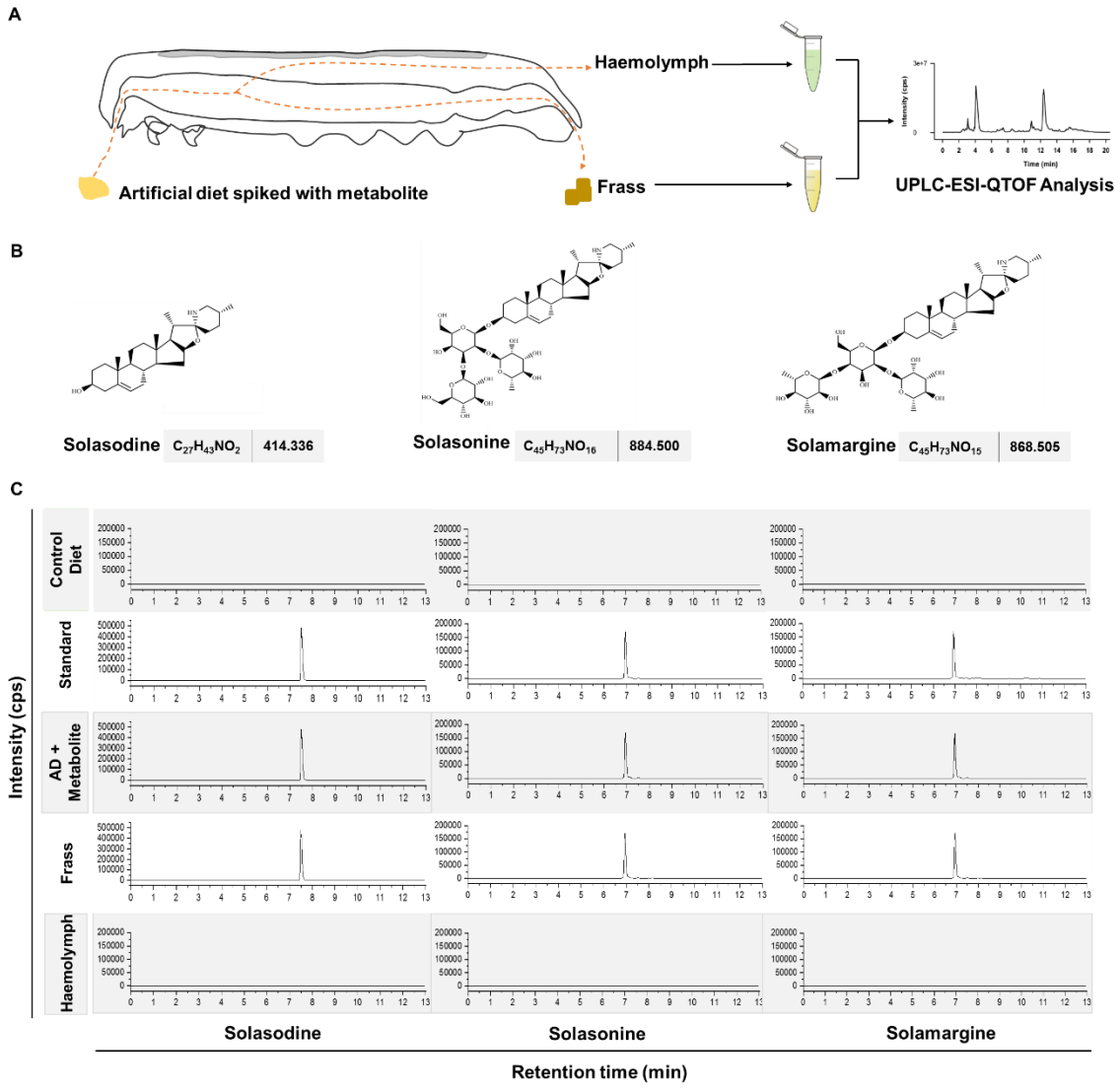


Figure 3 | UPLC-ESI-QTOF based analysis shows the inability of *S. litura* to metabolize steroidal alkaloids. (A) Haemolymph and frass samples collected from the *S. litura* larvae fed on artificial diet (AD) spiked with solasodine, solasonine and solamargine used for UPLC-ESI-QTOF analysis. **(B)** Structure, molecular formula and molecular weight of steroidal alkaloid solasodine and its glycosylated forms solasonine and solamargine. **(C)** Extracted ion chromatograms (XICs) of solasodine, solasonine and solamargine from the TIC (total ion chromatogram) of control diet, metabolite standards, artificial diet spiked with metabolites, frass and haemolymph; XICs show no presence of any metabolite on control diet and haemolymph; n = 5 for each treatment.

Concentration of SGA excreted through the frass is equal to the concentration of SGAs ingested

To understand the difference between the concentrations of metabolite ingested with the concentration of metabolite excreted, excretion efficiency determination assay was

performed with *S. litura* larvae. In this, the larvae were fed on a measured quantity of artificial diet spiked with physiological concentration of respective SGAs for 24 hrs. After 24 hrs the frass was collected, and U(H)PLC-ESI-QTOF based analysis of SGAs in the frass and diet was carried out. The quantity of SD, SM and SN ingested by the larvae were equal to the quantity of SD, SM and SN excreted by the larvae (Figure 4-B, C, D).

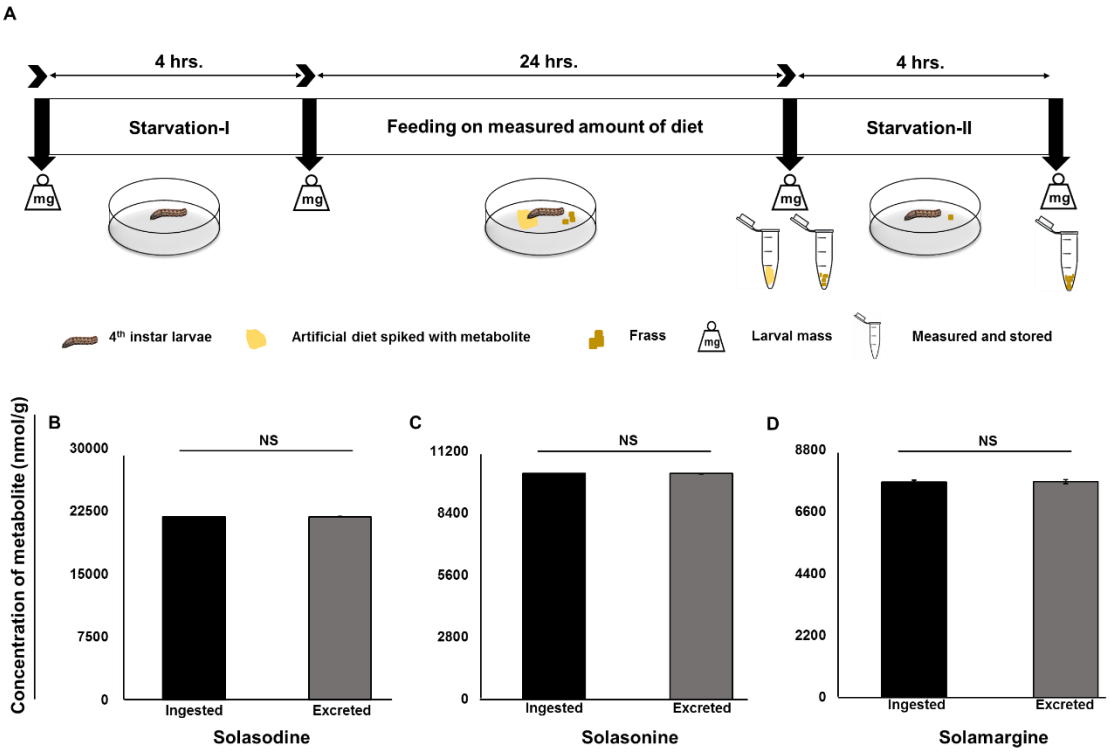


Figure 4 | Excretion efficiency determination assay shows steroidal alkaloids are excreted without any metabolism. (A) Schematic showing timeline of excretion efficiency determination assay used to quantify ingested and excreted solasodine, solasonine and solamargine; 4th instar larvae were used for all the assays; artificial diets(AD) were spiked with physiological concentration of solasodine (0.5 ng/g of AD), solasonine (10 ng/g of AD) and solamargine (10 ng/g of AD); diet and pooled frass (after and before starvation-II) were measured and extracted using 70% methanol and analyzed by UPLC-ESI-QTOF. Concentration (nmol/g) of ingested and excreted steroidal alkaloids **(B)** solasodine **(C)** solasonine and **(D)** solamargine per gram of artificial diet spiked with respective metabolites by *S. litura* shows no significant difference. NS, not significant.

Effects of SD, SN and SM on *S. litura*

Effect of SD, SN and SM on *S. litura* were studied by using larval mass gain of *S. litura.* Feeding *S. litura* larvae on diets spiked with varying concentrations of SAs

showed a strong negative correlation between metabolite concentration and larval mass gain. Reduced larval mass gain was observed in larvae fed on diets spiked with physiological concentration of SD, SN and SM; least larval mass gain was observed with SN (Figure 5).

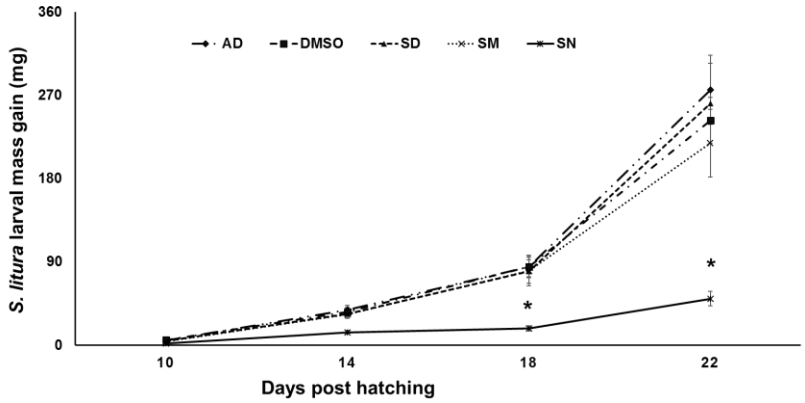


Figure 5 | Effects of steroidal alkaloids on *S. litura* in the form of reduced larval mass gain. Larval mass was measured on 10 dph (days post hatching) (), 14 dph, 18 dph and 22 dph after feeding *S. litura* larvae of 1 dph on artificial diet (AD) spiked with physiological concentration of solasodine (0.5 ng/g of AD), solasonine (10 ng/g of AD) and solamargine (10 ng/g of AD) with water and DMSO spiked diets as control; SN showed a significant reduced larval mass as compared to other treatments (10 dph: $f_{4,95} = 5.627$ $p = 0.0004$; 14 dph: $f_{4,95} = 6.063$ $p = 0.0002$; 18 dph: $f_{4,95} = 7.944$ $p = <0.0001$; 22 dph: $f_{4,95} = 20.17$ $p = <0.0001$; significant differences ($p \leq 0.05$) between means (± s.e.) determined by one-way ANOVA; n = 20 for each treatment). ANOVA, analysis of variance.

Discussion

Preliminary observations on the field showed that there was a differential occurrence of *S. litura* on different varieties of eggplant leaves. LCMS based metabolite profiling of various varieties of eggplant leaves showed the presence of steroidal alkaloids (SAs). The presence of SAs in Solanaceae and their role in deterring herbivory is well documented [10, 15, 16]. The most abundant steroidal alkaloids found in eggplant leaves were solasodine, solasonine, and solamargine. The highest physiological concentration of SAs was found in Kashi Taru variety with values as solasodine (0.5 ng/g of fresh leaves), solasonine (10 ng/g of fresh leaves) and solamargine (10 ng/g of fresh leaves). The correlation of concentration of SAs in different varieties of eggplant leaves showed a negative correlation with larval occurrence. This result

suggests that SAs have a role in eggplant and *S. litura* interaction. High larval mortality of early instar larvae was observed when larvae were reared on eggplant leaves. This may answer why early instar larvae are rarely found on eggplant leaves.

A reduced larval mass gain was observed on feeding *S. litura* larvae on diet spiked with solasonine. Solasonine has a higher toxic effect on *S. litura* larvae as compared to solasodine and solamargine. Aglycone viz. solasodine is comparatively less toxic and inactive as compared to their glycosylated parts viz. solasonine and solamargine [16]. Solasonine and solamargine are well known to show a detrimental effect on larval mass gain [17].

Further experiment of excretion efficiency determination assay showed that none of the SAs were being metabolized by *S. litura* larvae. This observation suggests that *S. litura* has not yet evolved with any counter adaptation mechanism to metabolize SAs and are excreted through the frass without any modification. Metabolite analysis of haemolymph and fat bodies of *S. litura* supports the fact that SAs are not getting sequestered. The excretion efficiency determination assay shows that the quantity of SGAs ingested by the larvae is equal to the quantity of SGAs excreted by the larvae, which also supports that the SGAs are excreted by the larvae without any metabolism. The concentration of SGAs Interestingly, on rearing *S. litura* neonate and 2nd instar larvae on artificial diet spiked with concentration gradient (10 µg/g of diet, 100 µg/g of diet and 1 mg/g of diet) of solasodine, solasonine and solamargine showed no mortality at 24 hrs 48 hrs, 72 hrs, and 96 hrs. It was interesting to observe that *S. litura* larvae on higher concentration, managed to survive by showing cannibalistic behavior and strategically avoid ingesting higher concentrations of SAs. *S. litura* at their larval stage is known to show intraspecific predation upon starvation [19].

The synergistic effect of steroidal glycoalkaloids is well known in Solanaceae family [11, 17]. Solasonine and solamargine when combined, could even kill snails [17]. This gives a way to study the combined effect of SGAs on *S. litura*.by considering their synergic effect in toxicity. Also, the presence of solasonine and solamargine could be one of the reasons why *S. litura* does not prefer to feed on eggplant leaves during its early stages.

Although SAs have a deterrent effect on herbivores, it has many beneficial effects on humans. SAs are known for its pharmaceutical benefits. Solasodine is known for its anti-cancer properties, as it specifically binds to cancer cell receptors and induces apoptosis and necrosis. Apart from the anti-parasitic effect, solamargine and

solasonine have the potential to treat different types of cancer like gastric cancer, lung cancer, liver cancer, etc. [20]. This dual property of steroidal alkaloids of playing the role of plant defense metabolite and nutritional benefits to humans may be taken as a good opportunity to generate a functionally modified eggplant crop with varying levels of SAs. This may improve the plant defense of eggplant crop against voracious feeders like *S. litura* and help in increasing the nutritional value for human consumption as well.

Future directions

The insecticidal property of SN needs to be further studied on other species of *Spodoptera.* The effects of steroidal alkaloids should be studied in the context of a third trophic level, which are the predator of *S. litura.*

Materials and methods

Plants

Seeds of 9 different varieties (3 pure-line breed and 6 hybrid) of *Solanum melongena* (eggplant) were germinated in red soil, vermiculite, perlite and cocopeat soil mixture in 3:1:1:1 ratio in controlled conditions (Relative humidity: 70-80%; Photoperiod: 16 hr light-8 hr dark; Temperature: 22 ± 2°C). Plantlets were transferred to pots after 15 days and were grown under natural conditions for 10 days followed by plantation in a randomized way in an open field at IISER, Pune. Plants were provided with macroelements and microelements (HiMedia) regularly for their better growth and development.

Insects

Spodoptera litura larvae were collected from various crop fields in and around Pune, India. Larvae were reared on artificial diet (minimal nutrition required for insect survival) [14] under controlled conditions (Relative humidity: 65%; Photoperiod: 16 hr light-8 hr dark; Temperature: 25 ± 2°C). Moths were provided with 10% sucrose solution and were reared in dark conditions. Eggs and early instar larvae from culture were transferred to artificial diet spiked with metabolites for all the experiments in this study. Fresh diet was provided to the larvae every alternate day until pupation.

Larval performance on eggplant leaves

Eggs of *S. litura* were transferred on eggplant leaves of 9 different varieties. Neonate mortality was recorded after egg hatching (n= 150). Since neonate mortality was 100%, 2nd and 3rd instar larvae were used for larval performance on eggplant leaves. Larval mass was recorded at the interval of every 4th day for four times (n=50). Larval frass and haemolymph were snap frozen and stored in -80 °C for LCMS based metabolite analysis.

Larval performance assay

To study the larval performance, freshly hatched neonates were fed on artificial diets spiked separately with physiological concentration: SD (0.5 ng/g of diet), SM (10 ng/g of diet), and SN (10 ng/g) (n=20 for each treatment). Freshly spiked diet was provided every alternate day to rear larvae till pupation. Larval mass was recorded for four times at interval of every 4th day. Frass and haemolymph was collected for UPLC/ESI/QTOF based analysis. To calculate larval performance upon synergic effect of SN and SM, neonates were fed on diets spiked with SN and SM together of 1:1 concentration of each. The concentration taken was 10 µg/g of diet, 100 µg/g of diet and 1 mg/g of diet each of SN and SM.

Extraction of metabolites and UPLC/ESI/QTOF (LCMS) based analysis

For metabolite analysis, 200 mg of leaf tissues were fine crushed and extracted in 1 mL of 70% methanol. Cell debris was removed by centrifugation (12000 x g, 10 min). Supernatant was collected and incubated at -80˚C overnight for protein precipitation. Samples were centrifuged again (12000 x g, 20 min, 4˚C). Frass and haemolymph samples were also extracted in a similar manner, using 70% methanol in the ratio of 100 mg of frass in 1 ml of extraction buffer and 10ul of haemolymph in 100ul of extraction buffer. Metabolite extracts from all tissues were analysed on AbSCIEX-X500R-QTOF (UPLC/ESI-QTOF-MS). Samples were separated on a Phenomenex Gemini® C18 column (50x4.6 mm, 5µm, 118Å), using Millipore water with 0.1% formic acid and methanol with 0.1% formic acid as mobile phase. A 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 0.5 ml/min was used. Compounds were analysed in positive ion mode (capillary voltage, 4,500 V; capillary exit, 130 V; dry gas temperature, 200 °C).

Excretion efficiency determination assay (Waldbauer assay)

Ingestion of metabolite spiked diet and excretion by *S. litura* larvae were budgeted using an excretion efficiency determination assay, following standardization [6]. Freshly molted larvae into 4th instar were first starved for 4 hrs, and then fed on measured amount of artificial diets spiked with SD, SN and SM for 24 hrs. After 24 hrs of feeding, larvae were again starved for 4 hours. Mass of larvae and frass was measured before and after every starvation. Frass of larvae before starvation and after starvation was pooled, and then were analysed by UPLC-ESI-QTOF.

Statistical analysis

All quantitative data in this study (metabolites in leaf tissue, larval tissues, and larval frass and haemolymph) were analysed by one-way ANOVA and the statistical significance ($p \leq 0.05$) was determined by Fisher's least significant difference or Games Howell post hoc test. Significance ($p \leq 0.05$) of the results of all larval performance, mortality and survivorship assays was evaluated using one-way ANOVA and the statistical significance ($p \leq 0.05$).

References

1. Wu, Jianqiang, and Ian T. Baldwin. "New insights into plant responses to the attack from insect herbivores." *Annual review of genetics* 44 (2010): 1-24.
2. Pentzold, Stefan, et al. "How insects overcome two-component plant chemical defence: plant β-glucosidases as the main target for herbivore adaptation." *Biological Reviews* 89.3 (2014): 531-551.
3. Friedman and Mendel. "Potato glycoalkaloids and metabolites: roles in the plant and in the diet." *Journal of Agricultural and Food Chemistry* 54.23 (2006): 8655-8681.
4. Blankemeyer and J. T., et al. "Developmental toxicology of solamargine and solasonine glycoalkaloids in frog embryos." *Food and chemical toxicology* 36.5 (1998): 383-389.
5. Sucha, Lenka, and Pavel Tomsik. "The steroidal glycoalkaloids from solanaceae: toxic effect, antitumour activity and mechanism of action." *Planta medica* 82.05 (2016): 379-387.
6. Harish, D. K., et al. *Growth and yield parameters in brinjal as influenced by organic nutrient management and plant protection conditions* (2011).
7. Choudhary, B. 1970. Vegetables. National Book Trust, India, 25-50 PP

8. Roddick, James G. "Steroidal glycoalkaloids: nature and consequences of bioactivity." *Saponins used in traditional and modern medicine*. Springer, Boston, MA, 1996. 277-295.
9. Tingey and Ward M. "Glycoalkaloids as pest resistance factors." *American Potato Journal* 61.3 (1984): 157-167.
10. Roddick and J. G. "The steroidal glycoalkaloid α-tomatine". *Phytochemistry* 13.1 (1974): 9-25.
11. Ghisalberti and Emilio L. "Steroidal glycoalkaloids: isolation, structure, analysis, and biosynthesis." *Natural Product Communications* 1.10 (2006): 1934578X0600101007.
12. Kolady, Deepthi Elizabeth, and William Lesser. "Potential welfare benefits from the public-private partnerships: A case of genetically engineered eggplant in India." *Journal of food, agriculture & environment* (2008).
13. Elmes, Graham W., J. C. Wardlaw, and J. A. Thomas. "Larvae of *Maculinea rebeli*, a large-blue butterfly and their Myrmica host ants: patterns of caterpillar growth and survival." *Journal of Zoology* 224.1 (1991): 79-92.
14. Nagarkatti, S., and Prakash, S. (1974). Rearing *Heliothis armigera* (Hubn.) on an artificial diet. *Technical bulletin commonwealth institute of biological control*.
15. Ripperger, H., & Schreiber, K. (1981). *Solanum* steroid alkaloids. In *The alkaloids: chemistry and physiology* (Vol. 19, pp. 81-192). Academic Press.
16. S. Al Sinani and E.A. Eltayeb he steroidal glycoalkaloids solamargine and solasonine in *Solanum* plants.
17. Weissenberg, M., Levy, A., Svoboda, J. A., & Ishaaya, I. (1998). The effect of some Solanum steroidal alkaloids and glycoalkaloids on larvae of the red flour beetle, Tribolium castaneum, and the tobacco hornworm, Manduca sexta. *Phytochemistry*, *47*(2), 203-209.
18. Cárdenas, P. D., Sonawane, P. D., Heinig, U., Bocobza, S. E., Burdman, S., & Aharoni, A. (2015). The bitter side of the nightshades: Genomics drives discovery in Solanaceae steroidal alkaloid metabolism. *Phytochemistry*, *113*, 24-32.
19. Ryuda, M., Nakayama, H., & Hayakawa, Y. (2008). A novel gene associated with intraspecific predation in Spodoptera litura larvae. *Applied entomology and zoology*, *43*(4), 563-568.

20. Cham, Bill E. "Solasodine rhamnosyl glycosides specifically bind cancer cell receptors and induce apoptosis and necrosis. Treatment for skin cancer and hope for internal cancers." *Research Journal of Biological Sciences* 2.4 (2007): 503-514.
21. Liu, Su, et al. "Identification of putative carboxylesterase and glutathione S-transferase genes from the antennae of the Chilo suppressalis (Lepidoptera: Pyralidae)." *Journal of Insect Science* 15.1 (2015).
22. Liu, Haiping, et al. "Virus-induced gene silencing in eggplant (Solanum melongena)." *Journal of integrative plant biology* 54.6 (2012): 422-429.
23. Felton, G. W., et al. "Impact of oxidized plant phenolics on the nutritional quality of dietar protein to a noctuid herbivore, *Spodoptera exigua*." *Journal of Insect Physiology* 38.4 (1992): 277-285.
24. Felton, G. W., et al. "Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores." *Journal of Chemical Ecology* 15.12 (1989): 2667-2694.
25. Hurrell, R. F., P. A. Finot, and J. L. Cuq. "Protein-polyphenol reactions: 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein." *British Journal of Nutrition* 47.2 (1982): 191-211.
26. Cheng, Tingcai, et al. "Genomic adaptation to polyphagy and insecticides in a major East Asian noctuid pest." *Nature ecology & evolution* 1.11 (2017): 1747-1756.
27. Ramakrishnan, N., V. S. Saxena, and S. Dhingra. "Insecticide-resistance in the population of *Spodoptera litura* (F.) in Andhra Pradesh." *Pesticides* 18.9 (1984): 23-27.
28. Daniel, Otto, et al. "Selected phenolic compounds in cultivated plants: ecologic functions, health implications, and modulation by pesticides." *Environmental Health Perspectives* 107.suppl 1 (1999): 109-114.
29. Dakora, F. D., and D. A. Phillips. "Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins." *Physiological and Molecular Plant Pathology* 49.1 (1996): 1-20.
30. Langcake, P., J. A. Irvine, and M. J. Jeger. "Alternative chemical agents for controlling plant disease." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* (1981): 83-101.

31. Ivashuta, Sergey, et al. "Environmental RNAi in herbivorous insects." *Rna* 21.5 (2015): 840-850.
32. McLoughlin, Austein G., et al. "Identification and application of exogenous dsRNA confers plant protection against Sclerotinia sclerotiorum and Botrytis cinerea." *Scientific reports* 8.1 (2018): 1-14.
33. Kundu, Anish, and Jyothilakshmi Vadassery. "Chlorogenic acid-mediated chemical defence of plants against insect herbivores." *Plant Biology* 21.2 (2019): 185-189.
34. Siddiqi, Azra I., and Samuel O. Freedman. "Identification of chlorogenic acid in castor bean and oranges." *Canadian journal of biochemistry and physiology* 41.4 (1963): 947-952.
35. Wu, Jianqiang, and Ian T. Baldwin. "New insights into plant responses to the attack from insect herbivores." *Annual review of genetics* 44 (2010): 1-24.
36. Harish, D. K., et al. *Growth and yield parameters in brinjal as influenced by organic nutrient management and plant protection conditions*. No. RESEARCH. 2011.
37. Kalawate, A., & Dethe, M. D. (2012). Bioefficacy study of biorational insecticide on brinjal. *Journal of Biopesticides*, *5*(1), 75.

APPENDIX 1: List of symbols and abbreviations

S. litura	*Spodoptera litura*
SAs	Steroidal alkaloids
SGAs	Steroidal glycoalkaloids
SD	Solasodine
SN	Solasonine
SM	Solamargine
CGA	Chlorogenic acid
CFA	Caffeic acid
QNA	Quinic acid
dph	Days post hatching
COE	*Carboxylesterase*
ANOVA	Analysis of variance
ACTB	Beta actin
UCCR	Ubiquinol-cytochrome c reductase
LCMS	Liquid chromatography
U(H)PLC-ESI-QTOF	Ultra performance liquid chromatography-electrospray ionisation- quantitative time of flight
RT	Retention time
TIC	Total ion chromatogram
XIC	Extracted ion chromatogram
PMRi	Plant mediated RNA interference
VIGS	Virus induced gene silencing
AD	Artificial diet

APPENDIX

APPENDIX 2: Artificial diet compositions (1000 mL)

1. Wheatgerm: 26 g
2. Kidney bean flour: 51.3 g
3. Chick pea flour: 56 g
4. Dried yeast powder: 31.6 g
5. Casein: 15.2 g
6. L-Ascorbic acid: 3.2 g
7. Cholesterol: 0.5 g
8. Multivitamin multi-mineral tablet: 2 tablets
9. Vitamin E capsule: 1 capsule
10. Linseed oil: 1 ml
11. Methyl-p-hydroxybenzoate: 8 g
12. Sorbic acid: 1.3 g
13. Aureomycin sulphate: 0.25 g
14. Formaldehyde solution: 2 ml
15. Agar–agar: 16.4 g
16. Distilled water: 820 ml