Cytochrome P450 isoforms transcriptional, larval growth and development responses to host allelochemicals in the generalist herbivore, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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***Helicoverpa armigera* (Hübner) is a polyphagous pest causing severe yield loss in many important crops. Host plants produce allelochemicals to deter insect pests and in response, insects deploy cytochrome P450 monooxygenases (P450s) to detoxify allelochemicals. Understanding the response of P450s to allelochemical exposure is key to effective pest management. We studied the response of seven *H. armigera* P450 isoforms to different concentrations of three allelochemicals (gossypol, tomatine and xanthotoxin) and their effects on insect growth and survival. Allelochemicals strongly induced overexpression of some P450s. *CYP6AE14* exhibited the highest overexpression in gossypol treatment. *CYP6AE14* and *CYP6B7* exhibited higher overexpression in xanthotoxin treatment and *CYP6B7* showed the highest overexpression in tomatine treatment. Overall, *CYP6AE14* and *CYP6B7* were induced by all three allelochemicals. Higher (0.5 and 1.0 µg) concentrations of allelochemicals caused significant larval growth retardation. Interestingly, gossypol showed a hormetic effect, i.e. larval weight was approximately 10% higher at lower (0.025 µg) concentration. Highest larval mortality (53%) was observed in tomatine treatment. These findings would help in identifying suitable P450 isoforms in the management of *H. armigera*.**

Keywords: Allelochemicals, cytochrome P450 isoforms, *Helicoverpa armigera*, pest management, real-time PCR.

HELICOVERPA armigera (Hübner) is one of the most important biotic constraints to agriculture across the world and causes severe yield loss in many important crops like cotton, tomato, pigeon pea, chickpea, etc.[1]. The wide adaptability of *H. armigera* to different host plants could lead to huge economic loss if left uncontrolled. This calls for the intensive use of pesticides that has consequently lead to the development of pesticide resistance in *H. armigera*[2]. Therefore, there is an urgent need to explore better alternates for management of *H. armigera*.

Plants produce a variety of secondary metabolites or allelochemicals to deter herbivorous insects. The target pest, *H. armigera* encounters a large number of different allelochemicals compared to mono/oligophagous insects, which encounter a limited range of allelochemicals[3,4]. *H. armigera* is prevalent on cotton and causes severe yield loss[5]. Cotton plants produce allelochemicals, predominantly a terpenoid aldehyde called gossypol. This can readily penetrate the biological membranes and form covalent bonds with amino acids, especially with lysine, and as a dimer it can cross-link with proteins[6]. Gossypol is also involved in the chelation of iron and other metal ions that consequently affect different classes of enzymes[7]. The other allelochemical, tomatine is a glycoalkaloid produced by tomato (*Solanum lycopersicum*). Tomatine disrupts the cell membrane by hydrolysing liposomes present in the membrane and interrupts the active transport of sodium and potassium ions[8]. Tomatine is also known to be a fungi-toxic, inhibiting the growth of fungal pathogens such as *Septoria lycopersici* and *Cladosporium fulvum*[9]. Xanthotoxin (a furanocoumarin) is one of the major allelochemicals produced by members of the Apiaceae and Rutaceae family (e.g. *Cuminum cyminum*, *Foeniculum vulgare*, *Citrus aurantifolia* and *Zanthoxylum simulans*). Xanthotoxin cross-links with DNA and also modifies the proteins that become toxic to the insect[10]. We studied the response of *H. armigera* to naturally occurring allelochemicals such as frequently encountered (gossypol), moderate to frequently encountered (tomatine) and least encountered (xanthotoxin).

Insects have developed detoxification mechanisms to cope with a variety of allelochemicals. Among several detoxification systems, cytochrome P450 monooxygenases (P450s) are a major class of detoxification enzymes involved in metabolizing a broad range of plant allelochemicals and xenobiotics, including insecticides[11,12]. Broad substrate processing capacity, high diversity and stringent transcriptional regulation of P450s have made them unique players in the evolution of complex detoxification systems which eventually helped the insects to adapt to a wide range of host-plant allelochemicals[10–13]. The extent of detoxification is known to be highly correlated with the transcript levels of P450s that determine the insect resistance to allelochemicals and insecticides[13]. Currently, the number of P450 genes identified in insects is expanding and are grouped into many families[14,15]. Among the P450 families, CYP6 and CYP9 are known to be predominantly involved in detoxification of allelochemicals[16]. Therefore, it is pertinent to identify which isoform/s of P450 is/are involved in metabolizing these allelochemicals. Thus, understanding the responses of P450s to these allelochemicals could help in devising effective pest management strategies.

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In this study, we aimed at identifying the P450 isoforms predominantly expressed in response to the above allelochemicals. Based on the information available in other insects[17–22], seven P450 isoforms (belonging to CYP6 and CYP9 families) were selected to study their response to these allelochemicals. Further, we wanted to know what concentration of these allelochemicals affects the growth and development of *H. armigera*. Additionally, all three allelochemicals induced P450s expression across the developmental stage of *H. armigera* was analysed in order to know the expression pattern of these P450s at un-induced state.

Neonate larvae of *H. armigera* were obtained from the National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. The larvae were reared on a chickpea-based semi-synthetic diet[23] and formed pupae were transferred to empty petri dishes. Eclosed moths were transferred to insect-rearing cages and 5% honey was provided to them through cotton wads. All insects were maintained at 27° ± 2°C temperature, 65% ± 5% relative humidity and 16:8 h of light and dark cycle.

To clone P450 genes, total RNA was extracted from seven-day-old (approximately third instar) single larva of *H. armigera* using the ISOLATE II RNA Mini Kit following the manufacturer's instructions (Bioline Reagents Ltd, UK). Complementary DNA (cDNA) was synthesized by reverse transcribing 2 µg of total RNA using the Tetro cDNA synthesis kit following the manufacturer's protocol (Bioline Reagents Ltd, UK).

Full-length genes of *CYP6B6*, *CYP6B7*, *CYP6AE12*, *CYP6AE14*, *CYP9A14*, *CYP9A17* and *CYP9A18* were amplified using specific primers designed based on the sequences retrieved from NCBI database (see Supplementary material, Table S1, online). For second-strand cDNA synthesis, PCR was performed in a 50 µl reaction comprising 5 µl 10X LA PCR buffer, 5 µl dNTP mix (2.5 mM), 1 µl each of forward and reverse primers (10 mM), 5 µl of 1 : 10 diluted first-strand cDNA as template and 2 U TaKaRa LA *Taq* HS (Takara Bio Inc, India), and final volume was made to 50 µl with PCR-grade water. PCR amplification was performed according to the manufacturer's recommended protocol (Takara Bio Inc, India). The PCR products were purified by gel elution (NucleoSpin Extract II kit – Macherey-Nagel, Germany) and cloned in TA cloning vector pTZ57R/T according to the manufacturer's instructions (Thermo Fisher Scientific, India). Recombinant clones obtained from the transformation were sequenced and the sequence was confirmed by performing NCBI-BLAST analysis.

Analytical-grade purified plant allelochemicals, gossypolacetic acid (HPLC ~95%) and xanthotoxin (GC ≥ 98%) were procured from Sigma-Aldrich, India while tomatine was from MP Biomedicals, USA. The stocks of these allelochemicals were prepared using acetone solvent.

To study the transcriptional response of the P450 isoforms to gossypol, tomatine and xanthotoxin, we tested eight concentrations of each of the allelochemicals, viz. 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 µg/g diet. For insect bioassay, allelochemicals supplemented with 1 ml of semi-synthetic diet were dispensed into each well of the bioassay plate. Seven-day-old larvae were selected for similar size and 30 larvae for each treatment were released individually into the wells of the bioassay plate to avoid cannibalism. Control larvae were fed on media containing only acetone solvent and all these were maintained until the end of larval period. Simultaneously, two independent experiments were performed. To assess the toxicity of allelochemicals on the insect, larval weight was recorded on the seventh day of allelochemicals treatment and mortality was recorded every day till the end of the larval period.

Reverse transcription-quantitative PCR (RT-qPCR) primers were designed using Beacon Designer 7 Software (Premier Biosoft International). The expression of *CYP6B6*, *CYP6B7*, *CYP6AE12*, *CYP6AE14*, *CYP9A14*, *CYP9A17* and *CYP9A18* was analysed after 24 h of allelochemicals treatment. The total RNA was extracted separately from three individual larvae from each treatment and control, and this experiment was repeated twice. cDNA was synthesized using 2 µg of the total RNA. RT-qPCR assays were performed in a total reaction volume of 20 µl comprising 10 µl SYBR Green I Master mix (LightCycler® 480 SYBR Green I Master), 0.5 µl (10 mM) each of forward and reverse primers (see Table S1, Supplementary Material online) and 5 µl of 1 : 10 diluted cDNA following the manufacturer's instructions (Roche Applied Science, Switzerland). Effect of each treatment was assessed with three biological replicates of larvae (two technical replicates for each biological replicate) and no-template controls were included. To minimize the sampling errors, 18S rRNA housekeeping gene was used as reference gene for normalization of RT-qPCR data. In a previous study, 18S rRNA exhibited least expression variations under various treatment conditions in *H. armigera*[24]. Additionally, β-tubulin expression was assessed in these samples in order to know whether the variations in P450s expression are indeed induced by allelochemicals or attributed due to biological and/or experimental artefacts. The relative gene expression levels were calculated using the Livak and Schmittgen method[25].

The expression of *CYP6B7* and *CYP6AE14* (which are induced by all three allelochemicals) was analysed on one-, three-, six-, nine-, 12- and 14-day-old larva (larvae reared on chickpea-based semi-synthetic diet without allelochemicals supplement) and three-day-old pupa and moth samples in order to understand the expression pattern of these two P450s during different developmental stages of *H. armigera*. The total RNA was extracted separately from three individual (above mentioned old) larva/pupa/moth samples. To minimize the sampling errors, β-tubulin housekeeping gene was used as reference

gene for normalization of RT-qPCR data. In a previous study, β-tubulin gene exhibited least expression variations across the developmental stages of *H. armigera*[24]. Remaining steps such as cDNA synthesis, RT-qPCR assays and data analysis were performed as described earlier.

The differences in the weight of the larvae between control and treatments were statistically analysed using ANOVA (GraphPadPrism v.5 – GraphPad Software, Inc, USA). Percentage of larval mortality was calculated, and LC_{50} and LC_{90} values were obtained using Probit model with IBM SPSS v22.0 software.

The full-length genes of seven different P450 isoforms, viz. *CYP6B6*, *CYP6B7*, *CYP6AE12*, *CYP6AE14*, *CYP9A14*, *CYP9A17* and *CYP9A18* were obtained from cDNA amplification with suitable primers. Sequencing of the cloned products showed sequence lengths of 1515, 1515, 1572, 1581, 1593, 1596 and 1593 bp for *CYP6B6*, *CYP6B7*, *CYP6AE12*, *CYP6AE14*, *CYP9A14*, *CYP9A17*, and *CYP9A18* respectively.

Different allelochemicals induced the expression of different P450s and higher concentrations of these allelochemicals affected the growth and development of *H. armigera* and caused larval mortality.

Gossypol induced overexpression of five P450s, viz. *CYP6B6*, *CYP6B7*, *CYP6AE14*, *CYP6AE12* and *CYP9A14*. Notably, the highest overexpression (31-fold) was observed in *CYP6AE14* at the highest concentration (1.0 µg/g). Further, its expression levels were positively correlated with the concentration of gossypol. While the other P450s (*CYP6B6*, *CYP6B7*, *CYP6AE12* and *CYP9A14*) showed a maximum of 5–10-fold overexpression (Figure 1). Lower concentrations of gossypol were ineffective in reducing the larval weight. Interestingly, 0.025 µg/g gossypol treatment led to 10% higher larval weight gain compared to control. In contrast, highest reduction (47%) in larval weight (Figure 2 *a*) and the highest larval mortality (23%) were observed in 1.0 µg/g of gossypol treatment (Table 1).

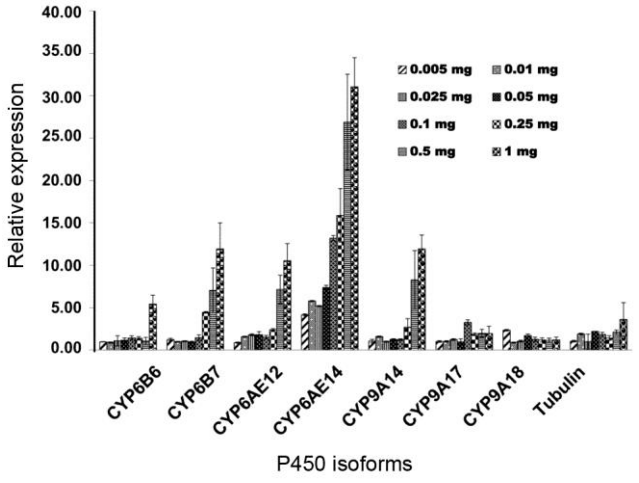

Figure 1. Effect of gossypol on the expression of P450 isoforms. Expression analysis was performed after 24 h of allelochemicals treatment. Error bars indicate standard error of six biological replicates of two experiments (each biological replicate has two technical replicates).

Tomatine induced overexpression of three P450s, viz. *CYP6B6*, *CYP6B7* and *CYP6AE14*. Notably, the highest overexpression (48-fold) was observed in *CYP6B7* followed by *CYP6B6* (35-fold) at the highest concentration (1.0 µg/g). The expression levels of these isoforms positively correlated with the concentration of tomatine. The expression of *CYP6AE14* was markedly induced only in the highest (1.0 µg/g) concentration of tomatine treatment. The expression of other P450s did not show any significant differences (Figure 3). The average larval weights were significantly lower in 0.25, 0.5 and 1.0 µg/g tomatine treatments (Figure 2 *b*). The highest reduction (60%) in larval weight and the highest larval mortality (53%) were observed in 1.0 µg/g tomatine treatment (Table 1).

Xanthotoxin strongly induced overexpression of *CYP6AE14* and *CYP6B7*, whereas *CYP6AE12*, *CYP9A14* and *CYP9A18* isoforms showed marginal overexpression. The highest induction ten-fold was observed in *CYP6AE14* at 0.25 µg/g treatment. Interestingly, mid-concentrations (0.05–0.25 µg/g) of xanthotoxin induced higher expression of P450s (Figure 4). The average larval weights were significantly lower in 0.5 and 1.0 µg/g treatments (Figure 2 *c*) and the highest reduction (44%) in larval weight and highest mortality (20%) were observed in 1.0 µg/g xanthotoxin treatment (Table 1).

Overall, the expression of *CYP6AE14* and *CYP6B7* was induced by all three allelochemicals. However, only the highest concentration (1.0 µg/g) of tomatine was able to induce the expression of *CYP6AE14*.

The basal expression analysis of *CYP6AE14* and *CYP6B7* revealed that the expression of these isoforms was evident only in the larval stages compared to pupa and moth stages of *H. armigera* (Figure 5). The expression of *CYP6AE14* was predominant over *CYP6B7* during the developmental stages of the larva. The highest expression levels of both *CYP6AE14* and *CYP6B7* were observed in the fifth instar larvae.

The cytochrome P450 monooxygenases are important metabolic enzymes involved in catabolizing plant allelochemicals and xenobiotic compounds, including insecticides[26]. Understanding the response of different P450s to allelochemicals would help in designing effective pest management strategies. The expression analysis of different P450s revealed that many isoforms were induced by the tested allelochemicals and the expression levels of P450s varied with the allelochemicals. Gossypol induced five P450s and *CYP6AE14* showed the highest overexpression (31-fold) and its expression levels correlated with the concentration of gossypol. These observations were congruent with a previous study which showed that

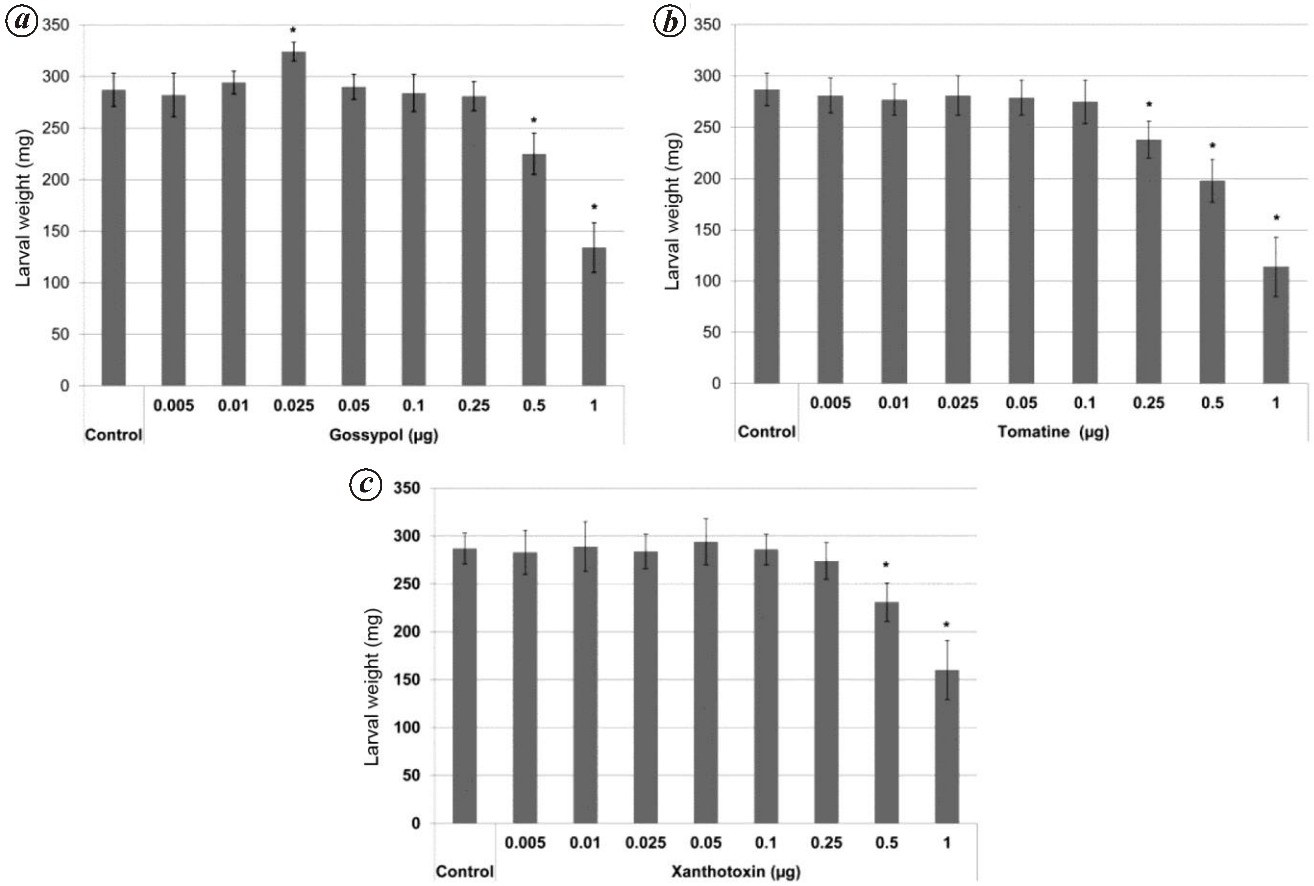

Figure 2. Effect of allelochemicals on larval weight. ***a***, Effect of various concentrations of gossypol on larval weight. ***b***, Effect of various concentrations of tomatine on larval weight. ***c***, Effect of various concentrations of xanthotoxin on larval weight. The final weight of the larvae was recorded on the seventh day of allelochemicals treatment. Each treatment comprised of 30 larvae. Asterisk (*) indicates significant differences in larval weight between control and treatments analysed using one-way ANOVA at $P < 0.05$.

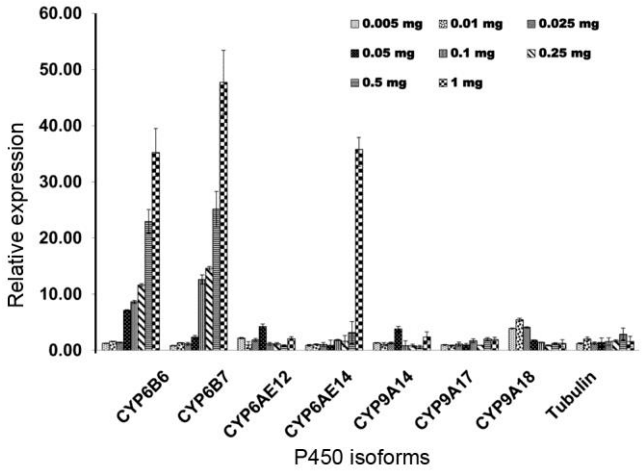

Figure 3. Effect of tomatine on the expression of P450 isoforms. Expression analysis was performed after 24 h of allelochemicals treatment. Error bars indicate standard error of six biological replicates of two experiments (each biological replicate has two technical replicates).

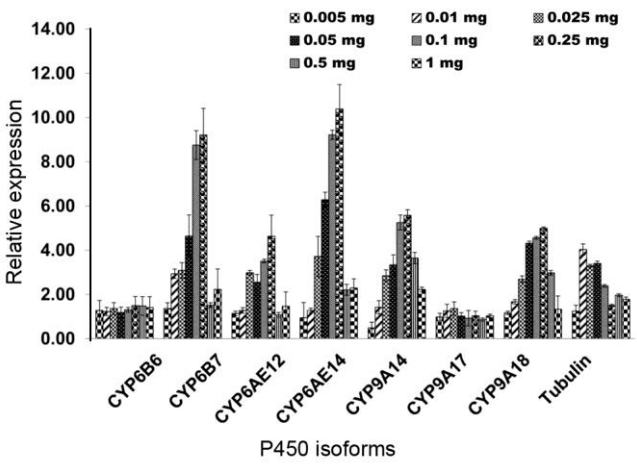

Figure 4. Effect of xanthotoxin on the expression of P450 isoforms. Expression analysis was performed after 24 h of allelochemicals treatment. Error bars indicate standard error of six biological replicates of two experiments (each biological replicate has two technical replicates).

Table 1. Larvicidal activity (%) of allelochemicals against *Helicoverpa armigera*

Allelochemicals concentration (μg)	Per cent mortality	95% CL for log (concentration)						Slope ± SE	χ^2
		LC_{50} value	Lower	Upper	LC_{90} value	Lower	Upper		
Control									
0	0	–	–	–	–	–	–	–	–
Gossypol									
0.25	3.3 ± 1.0	0.545	0.172	3.053	1.278	0.607	6.111	$Y = -0.952 + 1.748 \pm 0.679$	4.82
0.5	10.0 ± 1.8								
1	23.3 ± 2.3								
Tomatine									
0.25	6.67 ± 2.7	0.145	–0.337	3.406	1.052	0.274	8.695	$Y = -0.205 + 1.413 \pm 0.253$	21.604
0.5	16.6 ± 4.4								
1	53.3 ± 3.1								
Xanthotoxin									
0.25	0.00 ± 0.0	0.681	0.186	4.701	1.734	0.896	7.674	$Y = -0.797 + 2.832 \pm 1.116$	3.017
0.5	6.6 ± 1.2								
1	20.0 ± 3.9								

Note: The lower (0.005, 0.01, 0.025, 0.05 and 0.1 μg) concentrations of allelochemicals did not cause larval mortality and were on par with the control. In contrast, the higher concentrations (025, 0.5 and 1 μg) of allelochemicals caused larval mortality. Hence, we have provided details wherever larval mortality was observed. The values obtained after Abbott's correction are represented.

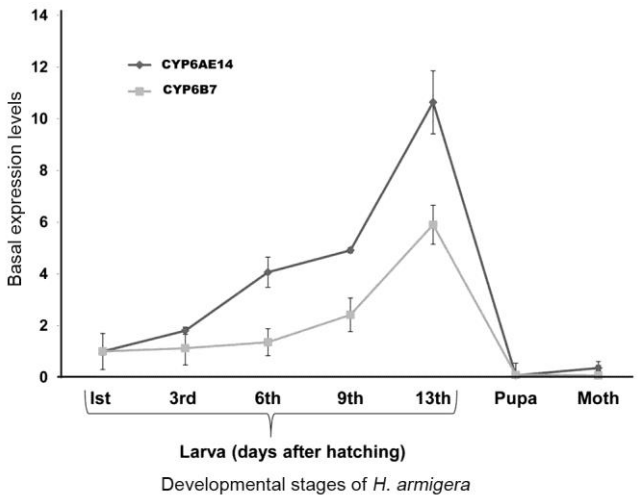

Figure 5. Basal expression levels of *CYP6B7* and *CYP6AE14* genes across various developmental stages of *Helicoverpa armigera*. Expression levels of the above genes in the above mentioned day old larvae and three-day-old pupa and moth. Error bars indicate standard error of the three biological replicates.

gossypol was able to induce the expression of *CYP6AE14* in *H. armigera*[17]. Tomatine markedly induced the expression of *CYP6B6* and *CYP6B7* isoforms, while the expression of other isoforms remained largely unaltered, except *CYP6AE14* which was induced only in the highest concentration of tomatine treatment. The expression levels of *CYP6B7* and *CYP6B6* correlated with the concentration of tomatine. Xanthotoxin strongly induced the expression of *CYP6AE14* and *CYP6B7*, whereas the other isoforms were overexpressed marginally. Interestingly, mid-concentrations of xanthotoxin induced higher expression of P450s compared to lower and higher concentrations. The highest overexpression (ten fold) was exhibited by *CYP6AE14* in 0.25 μg/g xanthotoxin treatment. Therefore, comparison of the expression levels of allelochemicals-induced P450s revealed that frequently to moderately encountered (gossypol and tomatine) allelochemicals more efficiently induced the expression of P450s compared to occasionally encountered (xanthotoxin) allelochemicals. Overall, *CYP6AE14* and *CYP6B7* were induced by all three allelochemicals.

Further, to understand the expression pattern of *CYP6AE14* and *CYP6B7* during different developmental stages of the insect, we performed basal expression analysis of these isoforms in various larval instars, pupa and adult stages of *H. armigera* fed on a control diet. The analysis revealed that these isoforms were expressed only in the larval stage compared to pupa and moth stages. Within the larval stages, 13-day-old (approximately corresponding to fifth instar) larvae had relatively higher transcript levels of these P450s.

Higher concentrations (0.5 and 1.0 μg/g) of allelochemicals led to larval growth retardation, despite higher expression levels of P450s. Notably, severe larval growth retardation was evident in tomatine treatment and the highest reduction (60%) in the larval weight was observed in 1 μg/g treatment compared to other allelochemicals (Figure 3). It is also evident that higher concentrations (0.5 and 1.0 μg/g) of allelochemicals eventually cause larval mortality. Notably, the highest mortality (53%) was recorded in tomatine treatment, whereas it was 23% and 20% in gossypol and xanthotoxin respectively, in case of 1 μg/g treatment. These results showed that *H. armigera* was able to tolerate relatively low concentrations of allelochemicals without affecting growth and development of the larvae.

Overall, *CYP6AE14* and *CYP6B7* were induced by all three allelochemicals (gossypol, tomatine and xanthotoxin). Therefore, these P450s may contribute to the adaptation of *H. armigera* to gossypol (cotton), tomatine (tomato) and xanthotoxin (Apiaceae and Rutaceae family). The results of this study would help in designing effective pest management strategies by attempting to reduce the natural fitness of this pest to feed on the respective host plants. In this regard, these P450s may be suitable target genes for RNA interference-mediated management of *H. armigera*.

Conflict of interest: Authors declare that there is no potential conflict of interest.

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