Genome mining and functional analysis of cytochrome P450 genes involved in insecticide resistance in *Leucinodes orbonalis* (Lepidoptera: Crambidae)

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Abstract

Genome-wide analysis of cytochrome P450 monooxygenase (CYP) genes from the advanced genome project of the *Leucinodes orbonalis* and the expression analysis provided significant information about the metabolism-mediated insecticide resistance. A total of 72 putative CYP genes were identified from the genome and transcriptome of *L. orbonalis*. The genes were classified under 30 families and 46 subfamilies based on the standard nomenclature. In the present study, a novel CYP gene, *CYP324F1*, was identified and it has not been reported from any other living system so far. Biochemical assays showed enhanced titers (5.81–18.5-fold) of O-demethylase of CYP in five field-collected populations. We selected 34 homologous CYP gene sequences, seemed to be involved in insecticide resistance for primer design and quantitative real-time PCR studies. Among the many overexpressed genes (>10 fold), the expression levels of *CYP324F1* and C*YP306A1* were prominent across all the field populations as compared with the susceptible iso-female line. Oral delivery of ds-*CYP324F1* and ds-C*YP306A1* directed against *CYP324F1* and C*YP306A1* to the larvae of one of the insecticide resistance populations caused reduced expression of these two transcripts in a dose-dependent manner (53.4%–85.0%). It appears that the increased titer of O-demethylase is the result of increased transcription level of CYP genes in resistant populations. The data provide insight for identifying the novel resistance management strategies against *L. orbonalis*. Volume 0, Number 0, Pages 1–12, 2020

Keywords: *Leucinodes orbonalis, insecticide resistance, genome, cytochrome P450, gene expression, RNAi*

1. Introduction

The ability of the insect pests to develop resistance against multiple insecticidal molecules hampered the insect control program [1]. The eggplant shoot and fruit borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae), a monophagous insect pest, defy all the chemical control measures due to the development of insecticide resistance [2, 3]. Repeated and indiscriminate application of many chemicals also fostered several environmental and health concerns, including disruption of natural biological control systems [4, 5, 6].

Pesticide detoxification often occurs when toxins are modified into nontoxic compounds through reduction, oxidation, conjugation reactions, and enhanced excretion of the altered toxin molecules. The metabolic detoxification in insects generally involves three main groups of enzymes acting in three phases (I–III) against a number of insecticides [7, 8]. Among

Abbreviations: *cDNA, complementary DNA; CYP, cytochrome P450 monooxygenase; dsRNA, double-stranded RNA; Lo-S,* Leucinodes orbonalis*-susceptible; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference.*

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Highlights

- Enhanced activity of cytochrome P450 monooxygenase (CYP) in the midgut of *Leucinodes orbonalis*.
- Genome and transcriptome mining of *L. orbonalis* yielded 72 full-length putative CYP gene sequences.
- A novel CYP gene, *CYP324F1*, was identified.
- Overexpression of numerous CYP genes in field collected populations of *L. orbonalis*.
- Silencing of *CYP324F1* and *CYP306A1* resulted in reduced transcription activities in a dose-dependent manner.

all, the phase I reaction by cytochrome P450 monooxygenases (CYPs) constitutes the largest gene superfamilies in all living organisms and they are known to perform a large number of highly diverse physiological and biochemical functions [7, 8, 9]. In insects, more than 1,700 CYP genes have so far been identified and an insect genome normally contain from 10 to >100 CYP genes [7]. CYPs have long been of particular interest because they are critical for detoxification and/or activation of xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens, and mutagens. They are also involved in metabolizing endogenous compounds such as hormones, fatty acids, and steroids [8, 9, 10]. The insect CYPs can be grouped into four major phylogenetic clans *viz*., CYP2, CYP3, CYP4, and the mitochondrial [11, 12]. The CYP2 clan consists of CYP15, CYP18, CYP303–CYP307, CYP343, CYP359, and CYP369 families [8]. CYP306A1 is specifically involved in ecdysteroid biosynthesis in the prothoracic glands of *Bombyx mori* and *Drosophila melanogaster* [13]. Similarly, CYP3 clan is further subdivided into many families *viz*., CYP6, CYP9, CYP28, CYP308–310, CYP317, CYP321, CYP324, CYP329, CYP332, CYP336–CYP338, and CYP345–CYP348 [14]. Among all, the members of CYP324 family are involved in detoxification of xenobiotics, in turn associated with insecticide resistance mechanism [9, 12]. The basal and upregulation of CYP gene expression can significantly affect the disposition of xenobiotics or endogenous compounds in the tissues of organisms and thus alter their pharmacological/toxicological effects [15, 16]. It has been suggested that the induction of CYP and their activities in insects help them in adapting to changing environment and to cope with toxic effect of insecticides [17].

The problem, together with the growing incidence of insect resistance, has called attention to the need for developing novel insecticides or management of insecticide resistance [18]. Hence, there is a quick need for more realistic and sustainable tool for insect pest control such as gene silencing. The RNA interference (RNAi) is one such method to develop target-specific insect control [19, 20]. This technique could silence the gene expression in nonmodel organisms, where the genetic information is scanty [21].

In this study, we examined the CYP genes and their potential role in detoxification of insecticides in field-collected insecticide-resistant populations of *L. orbonalis* for the first time. The quantitative real-time PCR (qRT-PCR) technique was used to assess the expression pattern of CYP genes in late second instar larvae. The functional validation of the highly expressed *CYP324F1* and *CYP306A1* genes was done by double-stranded RNA (dsRNA) feeding experiments.

2. Materials and Methods

2.1. Insecticide resistance and estimation of O-demethylase activity of CYPCYP

The base-line susceptibility data generated by the senior author to know the insecticide resistance status in different field collected populations of *L. orbonalis* was taken as a base for conducting biochemical assays and gene expression analysis. There was a large shift in the LC_{50} values against fenvalerate, phosalone, thiodicarb, emamectin benzoate, flubendiamide, and chlorantraniliprole, ranged from 1.6 to 534.6 in the field-collected populations of *L. orbonalis* over the laboratory-reared susceptible iso-female line (Lo-S) [22].

Twenty-five midguts from the starved 2nd instar larvae of *L. orobnalis* were used to prepare the microsomal fraction as per the standard procedure [23]. The O-demethylase activity, a marker enzyme for measuring the CYPs, was determined spectrophotometrically by analyzing the production of p-nitrophenol [24]. The 700 μL final reaction mixture in 50 mM phosphate buffer (pH 6.8) composed of 10 μg of midgut microsomal fraction, 2.9 mM p-nitroanisole (dissolved in dimethyl sulfoxide) and 3.3 mM nicotinamide adenine dinucleotide phosphate. The mixture was incubated for 60 Min at 30 °C. To this, 700 μL ice-cold acetone and 0.2 mL of 0.5 M glycine–NaOH buffer (pH 9.5) were added to stop the reaction. The supernatant after centrifugation was used to measure the absorbance at 410 nm. The enzyme activity was determined using p-nitrophenol standard curve [23].

2.2. Bioinformatics and naming of CYP genes

The CYP hmm model (CYP-PF00067) from the PFAM database was used to search *L. orbonalis* predicted protein sequences using the hmmsearch program from the HMMER3 software package. The CDHIT and CLUSTAL OMEGAR® were used to remove the redundancy and duplication. From the total (72) putative CYP genes, 34 were selected based on their known history of involvement in insecticide resistance in other insects based on BlastX analysis (NCBI) with 90% query coverage. The selected sequences were used for designing primers by Primer 3.0 plus software (Table 1). To remove the primer-dimer and confirm the efficiency, the OligoEvaluator™ (www.oligoevaluator.com) sequence analysis tool was used. Naming of all the putative CYP genes of *L. orbonalis* was performed by Dr. David R. Nelson, University of Tennessee Memphis, USA.

2.3. RNA isolation from *L. orbonalis* larvae

The field populations of *L. orbonalis* larvae were collected from intensive eggplant growing regions of India *viz*., Raichur

TABLE 1 *Primer details for amplification of cytochrome P450 genes of* **L. orbonalis**

Sl number	*Name of the gene*	*Primer sequence 5′ → 3′*	*Length*	*GC (%)*	T_m *(°C)*	*Sequence length*	*Product size*	
1.	CYP337B22	F	TTGATTTGTGTGTGGAACTGC	21	42.86	59.61	1,318	79
		R	TCGTCCGTAGGTTGTATCTGG	21	52.38	60.0		
2.	CYP6AE	F	GAGCCTTTTACGGGACAGAAC	21	52.38	60.12	852	57
		R	GTTTGATGATTTCGGGGTCTT	21	42.86	60.18		
3.	CYP6AE132	F	TTACACGCTGCCTACTGGTCT	21	52.38	59.95	952	103
		R	CTGCTCTGGGTTTGGGAAGT	20	55.0	61.98		
4.	CYP305B1	F	TCCGTTGGCGATATACACTTC	21	47.62	59.97	171	96
		R	ATTTTTCAATGCTCCATCGTC	21	38.12	59.04		
5.	CYP324F1	F	GCATCTGGCTGTTCTGGAG	19	57.89	59.52	207	50
		R	AATCTCTGGGGAGTTGACGA	20	50.0	59.62		
6.	CYP4G204	F	TCCACCAGTTCCTGTCATAGC	21	52.38	60.13	1,299	110
		R	GTATCCTGTAGGTGCCGATCA	21	52.38	59.97		
7.	CYP306A1	F	GGGTGATGAAGACGTTGACAG	21	52.38	60.56	1,155	88
		R	AACAGGGACGATAGACCGAAT	21	47.62	59.84		
8.	CYP4M67	F	GCCGTTGCTGCTATTTTAATG	21	42.86	59.77	774	101
		R	ACAGGTCCATTGTTACGCATC	21	47.62	59.87		
9.	CYP6AE132	F	CAACGTCAAAAATGGGAAGAA	21	38.10	59.96	1,060	74
		R	CCAAGCTCAAACCAACCAATA	21	42.86	59.98		
10.	CYP9A140	F	AATTGCTCAAGCCCTCATTTT	21	38.10	60.09	338	118
		R	TTCTTCAACCAGACGATCCTG	21	47.62	60.24		
11.	CYP4CG22	F	CCAGCGTTTCACTTCAACATC	21	47.62	60.68	1122	92
		R	CCGACTTCCTCCTGTATCCTC	21	57.14	60.08		
12.	CYP4M69	F	TGCCTCATGTTGTTAGCGAAT	21	42.86	60.65	748	79
		R	GGGAATCACCAAATACCTCGT	21	42.62	60.07		
13.	CYP6CT1	F	AACAAAGCCAAACTCCATCCT	21	42.86	59.99	209	64
		R	CGAGACATTTCCTTGGTCCTT	21	47.62	60.48		
14.	CYP6AB	F	TGGGCACCTTATGAGAGTTTG	21	47.62	60.12	694	82
		R	CATGGCTCTAACACCAGCATT	21	47.62	60.15		
15.	CYP4AU	F	TGGAATGTTAGCGACGAAAAG	21	42.86	60.25	522	77
		R	TGCGTTTGATTGGTTCTGATT	21	38.10	60.1		
16.	CYP4L49_Lo	F	CTCTTCGGTTGTACCCTTCG	20	55.0	59.73	352	71
		R	CCATCCTGCTATATCCGTGTC	21	52.38	59.42		

(*Continued*)

TABLE 1 *Continued*

Sl number	*Name of the gene*	*Primer sequence 5′ → 3′*	*Length*	*GC (%)*	T_m *(°C)*	*Sequence length*	*Product size*	
17.	CYP9A142 (1aa different)	F	TCGACGTAAAAGACCTGACCA	21	47.62	60.67	336	59
		R	AAAAGCGCATGTTGCTATGAC	21	47.86	60.29		
18.	CYP9A142 (1aa different)	F	CTGCATGAGCTTGCCATAAAT	21	42.86	60.24	222	84
		R	CTTGCCGTCCTTCTTTTCTTC	21	47.62	60.36		
19.	CYP6AB149	F	ACGTTAGTTGCTGCTCTCGAA	21	47.62	60.20	1,155	116
		R	TCATTTAGGGAATCTGCATCG	21	42.86	60.05		
20.	CYP367B1 (match with gap)	F	CCCTGAAAACACAAAGATCCA	21	42.86	60.12	1,354	119
		R	AGAACCTCCCTAAGCAATCCA	21	47.62	59.84		
21.	CYP6CT1 (another contig)	F	CCGAAGATGCACCAAAAACTA	21	52.38	60.1	207	88
		R	GATGGACTGGCACGGTATAAA	21	55.0	60.20		
22.	CYP324F1	F	TACGTGGGTATCTGGCTGTTC	21	42.86	59.44	376	57
		R	GATCTCTGGGGAGTTGACGA	20	55	59.87		
23.	CYP367A1	F	ACATTTACCAGAATGGCAGGA	21	47.62	59.95	611	107
		R	CCATATCAAGGTGGTGCAGTT	21	52.38	59.92		
24.	CYP9A141	F	TGGATATGGTGGTTTCAGAGC	21	52.38	60.0	631	111
		R	CCTCAGTAGCTTTGTCGTTGG	21	47.62	59.38		
25.	CYP337B22	F	CCAGATACAACCTACGGACGA	21	47.62	59.72	252	62
		R	CGCCAGCTACAAAGAAGAAGA	21	42.86	60.96		
26.	CYP354A23	F	GAGCGTTTTATGGACGATGAG	21	42.86	60.21	801	63
		R	AACACCGAAAGCGAGGAATAA	21	52.38	60.10		
27.	CYP304F24	F	GGAAATTGTGAAGGACGGTTT	21	42.86	60.24	1,207	118
		R	TAGGACGTGATAGTCGGATCG	21	42.86	60.25		
28.	CYP6AB150	F	GCACTTCAGATGGACGAAAAA	21	47.62	59.87	216	79
		R	CATCCTTAGCACCAAAGCAAA	21	36.36	59.30		
29.	CYP354A23 (another contig)	F	ATTTGGACGACGACACCATAG	21	38.10	58.16	238	120
		R	TTTGCATTTCAGGTTTTGTAGC	21	47.62	59.83		
30.	CYP367B1	F	GTCAAGAAGCATCAGCAAAAA	21	42.86	60.91	309	60
		R	CTGGGTGGTAAGCCATCATTA	21	47.60	60.08		

(*Continued*)

TABLE 1 *Continued*

Sl number	*Name of the gene*	*Primer sequence 5′ → 3′*	*Length*	*GC (%)*	T_m *(°C)*	*Sequence length*	*Product size*	
31.	CYP367B1	F	CGATTCAACCCTGAAAACACA	21	42.86	59.87	492	88
		R	AGAACCTCCCTAAGCAATCCA	21	52.38	60.32		
32.	CYP9A80	F	ACATTCGCAAAGGAGAAGTCA	21	52.38	59.72	1,343	66
		R	GATATAGCTCGGGATCGTGGT	21	42.86	60.05		
33.	CYP9A142	F	GAGTCCAGCATTCACCAGTTC	21	42.86	60.10	472	105
		R	TCCGCCTCCTTGATTCTATTT	21	47.62	59.91		
34.	CYP337B22 (2aa different)	F	CCAGATACAACCTACGGACGA	21	52.38	60.00	748	62
		R	CGCCAGCTACAAAGAAGAAGA	21	47.62	59.78		

(16.2120°N, 77.3439°E), Dharmapuri (12.0933°N, 78.2020°E), Bhubaneswar (20.2961°N, 85.8245°E), Pune (18.5204°N, 73.8567°E), and Varanasi (25.3176°N, 82.9739°E). Insects were reared under laboratory conditions at 27 ± 2 °C, 60%–70% RH and photoperiod of 14:10 H (L:D) on natural diet and the F_1 individuals were used for isolation of RNA for gene expression studies. Iso-female line designated as Lo-S (National Accession number: NBAIR-IS-CRA-01a) derived from Bengaluru population (Karnataka, Bengaluru: 12.9716°N, 77.5946°E), being maintained since October 2012 at Insect Genomic Resources Laboratory of ICAR-NBAIR, Bengaluru was used as susceptible control.

Total RNA was isolated from late second instar larvae using ISOLATE II RNA mini kit by following the manufacturer guidelines (Bioline, Taunton, Massachusetts, USA). To remove the DNA contamination, it was treated with RNase free DNase I (Fermentas Life Sciences, Waltham, Massachusetts, USA) and purified through Phenol-Chloroform (25:25) extraction [25]. The purity and concentration of total RNA was measured using spectrophotometer (NanoDrop Lite-1000; Thermo Scientific, Vilnius, Lithuania, Germany) and denatured agarose gel [26].

2.4. cDNA synthesis

RNA samples with an A260/A280 ratio ranging from 1.8 to 2.0 and A260/A230 ratio >2.0 were used for the preparation of complementary DNA (cDNA). The first strand of cDNA was synthesized from 4 μg of total RNA along with Oligo $(dT)_{18}$ primer and nuclease-free water using Revert-AID first-strand cDNA synthesis kit (Thermo Scientific™, Vilnius, Lithuania, Germany) following the supplier's guidelines and stored at −80 °C for future use.

2.5. qRT-PCR studies

The synthesized cDNA was diluted 10 times prior to using it for qRT-PCR. The relative expression of target gene was studied using qRT-PCR with 20 μL reaction consisted of 10 μL 2× SYBR® Premix EX Taq™ II (Tli RNaseH Plus; TAKARA®, Nojihigashi, Shiga, Japan), 1 μL cDNA, 1 μL of gene-specific primer each (forward and reverse), and make up the volume by nuclease-free water. The thermal condition used for qRT-PCR as follows: one cycle of 95 °C for 5 Min; 35 cycles of 95 °C for 30 Sec, 53 °C for 45 Sec, and 72 °C for 1 Min; a final cycle of 72 °C for 10 Min using Light Cycler 480II (Roche Applied Science, Basel, Switzerland). Each sample including control (no template) and internal control was performed in triplicates. The resultant PCR products were electrophoresed on 1.5% agarose gel in a 1.0× TAE buffer. Relative expression levels for the CYP genes were calculated by the $2^{-\Delta\Delta CT}$ method [27]. The *28SR3* (28S ribosomal protein S3 mitochondrial) gene was used as an internal control to normalize the expression of target genes [28]. Procedure and protocol followed was amenable with Minimum Information for Publication of Quantitative Real-Time PCR (MIQE) [29].

2.6. Cloning and synthesis of dsRNA of target genes

The coding sequences of *CYP324F1* and *CYP306A1* were amplified using first-strand cDNA (1:10 diluted) as a template with PCR master mix of 25 μL composing 10× Taq buffer, 2.5 mM dNTPs, 10 picomoles of forward and reverse primers (*CYP324F1*-F, *CYP324F1*-R and *CYP306A1*-F, *CYP306A1*-R) for corresponding gene (Table 2), 1 unit of Taq DNA polymerase (TaKaRa, Nojihigashi, Shiga, Japan) and nuclease-free water. PCR was performed in a thermal cycler (AB-Applied Bio Systems, Waltham, Massachusetts, USA) with parameters used as follows: initial denaturation at 94 °C for 5 Min, 35 cycles of 94 °C for 35 Sec; annealing for 30 Sec (*CYP324F1*—58.35 °C and *CYP306A1*—61.75 °C); extension at 72 °C for 1 Min, and final cycle of 72 °C for 10 Min with a no template control. PCR amplicons were eluted from 1.5% agarose gel using NucleoSpin Extract II (Macherey-Nagel, GmbH & Co. KG, Düren,

TABLE 2 *Primer details for upregulated genes, internal target/positive control genes used in dsRNA experiments*

Sl number	*Name*	*Primer sequence 5′ → 3′*	*Length*	*GC (%)*	T_m *(°C)*	*Sequence length*	*Product size*	
qRT-PCR Primers								
1.	*CYP324F1*	F	GCATCTGGCTGTTCTGGAG	19	57.89	59.52	207	50
		R	AATCTCTGGGGAGTTGACGA	20	50.0	59.62		
2.	*CYP306A1*	F	GGGTGATGAAGACGTTGACAG	21	52.38	60.56	1,155	88
		R	AACAGGGACGATAGACCGAAT	21	47.62	59.84		
3.	*LacZ*	F	ACAATTTCCATTCGCCATTCA	21	38.10	58.3	534	–
		R	ATGACCATGATTACGCCA	18	44.44	55.3		
4.	*28SR3*	F	CAAAACTGGATGTGTGGGAGT	21	47.62	60.1	224	71
		R	GTTAGCTGGTGGTTGCAGTGT	21	52.38	62.6		
dsRNA Primers								
5.	*CYP324F1*	ds-F	TAATACGACTCACTATAGGG AGAGAATTTGGATGCGCAAGCTG	43	44.6	72.8	207	203
		ds-R	TAATACGACTCACTATAGGG AGAAACAGTGAACAAGTTTAGTCC	44	40.48	69.5		
6.	*CYP306A1*	ds-F	TAATACGACTCACTATAGGG AGACTGGTGCTGCGGTTGACTTG	43	48.84	74.0	1,155	515
		ds-R	TAATACGACTCACTATAGGG AGAACGTGAGGATTCCTCGGCCT	43	48.384	72.2		
7.	*LacZ*	ds-F	TAATACGACTCACTATAGGG ACAATTTCCATTCGCCATTCA	41	39.02	70.0	534	452
		ds-R	TAATACGACTCACTATAGGG ATGACCATGATTACGCCA	38	42.11	69.5		

Germany), then ligated into TA cloning vector (pTZ57R/T) and transformed into DH5α strain (*Escherichia coli*) by following the manufacturer guidelines (Fermentas, St. Leon-Rot, Germany). Subsequent blue-white colony screening, the plasmids were isolated from the positive clones after overnight incubation using GenJETTM Plasmid MiniPrep kit (Fermentas, GmbH) and were confirmed from 1.5% agarose gel.

A unique dsRNA region of two target genes (*CYP324F1* and *CYP306A1*) was selected to minimize the off-target effect from the draft genome and sequence-specific primers were designed using Primer 3.0 Plus software and tailed with T_7 promoter region at 5' end. dsRNA template was synthesized by sequence-specific primer (ds-*CYP324F1* and ds-*CYP306A1*) along with the PCR master mix and cycling conditions as explained earlier. The PCR reaction volume was made up to 50 μL, the DNA template used in that was candidate CYP genes containing EcR plasmid clones (diluted 1:500 times, ~100 ng), with respective primer annealing temperature. The amplified product was resolved using 1.5% agarose gel. The DNA was eluted using NucleoSpin® Extract II (Macherey-Nagel, GmbH) and used as template (1 μg) for dsRNA synthesis with MEGAScript RNAi kit (Life Technologies, Darmstadt, Germany) following manufacturers guidelines. The quantification and integration of dsRNA were done by NanoDrop Lite-1000 (Thermo Scientific, Vilnius, Lithuania, Germany) and 1.5% agarose gel, respectively. The off-target control dsRNA, which is specific to bacterial *LacZ*, was synthesized using the similar protocol [30].

2.7. Oral delivery of dsRNA

Eggplant flower bud bioassay technique was employed for the oral delivery of dsRNA. An unopened flower bud (~1.5 cm) with slant cut tip was rinsed in 0.1% Triton X-100 (Sigma–Aldrich, St. Louis, Missouri, USA) and then washed twice in double distilled water. The stalks of the buds after shade drying were

TABLE 3 *Mixed function oxidase activity in midgut of* L. orbonalis

L. orbonalis *population source*	*GPS coordinates of the location collected*	*O-demethylase activity ug//min/ mg protein*	*Fold variation*
Raichur	16.2120°N, 77.3439°E	1341.6 ± 23.5	18.5
Dharmapuri	12.0933°N, 78.2020°E	873.6 ± 14.2	12.04
Pune	18.5204°N, 73.8567°E	396.2 ± 13.5	5.46
Varanasi	25.3176°N, 82.9739°E	421.8 ± 11.8	5.81
Bhubaneswar	20.2961°N, 85.8245°E	944.6 ± 26.6	13.02
Bangalore (Lo-R)	13.0358°N, 77.5970°E	72.5 ± 3.9	–

placed in 500 μL PCR tube containing known concentration of dsRNA. This technique allowed the entire flower bud contaminated with dsRNA. The 100 μL dsRNA solutions of 1, 0.5, 0.25, 0.1, and 0.05 $\mu g/\mu L$ were filled in the individual tubes. Freshly molted and starved second instar larva of insecticide-resistant Dharmapuri population was cautiously transferred to the treated buds individually under three replications. The bioassay procedure also included negative control (nuclease-free water) and positive off-target control (*LacZ*-dsRNA). The larval mortality was taken at 24, 48, 72, and 96 H.

2.8. qRT-PCR to measure gene knockdown

The silencing of target gene was examined by performing qRT-PCR. Total RNA from each treatment was isolated at 48 and 72 H of feeding from the surviving larvae. RNA extraction and cDNA synthesis were done as above. The 20 μL reaction composed of 10 μL 2× SYBR® Premix EX Taq™ II (Tli RNaseH Plus; TAKARA®, Nojihigashi, Shiga, Japan), 10 mM of each primers (qRT-*CYP324F1* and qRT-*CYP306A1*) (Table 2), and 2 μL of diluted cDNA (1 ng/μL) as a template. The *28SR3* was used as a reference gene [28]. The final volume was made up with adding nuclease-free water. For each target gene, no template control and off-target control under three technical replicates were performed. The qRT-PCR analysis was conducted by following cycles *viz*. 95 °C for 5 Min (denaturation); 40 cycles of 95 °C for 30 Sec and 60 °C for 1 Min for primer annealing in a Light Cycler 480 II (Roche Applied Science). The relative expression of the silenced target genes was calculated by $2^{-\Delta\Delta ct}$ method [27].

2.9. Statistical analysis

The data presented in the current study were analyzed using the IBM-SPSS 24.0 software package [31]. For dsRNA treatment, Student's *t*-test was used with significant at $P < 0.05$ statistical differences.

3. Results

3.1. Activities of detoxification enzymes

Differences in the titer of O-demethylase activity were analyzed using p-nitroanisole as a substrate. Significantly elevated activities (5.46–18.5-fold) were noted in the field collected populations over the susceptible Lo-S population of *L. orbonalis* (Table 3). Field populations collected from Raichur, Bhubaneswar, and Dharmapuri showed 18.5-, 13.0-, and 12.0-fold increased enzyme activity.

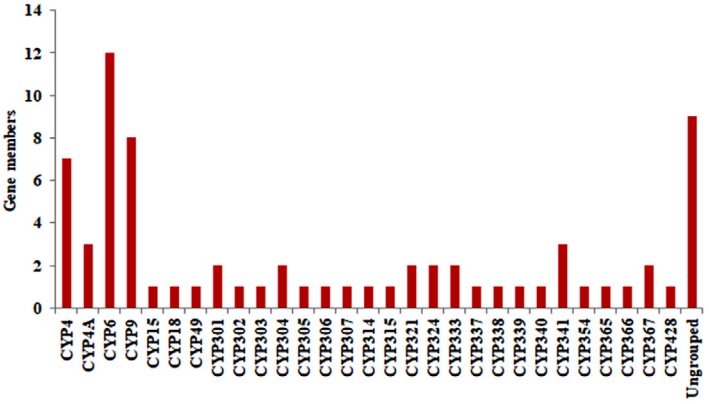

FIG. 1 *Distribution of CYP subfamilies under different families of CYP450.*

3.2. Bioinformatics and gene identification

Field populations of *L. orbonalis* were analyzed to see the expression pattern of CYP genes and compared with insecticide susceptible iso-female line. Through genome and transcriptome mining, 147 putative CYP genes were identified. The number of putative CYPs reduced from 147 to 72 after the removal of redundancy and repetition by CD-HIT and CLUSTAL OMEGA®. The CYP sequences were classified under Clan2 (eight), Clan3 (27), Clan4 (20), Mitochondrial CYP clan (10), and ungrouped (seven). Further, the genes were subdivided into 30 families and 46 subfamilies (Fig. 1). CYP6 clan has the highest number of CYP genes (nine) followed by CYP9 (eight). Eighteen families were represented by single gene. The gene sequences used in the present experiments for designing primers and performing expression analysis ranged from 171 to 1,354 bp. Thirty-four

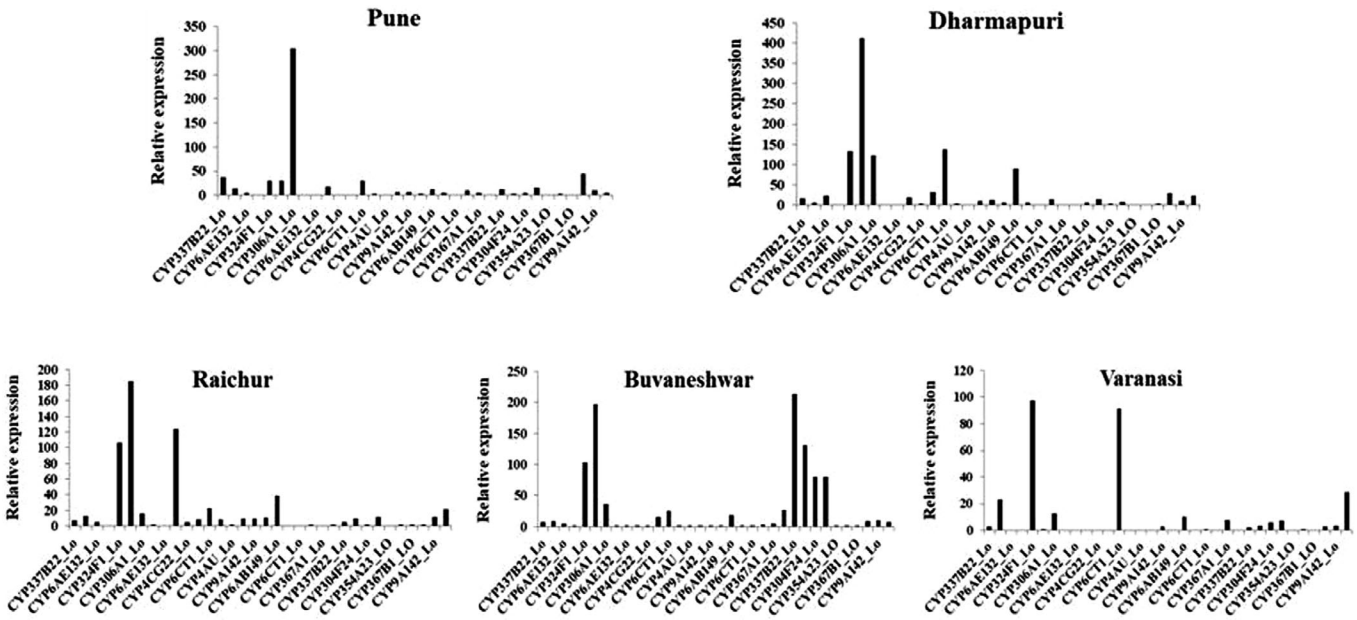

FIG. 2 *Transcription profiling of cytochrome P450 genes in field collected populations of* L. orbonalis *depicted as fold change over the susceptible Lo-S colony. (A) Pune, (B) Dharmapuri, (C) Raichur, (D) Bhubaneswar, and (E) Varanasi.*

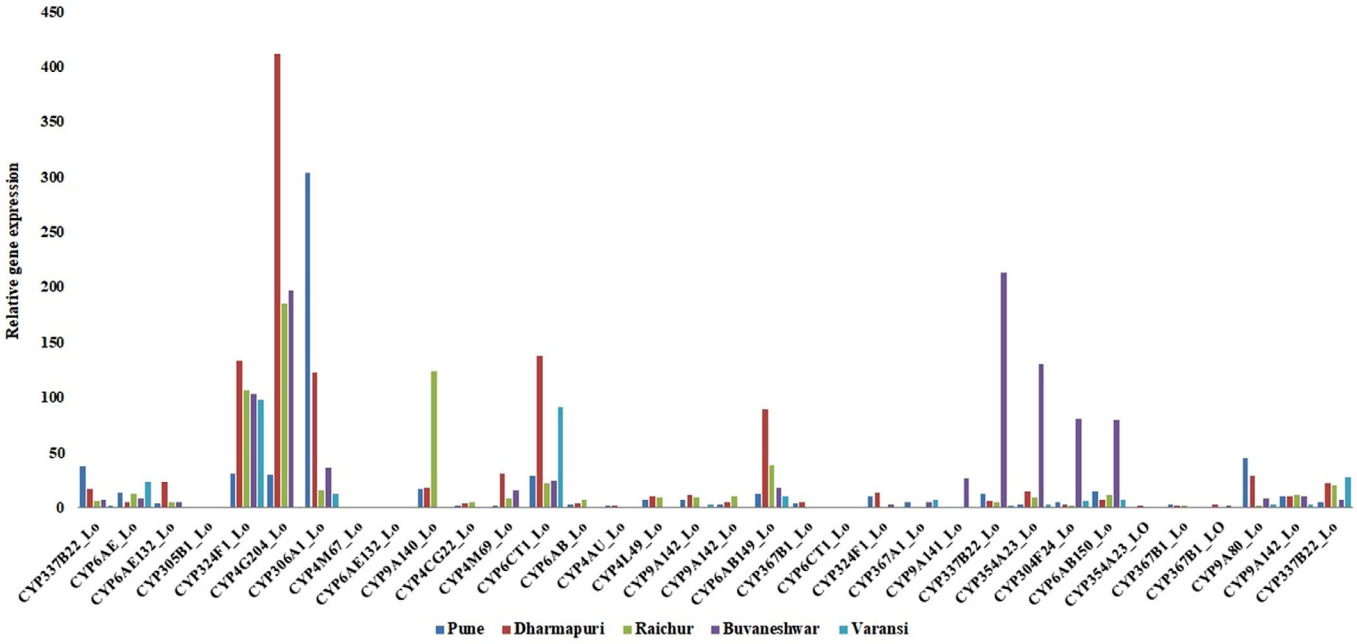

FIG. 3 *Differential expression of CYP450 genes in five field collected populations of* L. orbonalis *over susceptible populations.*

unigenes relating to insecticide resistance were identified based on BlastX search for genes from the closely related species in the NCBI database (having >90% identity). Of these, 18 belonged to CPY3 clan (mainly involved in metabolism of xenobiotics) and 10 belonged to CYP4 clan (having role in xenobiotic metabolism and physiological function).

3.3. Differentially expression of cytochrome P450 genes

Quantitative expression analysis was performed for 34 putative CYP genes using total RNA extracted from the late second instar larvae of five field-collected *L. orbonalis* populations along with susceptible Lo-S. Tenfold changes in the expression of four genes (*CYP6AB149*, *CYP6CT1*, *CYP306A1*, and *CYP324F1*) were

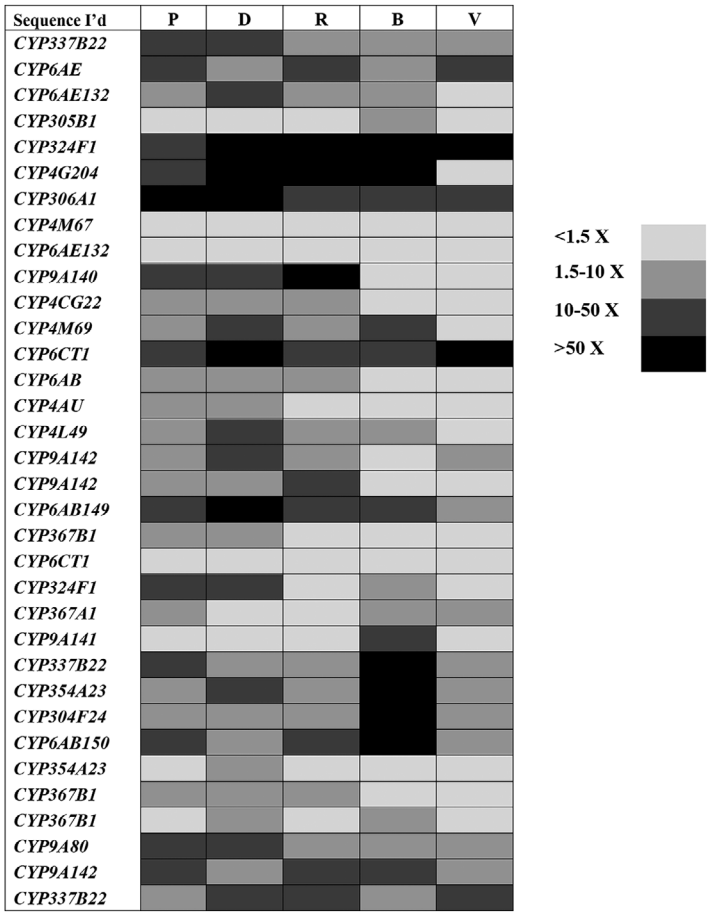

FIG. 4 *Expression profiles (fold changes over Lo-S population) of cytochrome P450 genes across field collected* Leucinodes orbonalis *populations using heat map (P, Pune; D, Dharmapuri; R, Raichur; B, Bhubaneswar; V, Varanasi). The fold changes are indicated in different shades indicate significant difference as per Mann–Whitney* U-*test* P *value* < 0.05.

observed over the cut-off value (Figs. 2 and 3). *CYP306A1* and *CYP324F1* expressed most abstemiously. Overexpression of *CYP306A1* (304.1-, 122.7-, 16.3-, 36.7-, and 12.6-fold) and *CYP324F1* (31.2-, 133.3-, 106.8-, 103.4-, and 97.8-fold) were observed in all the field collected *L. orbonalis* populations (Figs. 3 and 4).

3.4. Target gene silencing

The oral delivery of dsRNA (ds-*CYP306A1* and ds-*CYP324F1*) specific to *CYP306A1* and *CYP324F1* using five different concentrations *viz*., 1, 0.5, 0.25, 0.1, and 0.05 μg/μL caused dose-dependent larval mortality that ranged from 5.7% to 85.0% (Figs. 5A and 5B). There was no difference in the relative expression of target genes in the positive nontarget (ds-*LacZ*) and untreated control (Fig. 5).

Post-treatment observation of ds-*CYP324F1* resulted in 20.3% mortality with 1 μg/μL concentration at 96 H of feeding. Similarly, ingestion of ds-*CYP306A1* caused 33.4% mortality at 1 μg/μL concentration (Fig. 6). There was no significant mortality in case of off-target control (ds-*LacZ*) as well as untreated in control experiments (Figs. 5A and 5B). The molecular confirmation revealed that there was a complete silencing of the target genes after ingestion of ds-*CYP324F1* and ds-*CYP306A1* by the early second instar larvae over both the control populations after RNA extracted from dead larva (Figs. S1A, S1B, and S1C).

4. Discussion

L. orbonalis is the key pest of eggplant and one of the difficulties to control insect pests. Many field populations have developed multiple insecticide resistance and defy insecticidal treatments [32]. In insects, the metabolic resistance appears due to the quantitative mechanism *viz*., change in the level of production of transcripts [33, 34]. The increased production of O-demethylase, the marker enzyme for measuring the activity of CYP (5.46–18.5-fold), indicated the involvement of CYP genes in insecticide resistance in field-collected *L. orbonalis* populations. CYP-mediated insecticide resistance is not uncommon in insect pests [33].

Seventy-two putative CYP genes were identified and named from the genome and transcriptome dataset of *L. orbonalis* and classified under different clans. Similar to *L. orbonalis*, 90 CYP genes were reported from *D. melanogaster* [35], 111 from *Anopheles gambiae* [36], 48 from *Apis mellifera* [37], and 143 from *Tribolium castaneum* [38]. The number of CYP genes in *L. orbonalis* from the present study was also comparable to many other lepidopteran insects. Total of 84, 90, 72, and 36 CYP genes were identified from *B. mori*, *Plutella xylostella*, *Chilo suppressalis*, and *Cnaphalocrocis medinalis* [39, 40, 41,42].

CYP2 clan has diverse families *viz*., CYP15C, CYP18A, CYP 303A, CYP305A, CYP306A, and CYP307A. The genes *CYP306A1* and *CYP307A1* of CYP2 clan and *CYP314A1, CYP315A1*, and *CYP302A1* of mitochondrial P450 clan participate in the ecdysteroid biosynthesis [43]. The *CYP306A1* is specifically involved in ecdysteroid biosynthesis in the prothoracic glands of *B. mori* and *D. melanogaster* [43, 44]. Similarly, identification of *CYP307A1* has revealed the Black Box reaction involved in synthesis of a new signal compound essential for ecdysteroid biosynthesis [45]. *CYP306A1* gene was abstemiously expressed in all the five populations of the *L. orbonalis* (Figs. 2, 3, and 4).

The largest CYP3 clan consists of CYP6, CYP9, CYP28, CYP308-310, CYP17, CYP21, CYP324 families and CYP 395–400 families [9]. The family CYP6 (11) and CYP9 (9) constitute 27.77% of all the CYP genes in *L. orbonalis*. In the present study, *CYP324F1* was expressed uniformly with minimum of 10-fold changes over control in all the population (Figs. 2, 3, and 4). Similarly, *CYP6Bs* was involved in detoxifying diverse plant allelochemicals in various insects [46, 47, 48, 49]. *CYP6A2* and *CYP6G1* were reportedly involved in insecticides resistance in *D. melanogaster* [50, 51, 52, 53]. The overexpression of *CYP6AE14* and *CYP6B7* upon xanthotoxin and tomatine induction was

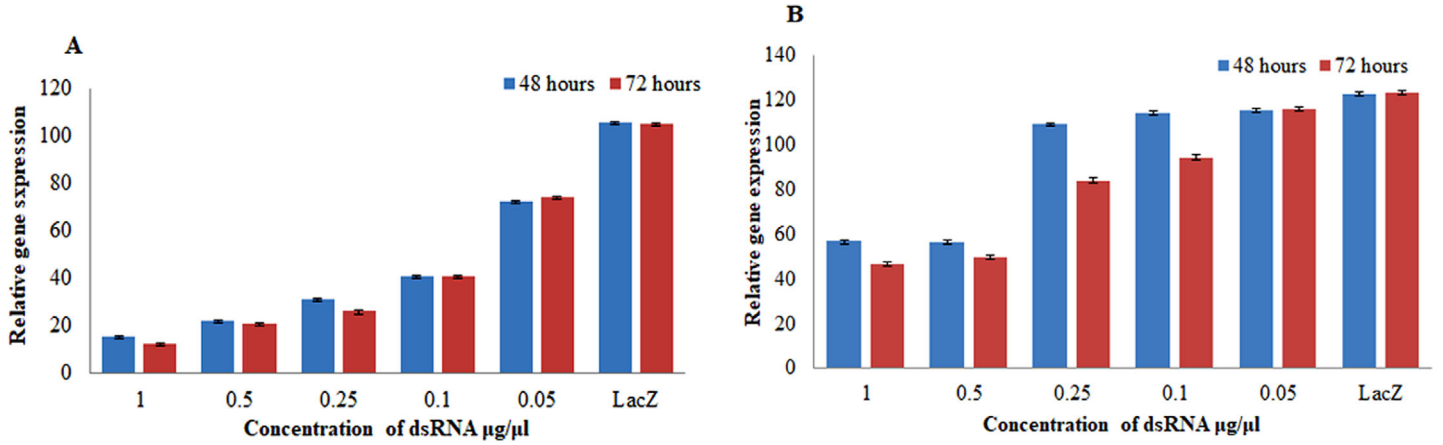

FIG. 5 *Relative expression of (A) CYP324F1 and (B) CYP306A1 after dsRNA treatment.*

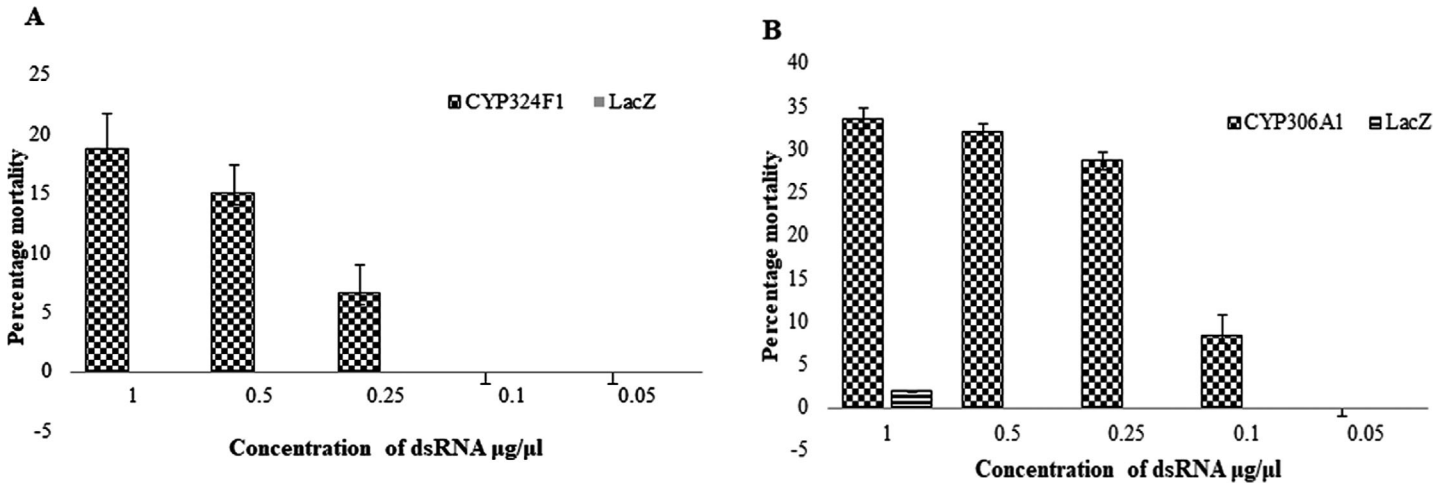

FIG. 6 *Percentage mortality of* L. orbonalis *after treating with dsRNA.*

observed in *Helicoverpa armigera* [54]. In the present study, a novel CYP gene, *CYP324F1* was identified from the genome of *L. orbonalis*. *CYP324F1* has not been reported from any other living system so far.

The RNAi efficiency varies among the insect species. A small amount of dsRNA induces a greater systemic response in *T. castaneum* and dipteran insects [21, 55, 56, 57, 58, 59, 60], whereas poor response was observed in case of lepidopteran insects [61, 62]. The ds-*CYP306F1* induced 33.4% larval mortality (Fig. 6B) with 5.7%–53.5% fold changes at different concentration of *L. orbonalis* (Figs. 5B and 6B). Similarly, silencing of *Lm-TSP* and *chitin synthase-1* genes induced the mortality in locusts [63, 64], *VTE* and *IAP* genes in *Acyrthosiphon pisum* [60]. The larval mortality due to ingestion of ds-*CYP324A1* was 20.28% and the reduction in gene expression was from 31.1% to 85% in a dose-dependent manner (Figs. 5A and 6A). Similarly, ingestion of ds-*CYP9A105* silenced the expression of *CYP9A105* and partly reversed the susceptibility of *Spodoptera exigua* larvae to the pyrethroid insecticides [62].

The partial silencing of two important CYP genes in the present study has the implications in possible restoration of susceptibility to insecticides. The possible role of CYP306A1 in ecdysterioid synthesis in the prothoracic gland of *L. orbonalis* also cannot be ruled out. The data obtained from the present study can be used for developing novel management tools to control the *L. orbonalis*.

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6. Conflict of Interest

The authors have no potential conflict of interest.

7. Authors Contributions

B.K. conducted the experiments and analysis, contributed in writing the hypothesis and manuscript, and worked on the materials and methods used for the study. P.A. contributed to the analysis, materials and methods, and corrections in the manuscript. R.A. carried out the analysis and technical suggestion, manuscript correction, and resource supply. G.R. performed the analysis, technical guidance and suggestions, and sorted out the reviews and literatures for this study. T.V. helped with the resources supply, technical guidance, and suggestions needed for carrying out this research. R.G.G. performed the analysis and provided technical guidance and suggestions. M.M. worked on designing the experiment, supplying resources, manuscript corrections, analysis, materials and methods, technical guidance, and suggestions.

8. References

[1] Ramkumar, G., and Shivakumar M. S. (2015) Parasitol. Res. 114, 2553–2560.
[2] Radhika, S., Reddy, K. D., and Subbarathnam, G. V. (1997) J. Res. 25(3), 16-14.
[3] Shirale, D., Patil, M., and Parimi, S. (2017) Can. Entomol. 00, 1–9.
[4] Yang, Y. C., Lee, S. G., Lee, H. K., Kim, M. K., Lee, S. H., and Lee, H. S. (2002) J. Agric. Food Chem. 50, 3765–3767.
[5] Lee, R.C., and Ambros, V. (2001) Science 294, 862–864.
[6] Kariyanna, B., Prabhuraj, A., Mohan, M., Bheemanna, M., Kalmath, B., Pampanna, Y., and Diwan, J. R. (2020). Plant Arch. 20(S2), 1255–1261.
[7] Nelson, D. R. 2009. Hum. Genomics 4, 59–65.
[8] Feyereisen, R., in Gilbert, L.I., Iatrou, K., Gill, S., Eds. (2005) Comprehensive Molecular Insect Science, vol. 4. pp. 1–77, Elsevier, Oxford.
[9] Feyereisen, R. (2011) Insect Molecular Biology and Biochemistry. pp. 236–295, Academic Press, INRA Sophia Antipolis, France.
[10] Scott, J. G. (1999) Insect Biochem. Mol. Biol. 29, 757–777.
[11] Feyereisen, R. (2013) Biochim. Biophys. Acta 1814, 19–28.
[12] Nelson, D. R. (2011) BBA Proteins Proteom. 1814, 14–18.
[13] Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., and Kataoka, H. (2004) J. Biol. Chem. 279, 35942–35949.
[14] Feyereisen, R. (2006) Biochem. Soc. Trans. 34, 1252–1255.
[15] Pavek, P., and Dvorak, Z. (2008) Curr. Drug Metab. 9, 129–143.
[16] Zhu, F., and Liu, N. (2008) Arch. Insect Biochem. Physiol. 67, 107–119.
[17] Terriere, L. C. (1984) Annu. Rev. Entomol. 29:771–788.
[18] Macedo, M. E., Consoli, R. A. G. B., Grandi, T. S. M., dos Anjos, A. M. G., de Oliveira, A. B., Mendes, N. M., Queiroz, R. O., and Zani, C. L. (1997) Mem. Inst. Oswaldo Cruz. 92, 565–570.
[19] Price, D. R., and Gatehouse, J. A. (2008) Trends Biotechnol. 26, 393–400.
[20] Palli, S. R. (2012) CAB Rev. 7, 004.
[21] Belles, X. (2010) Ann. Rev. Entomol. 55, 111–128.
[22] Kariyanna, B. (2019) Identification and functional analysis of genes involved in insecticide resistance in *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae). PhD Thesis. Submitted on September 2019. UAS, Raichur, Karnataka. p 123.
[23] Kranthi, K. R. (2005) CICR Technical Bulletin, vol. 143 p 155, ICAR, New Delhi.
[24] Kinoshita, F. K., Frawley, J. P., and Dubois, K. P. (1966) Toxicol. Appl. Pharm. 9, 505–513.
[25] Sambrook, J., and Russell, D. W. (2001) Molecular cloning: a laboratory manual. Woodbury: Cold Spring Harbor Laboratory. 3v, p 999.
[26] Masek, T., Vopalensky, V., Suchomelova, P., and Pospisek, M. (2005) Anal. Biochem. 336(1), 46–50.
[27] Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408.
[28] Kariyanna, B., Prabhuraj, A., Asokan, R., Babu, P., Jalali, S. K., Venkatesan, T., Gracy, R. G., and Mohan, M. (2019) Biologia. 75, 289–297.
[29] Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., and Kubista, M. (2009) Clin. Chem. 55, 611–622.
[30] Rebijith, K. B., Asokan, R., Ranjitha, H. H., Rajendra Prasad, B. S., and Krishna Kumar, N. K. (2015) Environ. Entomol. 1–8.
[31] IBM Corp. (2016) IBM SPSS Statistics for Windows. Version 24.0. IBM Corp, Armonk, NY.
[32] Prodhan, M. Z., Hasan, H. M. T., Chowdhury, M. M. I., Alam, M. S., Rahman, M. L., Azad, A. K., Hossain, M.J., Naranjo, S.E., and Shelton, A.M. (2018) PloS One, https://doi.org/10.1371/journal.pone.0205713.
[33] Mohan, M., Venkatesan, T., Sivakumar, G., Yandigeri, M.S., and Verghese, A. (2015) Fighting Pesticide Resistance in Arthropods. New Delhi: Westville Publishers.
[34] Feyereisen, R. (2015) Pest Manag. Sci. 71, 793–800.
[35] Tijet, N., Helvig, C., and Feyereisen, R. (2001) Gene. 262, 189–198.
[36] Ranson, H., Nikou, D., Hutchinson, M., Wang, X., Roth, C. W., Hemingway, J., and Collins, F. H. (2002) Insect Mol. Biol. 11, 409–418.
[37] Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R., and Oakeshott, J. G. (2006) Insect Mol. Biol. 15: 615–636
[38] Zhu, F., Moural, T., Shah, K., and Palli, S. R. (2013) BMC Genomics, 14, 174.
[39] Ai, J., Zhu, Y., Duan, J., Yu, Q., Zhang, G., Wan, F., and Xiang, Z.H. (2011) Gene 480, 42–50.
[40] You, M., Yue, Z., He, W., Yang, X., Yang, G., Xie, M., Zhan, D., Baxter, S. W., Vasseur, L., Gurr, G. M., and Douglas, C. J. (2013) Nat. Genet. 45, 220–225.
[41] Wang, B., Shahzad, M. F., Zhang, Z., Sun, H., Han, P., Li, F., and Han, Z. (2014) Biochem. Biophys. Res. Commun. 443, 756–760.
[42] Liu, S., Rao, X., Li M. Y., and Li, S. G. (2015) Entomol. Res. 45, 141–149.
[43] Rewitz, K. F., Rybczynski, R., Warren, J. T., and Gilbert, L. I. (2006) Biochem. Soc. Trans. 34, 1256–1260.
[44] Warren, J. T., Petryk, A., Marqués, G., Parvy, J. P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M. B., and Dauphin-Villemant, C. (2004) Insect Biochem. Mol. Biol. 34, 991–1010.
[45] Namiki, T., Niwa, R., Sakudoh, T., Shirai, K., Takeuchi, H., and Kataoka, H. (2005) Biochem. Biophys. Res. Commun. 337, 367–374.
[46] Hung, C. F., Harrison, T. L., Berenbaum, M. R., and Schuler, M. A. (1995) Insect Mol. Biol. 4, 149–160.
[47] Li, X., Berenbaum, M. R., and Schuler, M. A. (2000) Insect Biochem. Mol. Biol. 30, 75–84.
[48] Ma, R., Cohen, M. B., Berenbaum, M. R., and Schuler, M. A. (1994) Arch. Biochem. Biophys. 310, 332–340.
[49] Wen, Z. M., Pan, L. P., Berenbaum, M. R., and Schuler, M. A. (2003) Insect Biochem. Mol. Biol. 33, 937–947.
[50] Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., and ffrench-Constant, R. (2001) Mol. Genet. Genomics 266, 556–563.
[51] Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., and Feyereisen, R. (2002) Science 297, 2253–2256.
[52] Nicole, J., Ingolf, S., and Burkhard, S. (2010) Chem. Biodivers. 7, 722–734.
[53] Waters, L.C., Zelhof, A.C., Shaw, B.J., and Ch'ang, L.Y. (1992) Proc. Natl. Acad. Sci. USA. 89, 4855–4859.

[54] Sharath Chandra, G., Asokan, R., Manamohan, M., and Sita T. (2016) Curr. Sci. 111, 901–906.
[55] Winston, W. M., Molodowitch, C., and Hunter, C.P. (2002) Science 295, 2456–2459.
[56] Bucher, G., Scholten, J., and Klingler, M. (2002) Curr. Biol. 12, R85–86.
[57] Rogers, D.W., Baldini, F., Battaglia, F., Panico, M., Dell, A., Morris, H.R., and Catteruccia, F. (2009) PLoS Biol. 7, e1000272.
[58] Caljon, G., De Ridder, K., De Baetselier, P., Coosemans, M., and Van Den Abbeele, J. (2010) PLoS One 5, e9671.
[59] Torres, L., Almazán, C., Ayllón, N., Galindo, R.C., Rosario-Cruz, R., Quiroz-Romero, H., and de la Fuente, J. (2011) BMC Genomics 12, 105.
[60] Cao, M., Gatehouse, J. A., and Fitches, E.C. (2018) Int. J. Mol. Sci. 19, 1079.
[61] Marcus, J.M. (2005) Evol. Dev. 7, 108–114.
[62] Wang, R., Liu S., Baerson, S., Qin, Z., Ma. Z., Su, Y., and Zhang, J. (2018) Int. J. Mol. Sci. 19, 737.
[63] Wei, Z., Yin, Y., Zhang, B., Wang, Z., Peng, G., Cao, Y., and Xia, Y. (2007) Dev. Growth Differ. 49.
[64] Zhang, J., Liu, X., Zhang, J., Li, D., Sun, Y., Guo, Y., Ma, E., and Zhu, K.Y. (2010) Insect Biochem. Mol. Biol. 40, 824–833.