THE ENTOMOLOGICAL SOCIETY OF INDIA

New Delhi, 110 012

ESI- BEST Ph.D. THESIS AWARD 2021

1. Name of the applicant: **Kariyanna B.**

2. Year of the award: 2021

3. Date of birth: 15-07-1991

4. Field of specialization: **Agricultural Entomology**

5. Mode of award collection: In person

6. Postal address (with Phone/Fax/Email etc.): No. 102, 2nd cross, Near Hanuman Temple, Maruthi Nagar, Chikka Banavara (Post), Bengaluru 560090

7. Educational qualifications beginning with the first-degree or equivalent (in a tabular form)

Sl. No	Exams/ Degree	Name of Board or University	Year of Passing
1	B.Sc. (Agriculture)	University of Agricultural Sciences GKVK, Bengaluru, Karnataka	2014
2	M.Sc. (Agricultural Entomology	Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh	2016
3	Ph.D. (Agricultural Entomology)	University of Agriculture Sciences, Raichur, Karnataka	2019
4	PG-Diploma (IPR*)	Distance Education Annamalai University	2018
5	PG-Diploma (HRM*)	Distance Education Annamalai University	2019
6	PG-Diploma (RD*)	Distance Education Annamalai University	2020

*IPR: Intellectual property rights HRM: Human Resource Management RD: Rural Development

8. Date on which qualifying examination passed: **09th January 2018**

9. Transcript of the course work done, if any, as part of the Ph.D. degree programme. **(Photocopy enclosed)**

10. Was the thesis submitted in partial fulfillment of the requirement of the Degree? **Yes**

11. Date on which successfully completed thesis viva voce examination. **24th September 2019**

12. Date of the Degree/provisional certificate: **1st October 2020**

13. Have you received best thesis award earlier? **No**

14. Details of the thesis

(i) **Title of the thesis: Identification and functional analysis of genes involved in insecticide resistance in *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae).**

(ii) **Department/Division/Institution where research work was done:** Department of Agricultural Entomology, UAS, Raichur and Division of Genomic Resources, ICAR-NBAIR, Bengaluru.

(iii) Institution/University to which thesis was submitted as a part of the doctoral degree programme: University of Agricultural Sciences, Raichur

(iv) Name and Designation of the thesis supervisor

Dr. Prabhuraj A., Professor and Head, Department of Entomology, UAS, Raichur (Chairperson).

Dr. M. Mohan, Principal Scientist, Division of Genomic Resources, ICAR-NBAIR, Bengaluru (Co-chairperson).

(v) Research problem: Brief description, scientific, technological, socio-economic relevance and priority (limit to 1 page).

Brinjal is the second most important vegetable crop in the world. Brinjal is cultivated over 1.6 mha in India, which covers around 37 per cent of the world Brinjal growing area, with an average production of 50 million MT/ha and productivity of 18.5 MT/ha. Brinjal crop is habitually and concurrently attacked by 53 important insect pests, out of which the **shoot and fruit borer, *Leucinodes orbonalis* Guenee (Lepidoptera: Pyralidae) is the most destructive one**. The larvae bore into the young shoots and fruits resulting in wilting of shoot and withering of leaves. The damaged fruit loses its marketable value.

In India and Bangladesh, *L. orbonalis* causes severe yield losses up to 93 per cent despite best management practices. Incidence of *L. orbonalis* and fruit damage varies greatly depending on the prevailing climatic conditions and the nature of the cultivars. Surveys in India and Bangladesh indicated that farmers spray chemical insecticides up to **84 times during a 6-7 month cropping season**. A very high level of pesticide load at a concentration 40-450 times higher than the MRL in marketed brinjal fruits poses major health concern to the consumers as well as growers.

Although effective, repeated use of these controlling agents has fostered several environmental and health concerns, including disruption of natural biological control systems, outbreaks of other insect species, widespread development of resistance and undesirable effects on non-target organisms. *L. orbonalis* is also considered as a pest of quarantine significance to the number of countries outside its native range. *L. orbonlais* has high reproductive potential with overlapping generations. Studies reporting high level of insecticide resistance development in *L. orbonalis* might be attributed to the long-term indiscriminate use of various insecticides on brinjal

The present pest control strategies and transgenic approaches have certain limitations and are not completely successful in suppressing insect pests. However, the sequence specific gene silencing via RNA interference (RNAi) holds great promise for effective management of *L. orbonalis*. RNAi is naturally occurring conserved process responsible for gene regulation and defence against pests. The sprayable formulations of RNAi gene products are already being developed and tested for few pest species elsewhere and have great relevance in the present investigation. The outcome of the present investigation could lead to development of sustainable pest management strategies to address the socio-economic issues faced by the farmers.

(vi) What standard methods and modern procedures were used in the experimental work? (limit to one page).

Identification of cultivar resistance in brinjal against *L. orbonalis*:

Indigenous specialty cultivars such as Manjarigota, Mullubadane, Madhugiri Mulla, Eeranagere and Mattugulla and few popular hybrids (Gourav and Lalitha) of brinjal, were screened for their resistance / tolerance against *L. orbonalis* in open field condition for two seasons. Cumulative damage across the genotypes was analysed through SPSS and SAS to identify and extend the durability of cultivar resistance. Such cultivars become a base for developing molecular based pest management strategies.

Development of cost effective method of mass rearing brinjal shoot and fruit borer, *L. orbonalis:*

Potato based artificial rearing system has been standardised for easy rearing of larvae of *L. orbonalis* in large numbers for various studies. The mass production technology has been commercialized to private entrepreneurs and adopted by

students and researchers working on brinjal varietal and insecticide toxicity screening against *L. orbonalis*

Novel methods of detection of insecticide resistance in *L. orbonalis*:

Concentration-mortality bioassays for the insecticides *viz.*, fenvelarate (20% EC), emamectin benzoate (5% EC), phosalone (35% EC), thiodicarb (75% WP), flubendiamide (20% WG) and chlorantraniliprole (18.5% SC) were conducted based on their usage history on brinjal by farmers. **A novel filter paper residue bioassay** has been standardised for monitoring insecticide resistance on late second instar larvae of *L. orbonalis*.

Standardization of procedures for the detection of biochemical basis of insecticide resistance development in *L. orbonalis:*

Estimation of O-demethylase activity of cytochrome P450 monooxygenase was standardised by analysing the production of p-nitrophenol as per Kinoshita *et al.*, 1966. Estimation of carboxylesterase activity was determined using the method described by van Asperen (1962) with essential modifications and α-naphthyl acetate as a substrate. Estimation of glutathione S-transferase activity was determined using 1-chloro 2,4-dinitrobenzene (CDNB) as a substrate (Kao *et al.*, 1989) with essential modifications to suit the present assay conditions using ELISA reader. The native PAGE analysis of esterase isozymes with 10 per cent resolving gel was performed as per Davis (1964) to study the induction of esterase isozymes activities due to insecticide selection pressure.

Mining and identification of target genes and their gene families from the draft genome and transcriptome of the pest insect, *L. orbonalis:*

The in-house facility of HPC (High Performance Computing Facility) and CLC Genomics workbench available at **ICAR-NBAIR** have been utilised to handle the genome and transcriptome data. The cytochrome P450 monooxygenase (CYP), carboxylesterase (CE) and glutathione S-transferase (GST) hmm model (CYP-PF00067; CE-PF00135, PF02230 and GST-C: PF00043; GST-N: PF02798, GST-PF13417, PF14497, PF17171, PF17172) from the PFAM database (http://pfam.xfam.org) was used to search the *L. orbonalis* putative proteins using the hmmsearch program of the HMMER3 software package (http://hmmer.janelia.org). Based on multiple sequence alignment with CLUSTALW (https://www.genome.jp/tools-bin/clustalw) and a maximum

likelihood tree (with 100 bootstraps) was built using MEGA7.9 software, CLC workbench and TreeDyn online server with *D. melanogaster* genome as a reference. Naming of all the CYP genes (72 full length sequences) of *L. orbonalis* was performed by Dr. David R. Nelson, University of Tennessee Memphis, USA. The nomenclature, sub class assignment for GST (34 genes) and carboxylesterase (96 genes) were performed with the help of published literature.

Development and maintenance of homozygous insecticide susceptible and field populations of *L. orbonalis*

The insecticide susceptible laboratory **iso-female colony of *L. orbonalis* (Lo-S) (National Accession number: NBAIR-IS-CRA-01A)** used in this investigation was originally collected during 2012 from brinjal fields near Bengaluru, India (12.97°N and 77.58°N) and being maintained continuously on potato-based diet without exposure to any insecticides at ICAR-NBAIR, Bengaluru. The rearing conditions were 27±2ºC, 60-70 per cent RH, and a photoperiod of 14:10 h (L:D). The field populations of *L. orbonalis* were collected from Varanasi, Bhubaneswar, Pune, Raichur and Dharmapuri, where the brinjal is grown in a large scale. All the populations were maintained under laboratory conditions for various biological, biochemical and gene expression studies.

Validation of internal control genes for normalization of QRTPCR data

Gene expression profiles of a target gene can provide valuable clues towards the understanding of its biological function. Reverse transcription quantitative real-time PCR (qRT-PCR) is the best method for targeted gene expression analysis due to its sensitivity and reproducibility. However, calculating relative expression requires reference genes, which must be stable across various biological conditions. For this purposes, nine prospective genes namely, *actin, 28S ribosomal protein S18c, mitochondrial isoform X2 (28SM), calnexin, β-tubulin, 28S ribosomal protein S3 mitochondrial (28SR3), TATA box binding protein (TATA), elongation factor-1α (EF-1α), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH),* and *ubiquitin 60S ribosomal protein (Ubiquitin)* were evaluated for their potential use as reference genes using standard procedure. ***28SR3* and *GAPDH*** were found highly suitable based on their stability and stable expression pattern.

Selection of target genes for gene expression analysis and gene knock down studies

The metabolic genes (CPY, GST and CE) with known history of involvement in insecticide resistance in other insects were selected from the genome and transcriptome data sets of *L. orbonalis* and further confirmed with NCBI-BLASTX with 90% query coverage. The selected sequences were used for designing primers by Primer 3.0 plus software.

The two genes ***CYP324F1* and *CYP306A1*** were overexpressed in all the field population were picked up for RNAi knockdown experiments via dsRNA oral delivery.

Standardization of dsRNA delivery: Among the delivery routes studied, the oral feeding of dsRNA via brinjal flower bud dip assay proved efficient. An unopened flower bud with ~1.5 cm with slant cut tip was rinsed in 0.1 per cent Triton-X® (Sigma Aldrich, USA). The same was washed twice in double distilled water, after it was dried and placed in 500 µL PCR tube containing calculated **dsRNA** and wrapped with para-film to avoid the leakage. This technique makes the entire flower bud contaminate with dsRNA and facilitates the insects to feed on only on the treated bud with concentration of 1, 0.5, 0.25, 0.1 and 0.05 µg/µL. The bioassay procedure also included negative control (nuclease-free water) and positive off-target control (*LacZ*-dsRNA).

(vii) Briefly describe the significant results obtained? (Not more than 3 pages)

Brinjal shoot and fruit borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) is one of the difficult to manage insect pests despite best management practices. It is the major cause of loss of brinjal productivity in India, Bangladesh, Pakistan and Phillippines. It has complex genetic makeup, can develop resistance against insecticides quickly. Though brinjal is native to India and Sri Lanka, no true resistance source in the available cultivars has been documented due to the pest adaptation through co-evolution. The sole method of control adopted by farmers is the use of insecticides. Despite the heavy use of insecticides, more than one-third of the harvested fruits are unmarketable due to bore holes. Hence, the present investigation was undertaken to generate scientific data on the population dynamics

of the *L. orbonalis* on different varieties under the climate change era and to decipher the insecticide resistance status and associated resistance mechanisms.

Existence of very high levels of insecticide resistance against commonly used insecticides in certain field-collected populations were documented and the involvement of multiple metabolic enzymes in detoxification of the insecticidal molecules had been validated for the first time.

The repertoire of pesticide detoxification genes and their gene families were extracted by genome and transcriptome mining and their roles in insecticide degradation were characterized by quantitative gene expression studies. Silencing of two important *L. orbonalis* genes were undertaken to prove that these genes had important role in detoxification.

The following are the salient findings of the present investigation

I. Among traditional brinjal cultivar screened the **Eeranagere** cultivar were showed tolerance against *L. orbonalis*. Hence this variety will perform as a donor parents in breeding programme for the development of commercial cultivar.

II. The population dynamics of *L. orbonalis* showed a **bimodal distribution**.

III. The diploid genome size of *L. orbonalis* is 944.52 Mb, over chicken RBC (2.33 pg) as a reference standard. The relationship of genome size with the insect morphological traits and host plant range showed no evident correlation between them (Fig. 1). Estimation of the genome size is the pre-requisite for sequencing of the whole genome of *L. orbonalis.*

IV. The concentration-mortality bioassays revealed, 48.8 to 160 fold resistance to fenvalerate, 95.5 to 534.58 fold resistance to phosalone, 7.2 to 56 fold resistance to emamectin benzoate, 9. 4 to 22.8 fold resistance to thiodicarb, 29.5 to 303.0 fold resistance to flubendiamide and 1.6 to 8.60 fold resistance to chlorantraniliprole in different field populations of *L. orbonalis* tested as compared to laboratory-reared susceptible iso-female colony (Lo-S).

V. The biochemical assays on detoxification enzymes revealed significant over-production of CYP (O-demethylase) (5.4 to 18.5 fold), GST (4.1 to 8.9 fold) and CE (1.5 to 3.1 fold) in field-collected *L. orbonalis* populations as compared to susceptible Lo-S population. The study indicated the key role of biochemical factors in xenobiotic degradation, which results in moderate to very high level of resistance in field populations.

VI. Among the four major esterase activity bands (E_1 to E_4), the high molecular weight E_1 and E_2 bands were absent in Lo-S and Pune populations and faint in case of Varanasi population. However, the E_1 band was very prominent and intense in other field populations indicating its over-production in resistant populations (Fig. 2). Additional band indicated gene duplication in these populations that may be associated with insecticide resistance.

VII. Mining of the draft genome (*de novo*) and transcriptome dataset of *L. orbonalis* revealed that, 72, 94 and 34 full-length putative unigenes were coding for CYP, GST and CE respectively (Fig. 3,4 and 5).

VIII. A total of 72 CYP genes were identified and assigned under four CYP clades based on closest BLAST hits following the international CYP gene nomenclature rules. Among them ***CYP324F1*** novel gene was first ever reported for the living organism.

IX. Homologous search revealed that out of 72 CYP genes, **34** had a potential to metabolize insecticides. Among them, 18 and 10 belonged to CYP3 and CYP4 clades and these were involved in xenobiotic metabolism in insects (Fig. 3).

X. The unigenes coding for CE were classified under 10 families including unclassified group. The homologous search indicated that, out of 94 CE unigenes of *L. orbonalis*, **16** had the potential to metabolize insecticides (Fig. 4).

XI. The full-length unigenes coding GST were classified under six families. The homologous search indicated that out of 34 GST unigenes of *L. orbonalis*, **25** were uniquely involved in insecticide degradation in other insects (Fig. 5).

XII. Nine internal control genes were validated for their suitability in normalizing the target genes expression. *28S ribosomal protein S3 mitochondrial* (*28SR3*) and *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) were found highly suitable based on their stability and stable expression pattern for normalization (Fig. 6).

XIII. The quantitative gene expression studies with RT-qPCR revealed, differential expression of numerous genes under CYP and GST and few under CE in many of the field-collected *L. orbonalis* populations as compared to Lo-S population. A significant difference in expression pattern was noticed in the gene transcripts *CYP324F1, CYP306A1, CYP6AB149* and *CYP6CT1* (CYPs) (Fig. 7), *contig2323-0.22, contig14202-0.24, contig3755-0.39, contig3537-0.3, contig199-0.91, contig3023-0.16, contig3291-0.7, contig4841-1.1* and *contig2323-0.23* (GSTs) (Fig. 8) and *contig11486-0.8* and *contig4653-0.41* (CEs) (Fig. 9) compared to Lo-S. It indicated that evolution of multiple modes of insecticide resistance in *L. orbonalis*.

XIV. Employing RNA interference (RNAi) technique by silencing of *CYP324F1* and *CYP306A1* genes through oral feeding of dsRNA resulted, 53.4 to 85.0 per cent down-regulation of these transcripts (Fig. 10) with larval mortality ranging from 20.28 to 33.41 per cent at 1 µg/µL dsRNA concentration (Fig. 11).

The data generated here has immense value in designing new tactics for the management of insecticide resistance in *L. orbonalis*. This is the first comprehensive study in the country as well as in the world on brinjal shoot and fruit borer, *L. orbonalis*, reporting various aspects *viz.*, biological, biochemical and molecular phenomena holistically involved in insecticide degradation.

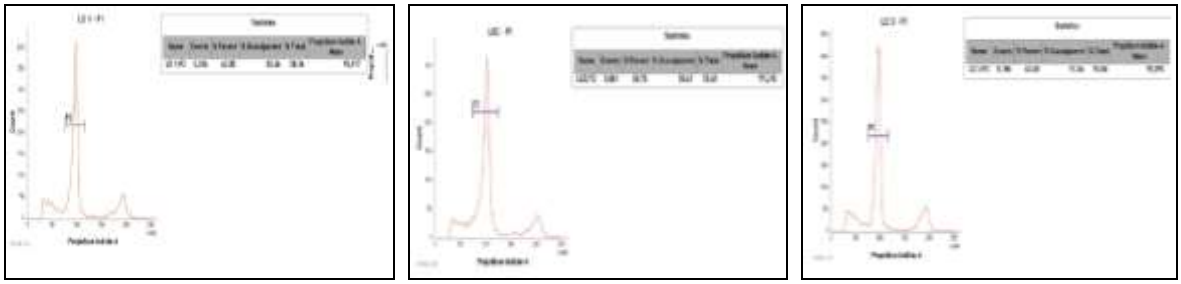

Fig. 1. Flow cytometric fluorescence histograms of *Leucinodes orbonalis* cells.

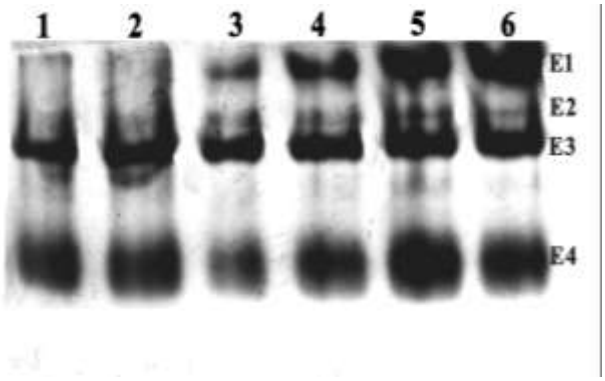

Fig. 2. The native PAGE of the esterase isozyme pattern of *Leucinodes orbonalis;* (1. Lo-S, 2. Pune, 3. Varanasi, 4. Raichur, 5. Bhubaneswar, 6. Dharmapuri).

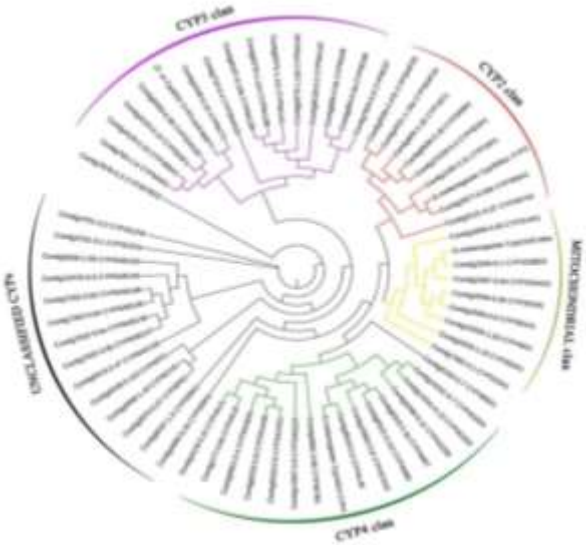

Fig. 3. Phylogenetic relationship of **72 CYP450 genes** of *L. orbonalis* with selected *D. melanogaster* genes representing different clans.

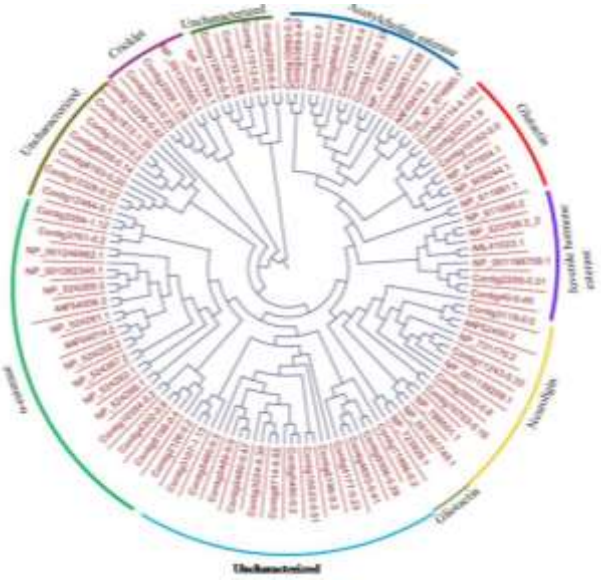

Fig. 4. Phylogenetic relationships of **94 carboxylesterases** genes of *Leucinodes orbonalis* with selected *Drosophila melanogaster* genes representing different classes.

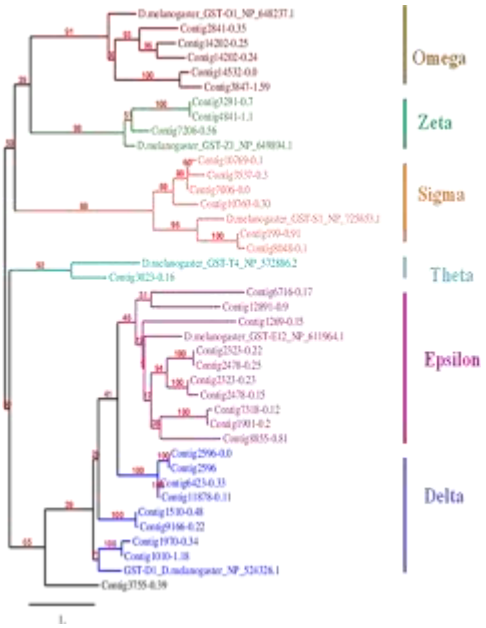

Fig. 5. Phylogenetic relationship of **34 glutathione S-transferase** genes of *Leucinodes orbonalis* with selected *Drosophila melanogaster* genes representing six classes.

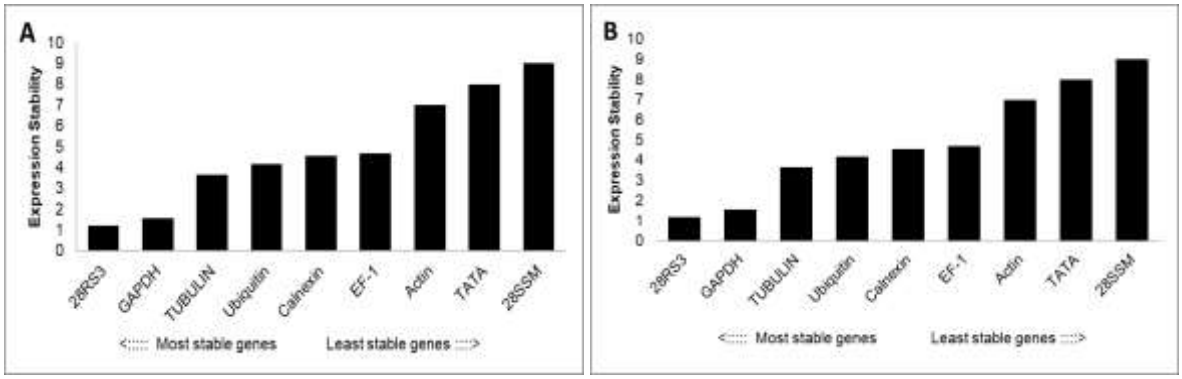

Fig. 6. Analysis of **gene expression stability** values and ranking of reference genes using RefFinder algorithm in *Leucinodes orbonalis*. A) Developmental stages (B) Populations.

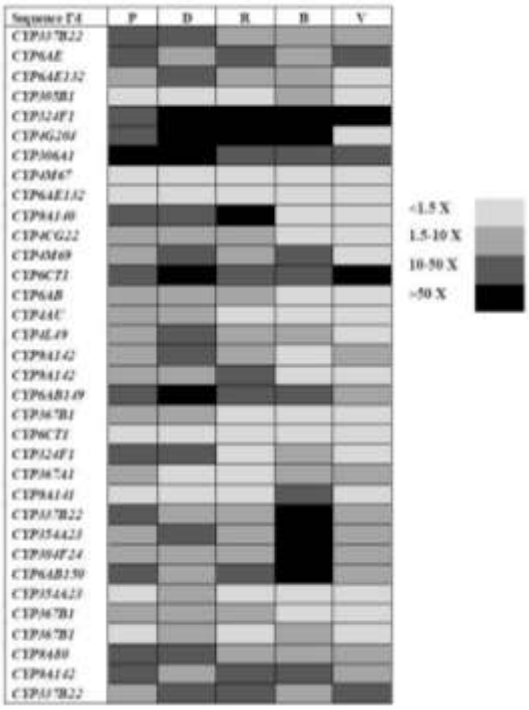

Fig. 7. Expression profiles (Fold changes over Lo-S population) of **cytochrome P450 genes** across field collected *Leucinodes orbonalis* populations using heat map (P-Pune; D-Dharmapuri; R-Raichur; B-Bhubeneswar; V-Varanasi). The fold changes are indicated in different shades indicate significant difference as per Mann-Whiteney U-test p-value<0.05.

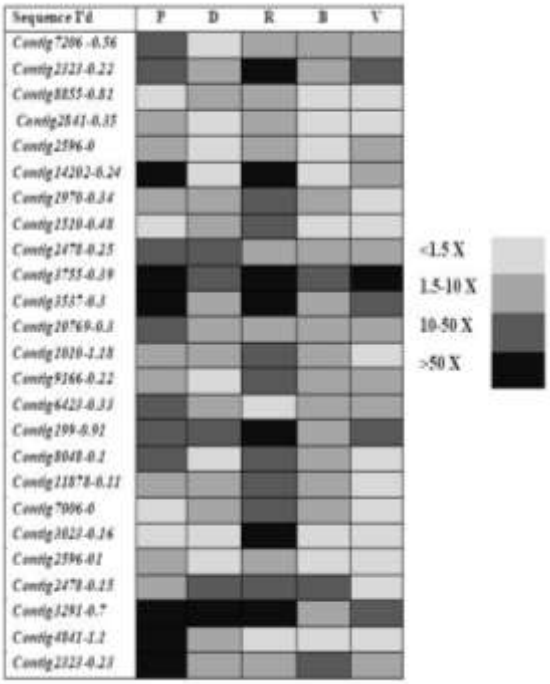

Fig. 8. Expression profiles (Fold changes over Lo-S population) of **glutathione S-transferase genes** across field collected *Leucinodes orbonalis* populations using heat map (P-Pune; D-Dharmapuri; R-Raichur; B-Bhubeneswar; V-Varanasi). The fold changes are indicated in different shades indicate significant difference as per Mann-Whiteney U-test p-value<0.05.

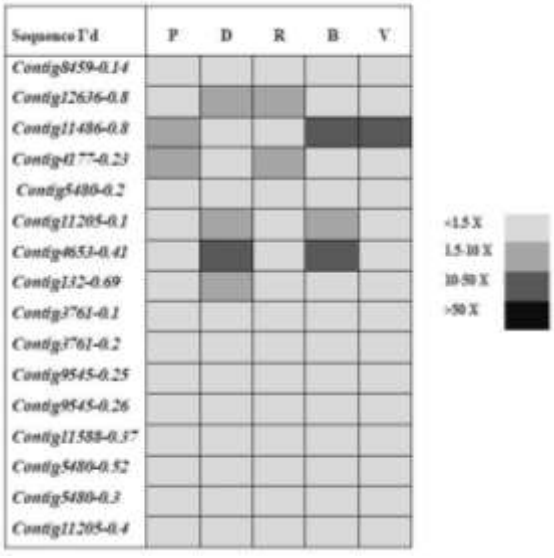

Fig. 9. Expression profiles (Fold changes over Lo-S population) of **carboxylesterases** using heat map (P-Pune; D-Dharmapuri; R-Raichur; B-Bhubeneswar; V-Varanasi). The fold changes are indicated in different shades indicate significant difference as per Mann-Whiteney U-test p-value<0.05.

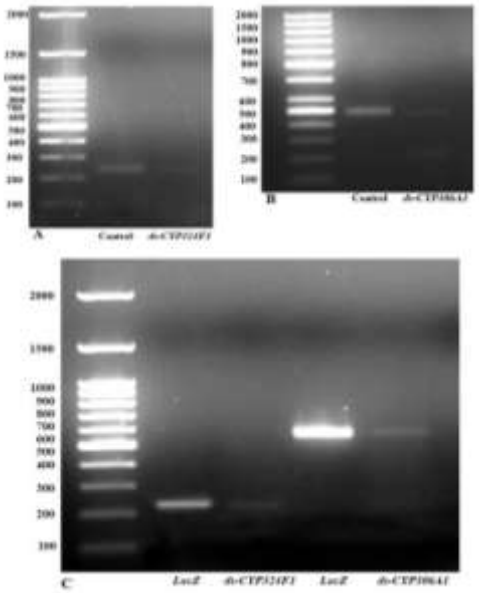

Fig. 10. Molecular confirmation of ds-RNA effect in larvae after 92 hours of feeding at 1µg/µl concentration: A. Control versus **ds-*CYP324F1***, B. Control versus **ds-*CYP306A1*** and C. ds-*LacZ* versus target genes**.**

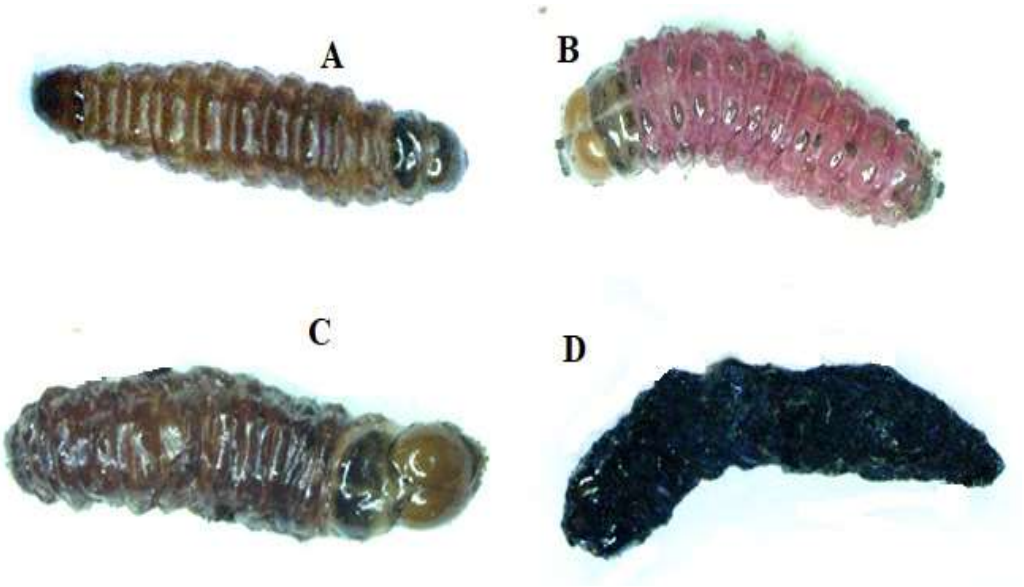

Fig. 11. Response of second instar larvae of *Leucinodes orbonalis* upon ds-RNA treatment; A. Control; B. *LacZ* control; C. ds-*CYP324F1* and D. ds-*CYP306A1*

(viii) In what way these results have made an original contribution to science and have impact

- The traditional cultivar **Eeranagere** was identified as a tolerant cultivar against the *L. orbonalis* and can be used as a donor to develop resistant variety in breeding programme.
- The integrated (biological, biochemical and molecular) analysis revealed and confirmed the existence of very high levels of **multiple insecticide resistance among the field populations of *L. orbonalis*.**
- The diploid genome size of *L. orbonalis* was estimated at **944.52 Mb**. The information on genome size was utilised in deciding the coverage and the platforms required for whole genome sequencing of *L. orbonalis*.
- The present investigation identified from total 74, 34 and 94 genes, among them **34** cytochrome p450 monooxygenese (CYP450), **25** glutathione S-transferaee (GST) and **16** carboxylesterase (CE) genes from the genome that were involved in insecticide resistance.
- The study identified a novel *cyp* gene, *CYP324F1* from the genome of *L. orbonalis*. This *cyp* gene has not been identified from any other living system so far.

- Gene knock down studies revealed the significant role played by two *cyp* genes *viz.*, *CYP306A1* and *CYP324F1* in degrading the insecticide molecules in addition to other physiological roles.

The present study will serve as a strong base for development of sprayable nanoformulations of RNAi gene products for this important insect pest. It could be a viable alternative to conventional methods of control as well as transgenic approach.

(ix) Publications (in journals/along with NAAS rating as on 1.1.2021, Seminar proceedings, University/Institute Newsletter, Lecture delivered, Book chapter etc. (publications only from the thesis to be included, only those publications in published/accepted/proof stage will be considered, no marks will be given for other publications not related with thesis works)

1. **Kariyanna B,** Prabhuraj A, Asokan R, et al., 2020. Genome mining and expression analysis of carboxylesterase and glutathione S-transferase genes involved in insecticide resistance in eggplant shoot and fruit borer, *Leucinodes orbonalis* (Lepidoptera: Crambidae). ***Frontiers in Physiology***, **11**: 594845. https://doi.org/10.3389/fphys.2020.594845 **(IF: 4.137).**

2. **Kariyanna B,** Prabhuraj A, Asokan R, Ramkumar G, Gandhi Gracy R, Venkatesan T, Mohan M, 2020. Genome mining and functional analysis of cytochrome P450 genes involved in insecticide resistance in *Leucinodes orbonalis* (Lepidoptera: Crambidae). ***Biotechnology and Applied Biochemistry***, https://doi.org/10.1002/bab.1997 **(IF: 1.638).**

3. **Kariyanna B,** Prabhuraj A, Asokan R, Prasad Babu, Jalali SK, Venkatesan T, Gandhi Gracy R, Mohan M, 2019. An identification of suitable genes for qRT-PCR normalization for eggplant shoot and fruit borer, *Leudcinodes orbonalis* Guenee (Lepidoptera: Crambidae). ***Biologia***, **75**: 289–297. https://doi.org/10.2478/s11756-019-00346-4 **(IF: 0.811 (2019).**

4. Gandhi Gracy R, Basavaarya BR, **Kariyanna B,** Arunkumara CG, Jalali SK, Venkatesan T, Mohan M, 2019. The relationship between genome size, morphological parameters and development period in four economically important insect species. ***Biocatalysis and Agriculture Biotechnology***, **20**: 101188. https://doi.org/10.1016/j.bcab.2019.101188. **(Science Citation Index: 2.38).**

5. **Kariyanna B,** Prabhuraj A, Bheemanna M, Basavaraj K, Pamapanna Y, Mohan M, Diwan JR, 2021. Influence of cultivars on population dynamics of brinjal shoot and fruit borer *Leucinodes orbonalis*. ***Indian Journal of Entomology***, **83**: 1-4. https://doi.org/10.5958/0974-8172.2021.00043.2 **(NAAS: 5.89).**

6. **Kariyanna B,** Prabhuraj A, Mohan M, Bheemanna M, Basavaraj K, Pampanna Y, and Diwan JR, 2020. Insecticide usage pattern and evolution of resistance in brinjal shoot and fruit borer, *Leucinodes orbonalis* (Lepidoptera: Crambidae) in India, ***Plant Archives***, **20** (S2): 1255-1261 **(NAAS: 4.41).**

(x) **Soft copy in PDF format needs to be submitted for the above including whole thesis Copy enclosed**

(xi) Whether any patents have been taken/applied for, **No**

(xii) Professional recognition, awards, fellowships received (In a separate sheet give full particulars such as the agency/ organization which gave the award, purpose, the nature of the award, etc.).:

- ✓ DST-Inspire Fellowship-2016 (IF-160900), Department of Science and Technology, Government of India (GOI), **2016-2019.**
- ✓ Gold Medal and First rank, Ph.D. (Agri.) Entomology, University of Agricultural Sciences, Raichur, **2019.**
- ✓ University Gold Medal, M.Sc. (Agri.) by Indira Gandhi Krishi Vishwavidyalaya, Raipur-492001, Chhattisgarh, **2016.**
- ✓ First rank and Silver Medal, Department of Entomology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, **2016.**

Best oral Presentation Awards

- ❖ **2020-Best oral Presentation Award:** XVII AZRA International conference on frontier research in applied zoology and insect pest management strategies: A way forward for food and nutritional security. University of Agricultural Sciences, Raichur, Karnataka, 12^{th}-14^{th} Feb, 2020.
- ❖ **2018-Best oral Presentation Award**: Third international Conference on food and agriculture, UPM, Kuala Lumpur, Malaysia. International Conference. 28-Nov-2018.
- ❖ **2018-Best oral Presentation** + Cash price (₹1000): First Post-Graduate Research Conference-2018. University of Agricultural Sciences, Raichur, Karnataka. 12^{th}-13^{th} June, 2018.

17. A concise statement (about 150 words) highlighting the most significant aspects of the research work done that you would like to see in your citation of award, if chosen.

Taking advantage of the whole genome and stage specific transcriptome sequence assemblies of brinjal shoot and fruit borer, *Leucinodes orbonalis* developed at ICAR-NBAIR, a genome-wide analysis of the genes and gene families involved in insecticide resistance was undertaken. The nomenclature, sub-class assignment and phylogenetic analysis of the genes under the gene families of cytochrome-p450-monooxygenase glutathone S-transferase and carboxylesterase were undertaken. The expression pattern of these genes were analysed through quantitative real time PCR for their suitability in development of RNAi based pest control. The cypome rich genome yielded a novel *cyp* gene *CYP324F1* that has not been reported from any other living organisms so far. Together with other genes, *CYP324F1* played a significant role in insecticide degradation in resistant field populations of *L. orbonalis*. The present study will serve as a strong base for development of sprayable nanoformulations of RNAi gene products for managing this dreadful insect pest. It could be a viable alternative to conventional methods of pest control as well as transgenic approach.

18. Certificate by the student that all the details given are true

Signature

19. Certificate by the supervisor/guide that the information submitted has been verified and true

Signature

Professor & Head
Dept. of Agril. Entomology
College of Agriculture
UAS, RAICHUR-584104

20. Forwarding note by the Head of the Department/professor

Name and Designation

Professor & Head
Dept. of Agril. Entomology
College of Agriculture
UAS, RAICHUR-584104

Seal

Application Form For ESI Best Ph.D. Thesis Award

Personal Information

Applicant's Name : 2021

Present Position/Designation : Agricultural Entomology

Applicant's Address : Plot no. 102, 2nd cross, Near Hanuman Temple Behind flour mill, Maruthi Nagar 560090 Bengaluru Urban Karnataka

DOB : 1991-07-15

Subject of Specialization : In person

Recommendations

Academic Record and Professional Attainments

Academic Record

SN	Degree	University/Institution	Year	Distinction
1.	B.Sc. (Agriculture)	UAS, GKVK, Bengaluru, Karnataka	2014	
2.	M.Sc. (Agricultural Entomology)	IGKV, Raipur, Chhattisgarh	2016	
3.	Ph.D. (Agricultural Entomology)	UAS, Raichur, Karnataka	2019	

Special attainments of the nominee

Publications

List 20 most important research publications in chronological order. For each publication, give name(s) of the author(s),indicate the corresponding author by'*' after his/her name, year of publication, full title of the paper, name of the journal, volume No. and page Nos.and NAAS rating during the 2021 forESI senior scientist award

SN	Details of Publications in the format given above	NAAS Journal ID and (Score)	Number of Citations

Print/Save as PDF

1.	Kariyanna B, Prabhuraj A, Asokan R, et al., 2020. Genome mining and expression analysis of carboxylesterase and glutathione S-transferase genes involved in insecticide resistance in eggplant shoot and fruit borer, Leucinodes orbonalis (Lepidoptera: Crambidae). Frontiers in Physiology, 11: 594845. https://doi.org/10.3389/fphys.2020.594845 (IF: 4.137).	1664-042X	
2.	Kariyanna B, Prabhuraj A, Asokan R, Ramkumar G, Gandhi Gracy R, Venkatesan T, Mohan M, 2020. Genome mining and functional analysis of cytochrome P450 genes involved in insecticide resistance in Leucinodes orbonalis (Lepidoptera: Crambidae). Biotechnology and Applied Biochemistry, https://doi.org/10.1002/bab.1997 (IF: 1.638).	0885-4513	1
3.	Kariyanna B, Prabhuraj A, Asokan R, Prasad Babu, Jalali SK, Venkatesan T, Gandhi Gracy R, Mohan M, 2019. An identification of suitable genes for qRT-PCR normalization for eggplant shoot and fruit borer, Leudcinodes orbonalis Guenee (Lepidoptera: Crambidae). Biologia, 75: 289–297. https://doi.org/10.2478/s11756-019-00346-4 (IF: 0.811).	0006-3088	13
4.	Gandhi Gracy R, Basavaarya BR, Kariyanna B, Arunkumara CG, Jalali SK, Venkatesan T, Mohan M, 2019. The relationship between genome size, morphological parameters and development period in four economically important insect species. Biocatalysis and Agriculture Biotechnology, 20: 101188. https://doi.org/10.1016/j.bcab.2019.101188. (Science Citation Index: 2.38).		
5.	Kariyanna B, Prabhuraj A, Bheemanna M, Basavaraj K, Pamapanna Y, Mohan M, Diwan JR, 2021. Influence of cultivars on population dynamics of brinjal shoot and fruit borer Leucinodes orbonalis. Indian Journal of Entomology, 83: 1-4. https://doi.org/10.5958/0974-8172.2021.00043.2 (NAAS: 5.89)	0367-8288	
6.	Kariyanna B, Prabhuraj A, Mohan M, Bheemanna M, Basavaraj K, Pampanna Y, and Diwan JR, 2020. Insecticide usage pattern and evolution of resistance in brinjal shoot and fruit borer, Leucinodes orbonalis (Lepidoptera: Crambidae) in India, Plant Archives, 20 (S2): 1255-1261 (NAAS: 4.41)	0972-5210	4

Externally Funded Projects

I certify that the information given in the form are correct.

Signature:	
Name:	2021
Address:	Plot no. 102, 2nd cross, Near Hanuman Temple Behind flour mill, Maruthi Nagar 560090 Bengaluru Urban Karnataka
Date of	5th October 2021

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Print/Save as PDF

UNIVERSITY OF AGRICULTURAL SCIENCES, RAICHUR, KARNATAKA, INDIA

DIRECTORATE OF POSTGRADUATE STUDIES

Sl. No. 90

OVERALL GRADE POINT AVERAGE CARD

Date: 01 OCT 2019

(From August 2016 to October 2019)

Name of the Student	KARIYANNA B.	I.D. No.	PHD16AGR6006
Father's Name	BHEERANNA	Year of Admission	2016
Degree Programme	Ph. D.	Year of Completion	2019
Subject	AGRICULTURAL ENTOMOLOGY		

COURSES COMPLETED

Courses No.	Title	Credit Hours	Grade Obtained
	I SEMESTER 2016-17		
AET 517	Vertebrate Pest Management	1+1	9.15
AET 520	Plant Quarantine	2+0	9.03
AET 521	Vermiculture and Vermicomposting	1+1	9.20
AET 602	Immature Stages of Insects	1+1	9.35
AGR 607	Advances in Integrated Farming Systems	2+0	9.09
GPB 611	Marker Assisted Selection in Crop Improvement	2+1	9.15
PAT 515	Biological Control of Plant Diseases	1+1	9.30
AST 517	Data Analysis	1+1	9.10
	II SEMESTER 2016-17		
AET 513	Storage Entomology	1+1	9.27
AET 604	Advanced Insect Ecology	1+1	8.68
AET 611	Molecular Approaches in Entomological Research	1+1	8.89
AET 612	Advanced Integrated Pest Management	2+0	9.20
AET 651	Seminar-I	1	9.16
AET 661	Research	2	Satisfactory
AMB 606	Advances in Microbial Biotechnology	3+0	9.55
BCH 501	Basic Biochemistry	2+1	8.55

P.T.O.

Name of the Student: KARIYANNA B. ID. No: PHD16AGR6006

Courses No.	Title	Credit Hours	Grade Obtained
	I SEMESTER 2017-18		
AET 651	Seminar-II	1	9.35
AET 661	Research	9	Satisfactory
AET 671	Qualifying Examination	5	9.05
	II SEMESTER 2017-18		
AET 651	Seminar-III	1	9.32
AET 661	Research	9	Satisfactory
	I SEMESTER 2018-19		
AET 651	Seminar-IV	1	9.53
AET 661	Research	9	Satisfactory
	II SEMESTER 2018-19		
AET 661	Research	9	Satisfactory

OVERALL GRADE POINT AVERAGE (OGPA)
91.20 PER CENT

9.12
10.00

REGISTRAR
REGISTRAR
University of Agricultural Sciences
RAICHUR-584 104 Karnataka (India)

Minimum OGPA required for pass:

(i) Masters Degree 7.00/10.00
(ii) Ph.D. Degree 7.50/10.00

UNIVERSITY OF AGRICULTURAL SCIENCES, RAICHUR

KARNATAKA, INDIA

DIRECTORATE OF POSTGRADUATE STUDIES

Sl. No. 90

Date: 0 1 OCT 2019

PROVISIONAL DEGREE CERTIFICATE

This is to certify that **Mr. KARIYANNA B. S/o BHEERANNA, Ph.D.** degree student bearing **ID. No. PHD16AGR6006** in the Department of **AGRICULTURAL ENTOMOLOGY** has successfully completed the requirement of the course work as well as the thesis prescribed for the award of **Ph.D.** degree during 2019.

He has secured an Overall Grade Point Average of **9.12/10.00** in 10.00 point scale (**91.20** per cent).

REGISTRAR

REGISTRAR
University of Agricultural Sciences
Raichur-584 104 Karnataka (India)

ಕೃಷಿ ವಿಜ್ಞಾನಗಳ ವಿಶ್ವವಿದ್ಯಾಲಯ, ರಾಯಚೂರು

ಕರ್ನಾಟಕ, ಭಾರತ

University of Agricultural Sciences, Raichur

Karnataka, India

IN7160302967

ಪ್ರಮಾಣ ಪತ್ರ

ಕರಿಯಣ್ಣ ಬಿ ತಂದೆ ಭೀರಣ್ಣ ಇವರು

೨೦೧೮-೧೯ ನೇಯ ಶೈಕ್ಷಣಿಕ ವರ್ಷದಲ್ಲಿ ಡಾಕ್ಟರ್ ಆಫ್ ಫಿಲಾಸೊಫಿ ಕೃಷಿ ಕೀಟಶಾಸ್ತ್ರ ಪದವಿಯಲ್ಲಿ ಹೆಚ್ಚಿನ ಅಂಕ ಪಡೆದು 'ಪ್ರಥಮ ಸ್ಥಾನ' ಗಳಿಸಿದ್ದರಿಂದ ಕೃಷಿ ವಿಜ್ಞಾನಗಳ ವಿಶ್ವವಿದ್ಯಾಲಯ, ರಾಯಚೂರು ಚಿನ್ನದ ಪದಕವನ್ನು ದಿನಾಂಕ ಒಂಬತ್ತನೇಯ ಜನವರಿ, ೨೦೨೧ ರಂದು ನಡೆದ ಘಟಿಕೋತ್ಸವದಂದು ನೀಡಲಾಗಿದೆ.

CERTIFICATE

Kariyanna B S/o Bheeranna

has been awarded the UNIVERSITY OF AGRICULTURAL SCIENCES, RAICHUR GOLD MEDAL for having secured the Highest Overall Grade Point Average and obtained 'First Rank' in Doctor of Philosophy in Agricultural Entomology Degree during the year 2018-19 on the Convocation Day on 9th January, 2021.

ಕುಲಸಚಿವರು
Registrar

ಕುಲಪತಿ
Vice-Chancellor

इंदिरा गांधी कृषि विश्वविद्यालय

कृषक नगर, रायपुर 492 012 (छत्तीसगढ़) भारत

INDIRA GANDHI KRISHI VISHWAVIDYALAYA

KRISHAK NAGAR, RAIPUR 492 012, CHHATTISGARH, BHARAT

F.No. 25/ACD-1/2019 / 8525

Raipur, Dated: 26/3/2019

आवश्यक सूचना विश्वविद्यालय पदक हेतु योग्य अभ्यर्थियों की अनंतिम सूची

शैक्षणिक सत्र 2015–16

1. यदि पदकों की अनंतिम सूची में कोई दावा–आपत्ती हो तो अभ्यर्थी दिनांक 29 मार्च 2019 के पूर्व समस्त दस्तावेजों के साथ अपना अभ्यावेदन/आवेदन प्रस्तुत करें। यह आवेदन स्पीड पोस्ट/ईमेल अथवा स्वयं उपस्थित होकर प्रस्तुत कर सकते है। अंतिम तिथि के पश्चात प्राप्त होने वाले आवेदनों पर विचार नहीं किया जावेगा।
2. समस्त अधिष्ठाता/प्राचार्य विद्यार्थियों के मध्य प्रचारित करें एवं महाविद्यालय सूचना फलक पर चस्पा करें।
3. महाविद्यालय के अधिष्ठाता/प्राचार्य भी अनंतिम सूची में दावा आपत्ति होने पर दिनांक 29 मार्च 2019 के पूर्व समस्त दस्तावेजों के साथ अभ्यावेदन कर सकते हैं।
4. अभ्यावेदनों का अंतिम निराकरण प्राप्त दस्तावेजों के परीक्षण उपरांत दिनांक 30 मार्च 2019 के बाद अंतिम सूची प्रकाशित कर दी जावेगी।

अभ्यर्थियों के चयन करने हेतु विश्वविद्यालय पदक समिति की अनुशंसा के पश्चात् निम्नलिखित अभ्यर्थियों का नाम विभिन्न पदकों हेतु अनंतिम रूप से योग्य पाया गया है:–

AGRICULTURE 2015-16						
	SN.	Medal	Name of Students	I.D. No.	OGPA	Name of College
B.Sc.	1.	GOLD	PAYAL SHARMA	110112045	8.28	COA, Raipur
	2.	SILVER	ANANNYA SUSAN	110412006	8.27	SG., COA, Jagdalpur
	3.	BRONZE	CHANCHAL PORTE	110712004	8.26	DKS, COA,Bhatapara
M.Sc.	1.	GOLD	करियन्ना बी	20141520302	8.86	कीट विज्ञान विभाग, कृ. महा. रायपुर
Ph.D.	1.	GOLD	कु. अर्चना दीक्षित	20141518600	8.86	उद्यानिकी विभाग, कृ.महा. रायपुर
HORTICULTURE 2015-16						
B.Sc.	1.	GOLD	JYOTI MISHRA	111112014	8.09	KLS. COH,Rajnandgaon
	2.	SILVER	JAYESH KHODIYAR	116112017	7.73	Danteshwari , COH,Raipur
	3.	BRONZE	RASHILA PAINKARA	116412035	7.63	K. L. COH, Dhamtari
M.Sc.	1.	GOLD	कु. सुनिधि मिश्रा	2014152044	8.61	सब्जी विज्ञान विभाग,कृ.महा. रायपुर
AGRIL. ENGINEERING 2015-16						
B.Tech	1	GOLD	SUBARNA GHOSH	210112043	8.02	BRSM,COAE, Mungeli
	2	SILVER	PRACHI YADAV	210112029	7.67	BRSM,COAE, Mungeli
	3	BRONZE	HEMANT KUMAR	210112015	7.30	BRSM,COAE, Mungeli
	4	BRONZE	GAURAV KUMAR	210112013	7.30	BRSM,COAE, Mungeli
M.Tech.	1.	GOLD	प्रवीण कुमार निषाद	2014152046	8.46	कृ. प्र. एवं खाद्य अभि, कृ. अभि. महा. रायपुर
Ph.D.	उद्यानिकी – कोई विद्यार्थी नहीं					
	कृषि अभियांत्रिकी – कोई विद्यार्थी नहीं					

व्ही पी शुक्ला स्मृति स्वर्ण पदक हेतु 2015-2016

पाठ्यक्रम	विद्यार्थियों का नाम	नामांकन क्रमांक	ओ.जी.पी.ए.	विभाग/महाविद्यालय का नाम
बी.एस.सी	कु. पायल शर्मा	110112045	8.28	कृ.महा. रायपुर
एम.एस.सी	करियन्ना बी	20141520302	8.86	कीट विज्ञान विभाग, कृ. महा. रायपुर

विभागवार – रजत पदक हेतु योग्य विद्यार्थियों की सूची

M.Sc. Agriculture 2015-2016

S.No.	विद्यार्थियों का नाम	विभाग का नाम	नामांकन क्रमांक	ओ.जी.पी.ए.	महाविद्यालय का नाम
1.	Banashri Lodh	Agronomy	20141520270	8.42	COA Raipur
2.	Sonal Upadhyaya	Genetics & P B	20141520335	8.67	COA Raipur
3.	Sateyendra Patle	Soil Science & Agri. Chemistry	20141520393	8.21	COA Raipur
4.	Kariyanna B.	Entomology	20141520302	8.86	COA Raipur
5.	Divya Chandrakar	Agri. Economics	20141520229	8.69	COA Raipur
6.	Ku. Jyoti	Agri. Extn.	20141520248	8.12	COA Raipur
7.	Aafreen Khan	Plant Pathology	20141520351	8.36	COA Raipur
8.	Seventika Tiwari	Pl. Mole. Bio. & Biotech.	20141520348	8.07	COA Raipur
	Geetanjali Verma		20141520345	8.07	
9.	Dhanni dev	Agri. Microbiology	20141520257	8.11	COA Raipur
10.	Komal Chawla	Agri. Statistics	20141520266	8.6	COA Raipur
11.		***Pl. Physiology***	***Not eligible – due to less OGPA, i.e., below 8.0.***		
12.	Ancy A S	Agri.Meteorology	20141520396	8.23	COA Raipur
13.	Veijaneng Haokip	Forestry	20141520458	8.58	COA Raipur
14.	Garima Agarwal	MBA	20151420406	8.16	COA Raipur
M.Sc. (Horticulture)					
15.	Sunidhi Mishra	Veg. Science	20141520449	8.61	COA Raipur
16.	Bhavna Panda	Fruit Science	20141520425	8.40	COA Raipur
17.	Neelima Netam	Floriculture & L S	20151420418	8.20	COA Raipur
M Tech. (Agriculture Engineering)					
18.	Chelapuri Ramalu	F M P&E	20141520171	8.21	SV COAE Raipur
19.	Rahul Raghuvanshi	S W & E	20141520490	8.44	
20.	Praween Kumar Nishad	A P F E	20141520436	8.46	

कुलसचिव

F.No. 25/ACD-1/2019 / 8526

Raipur,Dated: 26/3/2019

Copy to -

1. निदेशक शिक्षण एवं परीक्षा नियंत्रक, इंदिरा गांधी कृषि विश्वविद्यालय रायपुर।
2. अधिष्ठाता छात्र कल्याण, इंदिरा गांधी कृषि विश्वविद्यालय रायपुर।
3. अधिष्ठाता, कृषि महाविद्यालय, रायपुर/बिलासपुर/जगदलपुर/ अंबिकापुर, सरगुजा/कवर्धा/जांजगीर चांपा/ बलौदाबाजार–भाटापारा/ बेमेतरा कोरिया /कांकेर /रायगढ़ /राजनांदगांव ।
4. अधिष्ठाता, उद्यानिकी महाविद्यालय एवं अनुसंधान केन्द्र जगदलपुर/, राजनांदगांव।
5. अधिष्ठाता, उद्यानिकी महाविद्यालय एवं अनुसंधान केन्द्र, ।
6. अधिष्ठाता, बी.आर.एस.एम. कृषि अभियांत्रिकी एवं प्रौद्योगिकी महाविद्यालय, रायपुर/ मुंगेली ।
7. समस्त विभागाध्यक्ष,कृषि महाविद्यालय, ——।
8. प्राचार्य, भारतीय कृषि महाविद्यालय, पदमनाभपुर, पुलगांव चौक,दुर्ग /छत्तीसगढ़ कृषि महाविद्यालय, रिसाली (भिलाई) धनोरा रोड,पो०–हनौदा, जिला–दुर्ग / महामाया कृषि महाविद्यालय, नगरी रोड़,ग्राम–सियादेही, पो.– अरौद, धमतरी /श्रीराम कृषि महाविद्यालय, श्रीराम परिसर, ग्राम–ठाकुर टोला,पो.–सोमनी, राजनांदगांव, /कृषि महाविद्यालय, गायत्री मंदिर के पास, अम्बागढ चौकी, राजनांदगांव/भोरमदेव कृषि महाविद्यालय, ग्राम–घुघरी कला, खुटु नर्सरी के पास, कवर्धा/मार्गदर्शन संस्थान कृषि महाविद्यालय, रिंग रोड, चोपड़ा पारा अम्बिकापुर, सरगुजा/कृषि महाविद्यालय,चिता लंका कलेक्ट्रेट कार्यालय के सामने, दन्तेवाड़ा/कृषि महाविद्यालय, जोरापाली (केनापाली) रोड़, रायगढ़ (छ.ग.) ।
9. प्राचार्य, दन्तेश्वरी उद्यानिकी महाविद्यालय, मनोपचार हास्पिटल के पास माना बस्ती,रायपुर/रानी दुर्गावती उद्यानिकी महाविद्यालय, मेढुका,वि०ख०गौरेला,तह०–पेन्ड्रा रोड़,बिलासपुर /गायत्री उद्यानिकी महाविद्यालय, गोकुलपुर रूद्री रोड, धमतरी /के० एल० उद्यानिकी महाविद्यालय, गुण्डदेह रोड, पोटियाडीही, धमतरी (छ.ग.)।
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12. मान. कुलपति के निज सहायक, इंदिरा गांधी कृषि विश्वविद्यालय रायपुर।
13. सर्वर प्रभारी, इं.गां.कृ.वि.रायपुर। कृपया उपरोक्तानुसार सभी को ई–मेल करने का कष्ट करें।

कुलसचिव

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GOVERNMENT OF KARNATAKA

168691

1338701596

ಕರ್ನಾಟಕ ಪ್ರೌಢ ಶಿಕ್ಷಣ ಪರೀಕ್ಷಾ ಮಂಡಳಿ
Karnataka Secondary Education Examination Board

ಪ್ರಮಾಣ ಪತ್ರ
Certificate

ಈ ಕೆಳಗೆ ನಮೂದಿಸಿದ ಅಭ್ಯರ್ಥಿಯು ಎಸ್.ಎಸ್.ಎಲ್.ಸಿ. ಪರೀಕ್ಷೆಯಲ್ಲಿ ಕೆಳಗಿನ ವಿವರಗಳೊಂದಿಗೆ ತೇರ್ಗಡೆಯಾಗಿದ್ದಾನೆ/ದ್ದಾಳೆ ಎಂದು ಪ್ರಮಾಣೀಕರಿಸಲಾಗಿದೆ.

This is to certify that the candidate mentioned below has PASSED S.S.L.C. Examination with the following details.

ನೋಂದಣಿ ಸಂಖ್ಯೆ Register No. :	ತಿಂಗಳು/ವರ್ಷ Month/Year :	ಶಿಕ್ಷಣ ಮಾಧ್ಯಮ Medium of Instruction
20080824552	MARCH-2008	KANNADA

ಹೆಸರು Name : KARIYANNA B

ತಾಯಿಯ ಹೆಸರು Mother's Name : BHIMA BAI

ತಂದೆಯ ಹೆಸರು Father's Name : BHEERANNA

ಜನ್ಮ ದಿನಾಂಕ Date of Birth : 15-07-1991 FIFTEENTH JULY NINETEEN NINETY-ONE

ವಿಷಯಗಳು SUBJECTS	ಅಂಕಗಳು MARKS ಗರಿಷ್ಠ MAX.	ಕನಿಷ್ಠ MIN.	ಪಡೆದ ಅಂಕಗಳು MARKS OBTAINED	ಪಡೆದ ದರ್ಜೆ(ಶ್ರೇಣಿ) CLASS OBTAINED
ಪ್ರಥಮ ಭಾಷೆ/ FIRST LANGUAGE : KANNADA	125	44	115	
ದ್ವಿತೀಯ ಭಾಷೆ/ SECOND LANGUAGE : ENGLISH	100	35	085	
ತೃತೀಯ ಭಾಷೆ/ THIRD LANGUAGE : HINDI	100	35	072	
ಗಣಿತ/ MATHEMATICS	100	35	052	
ವಿಜ್ಞಾನ/ SCIENCE	100	35	088	
ಸಮಾಜ ವಿಜ್ಞಾನ/ SOCIAL SCIENCE	100	35	084	
ಒಟ್ಟು ಅಂಕಗಳು/ TOTAL MARKS	625	219	496	FIRST (79.36%) AN133

* ವಿದ್ಯಾರ್ಥಿಗಳು ಶೇ.85 ಮತ್ತು ಅದಕ್ಕಿಂತ ಹೆಚ್ಚಿನ ಅಂಕಗಳನ್ನು
* ಪ್ರಥಮ ದರ್ಜೆ: ಶೇ.60 ಮತ್ತು ಅದಕ್ಕಿಂತ ಹೆಚ್ಚಿನ ಅಂಕಗಳನ್ನು ಮತ್ತು ಶೇ.85ಕ್ಕಿಂತ ಒಳಗೆ.
* ದ್ವಿತೀಯ ದರ್ಜೆ: ಶೇ.50 ಮತ್ತು ಹೆಚ್ಚಿನ ಅಂಕಗಳನ್ನು ಮತ್ತು ಶೇ.60ಕ್ಕಿಂತ ಒಳಗೆ.
* ಒಂದೇ ಬಾರಿಗೆ ತೇರ್ಗಡೆ ಆದವರಿಗೆ ಮಾತ್ರ ದರ್ಜೆಯನ್ನು ನೀಡಲಾಗುವುದು.
* ಹೆಚ್ಚಿನ ಮಾಹಿತಿಗಾಗಿ
ಪ್ರತಿ ವಿಷಯದಲ್ಲಿ ಕನಿಷ್ಠ ಶೇ.30 ಅಂಕಗಳು ಮತ್ತು ಒಟ್ಟಾರೆ ಶೇ.35 ಅಂಕಗಳು.

* DISTINCTION : 85% AND ABOVE
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* SECOND CLASS : 50% AND ABOVE BUT BELOW 60%
* CLASS IS DECLARED FOR THOSE WHO PASS IN FIRST ATTEMPT ONLY.

PASS IN EXAMINATION :
30% MIN. IN EACH SUBJECT AND 35% IN AGGREGATE

KSEEB KSEEB KSEEB KSEEB KSEEB KSEEB KSEEB KSEEB KSEEB KSEEB

ಪಡೆದ ಒಟ್ಟು ಅಂಕಗಳು (ಅಕ್ಷರಗಳಲ್ಲಿ) TOTAL MARKS OBTAINED (In words) : FOUR HUNDRED NINETY-SIX ONLY

ಶಾಲೆಯ ಹೆಸರು ಮತ್ತು ವಿಳಾಸ
NAME OF THE SCHOOL & ADDRESS :
GOVERNMENT JUNIOR COLLEGE
PEENYA
BANGALORE NORTH

ದಿನಾಂಕ DATE : 5-MAY-2008

ಕಾರ್ಯದರ್ಶಿಗಳು
ಕರ್ನಾಟಕ ಪ್ರೌಢ ಶಿಕ್ಷಣ ಪರೀಕ್ಷಾ ಮಂಡಳಿ
SECRETARY
KARNATAKA SECONDARY EDUCATION EXAMINATION BOARD
ಬೆಂಗಳೂರು BANGALORE

No: DST/INSPIRE Fellowship/[IF160900]
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE and TECHNOLOGY
Department of Science and Technology
Technology Bhawan, New Mehrauli Road
New Delhi-110016

Date: 21 September, 2017

Subject: Award of INSPIRE Fellowship to the Research Students [IF160900]

Dear Kariyanna B ,

The Government of India has launched a unique Scheme **"Innovation in Science Pursuit for Inspired Research (INSPIRE)"** with several components. INSPIRE Fellowship provides fellowship in Basic and Applied Sciences. I am pleased to inform you that you have been Selected for the award of INSPIRE Fellowship to hostthe same at the University/Institute/College/National Laboratory as indicated in the application form of your subsequent admission.

The value of the Fellowship will be at Par with the Junior Research Fellowship (JRF)/ Senior Research Fellowship (SRF) of Government of India along with a Contingency grant. The Fellowship shall be available to you from INSPIRE Fellowship Effective Date (which will be communicated through 1st Sanction Order) for a period of five years or completion of your doctoral (PhD) program, whichever is earlier.

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Scientist 'C'

Kariyanna B
C/O : BHEERANNA
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State/UT: KARNATAKA - 560090

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Date: 21 September, 2017

GENERAL INSTRUCTIONS

Dear Kariyanna B ,

Please note that these documents are necessary to upload within one month from the date of this letter at INSPIRE Online Webportal only for financial release after final selection

1. Joining-cum-Acceptance (JCA) Letter for INSPIRE Fellowship (template available in your dashboard in online portal))
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3. Copy of Cancelled Blank Cheque of the Host University/ College/ Institute Account for transferring the fund (Account should be registered in Public Financial Management System: https://pfms.nic.in).
4. PFMS registration certificate, or Unique Agency Code in case of University/College/Institute is newly registered in Public Financial Management System.
5. Since the fund would be transferred under Science & Technology Institutional and Human Capacity Building (1817), Hence bank account number of Host University/College/Institute should be registered under this scheme in PFMS.
6. **Please note that these documents are necessary to upload within one month from the date of this letter at INSPIRE Online Webportal only, otherwise it will be treated as the candidate has not intrested in accepting the INSPIRE Fellowship.**

Thanking you,

Dr. S. Mallikarjuna Babu
Scientist 'C'

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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Additional supporting information may be found online in the Supporting Information section at the end of the article.

Received 5 April 2020; accepted 14 July 2020

DOI: 10.1002/bab.1997

Published online in Wiley Online Library (wileyonlinelibrary.com)

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.

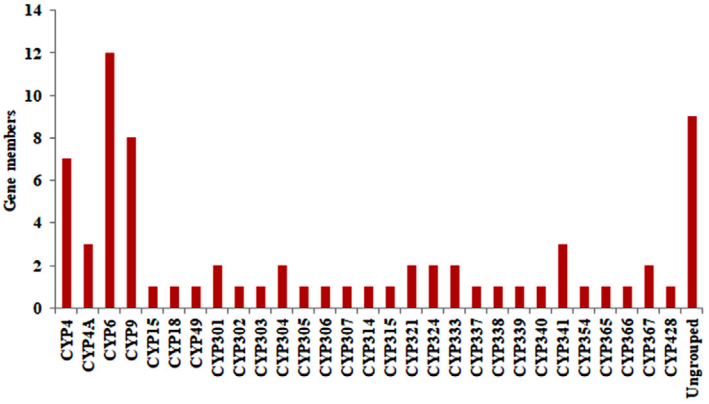

FIG. 1 *Distribution of CYP subfamilies under different families of CYP450.*

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

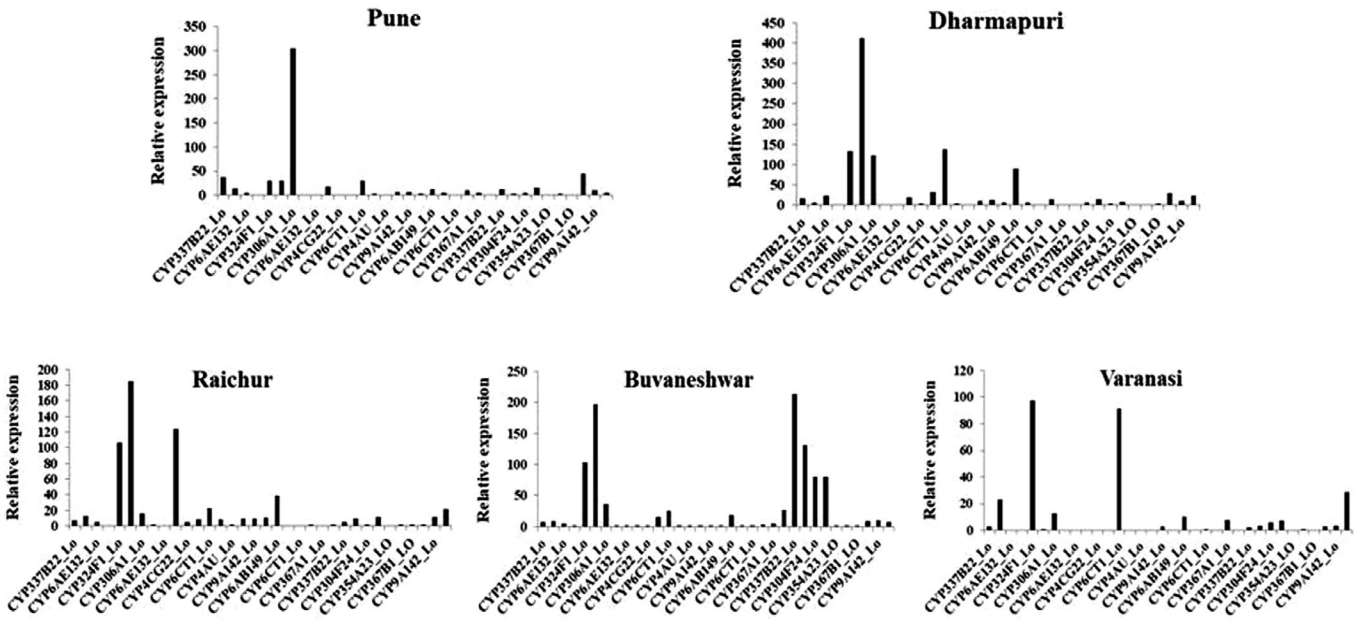

FIG. 2 *Transcription profiling of cytochrome P450 genes in field collected populations of* L. orbonalis *depicted as fold change over the susceptible Lo-S colony. (A) Pune, (B) Dharmapuri, (C) Raichur, (D) Bhubaneswar, and (E) Varanasi.*

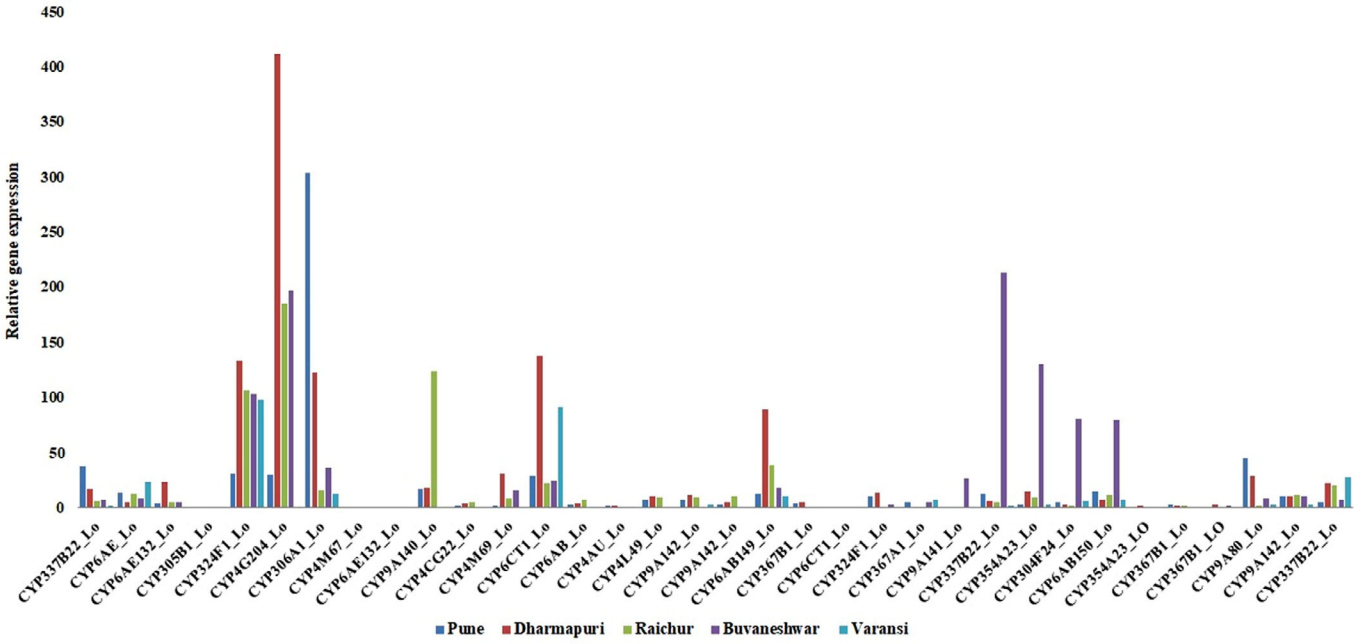

FIG. 3 *Differential expression of CYP450 genes in five field collected populations of* L. orbonalis *over susceptible populations.*

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

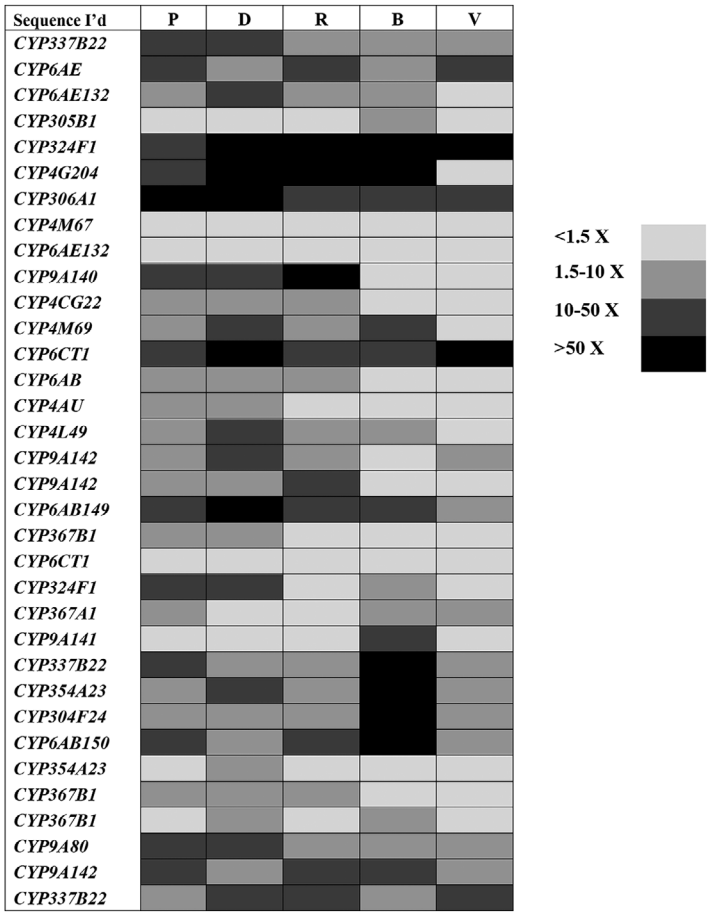

FIG. 4 *Expression profiles (fold changes over Lo-S population) of cytochrome P450 genes across field collected* Leucinodes orbonalis *populations using heat map (P, Pune; D, Dharmapuri; R, Raichur; B, Bhubaneswar; V, Varanasi). The fold changes are indicated in different shades indicate significant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

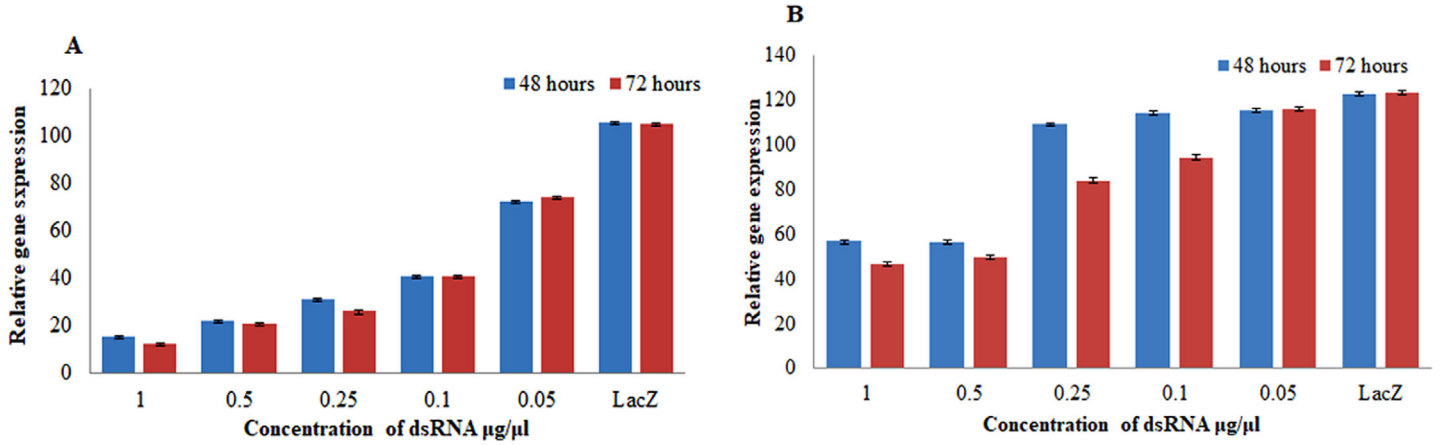

FIG. 5 *Relative expression of (A) CYP324F1 and (B) CYP306A1 after dsRNA treatment.*

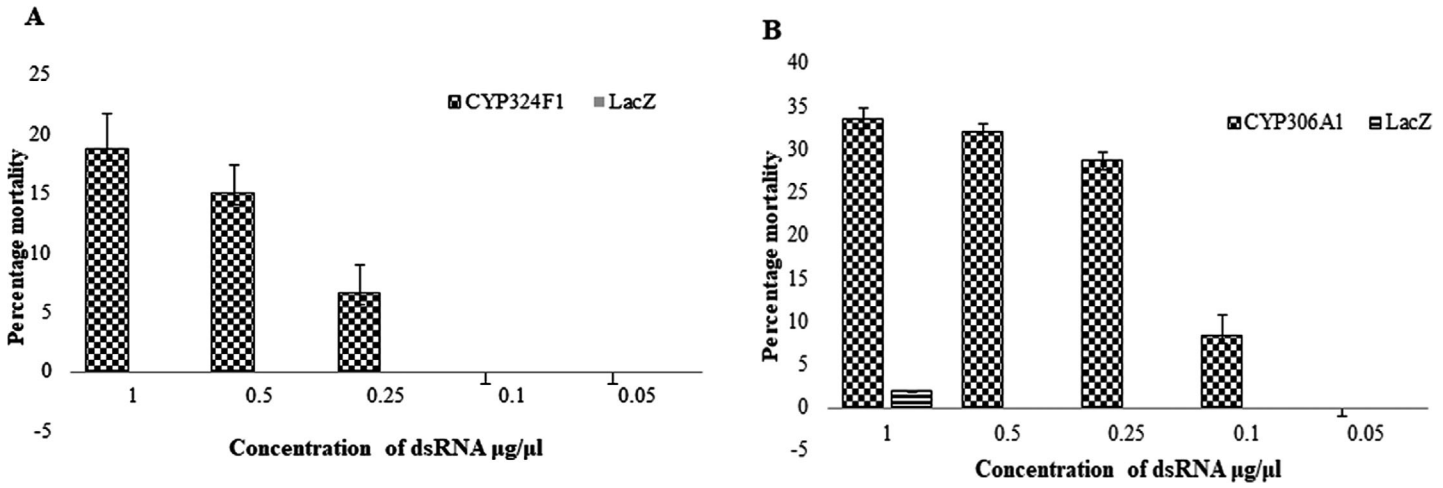

FIG. 6 *Percentage mortality of* L. orbonalis *after treating with dsRNA.*

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*.

5. Acknowledgements

The authors profusely thank Indian Council of Agricultural Research for generous funding under CRP Genomics project. The first author acknowledges the award of DST-Inspire fellowship (2016/IF160900). The authors acknowledge the help rendered by Dr. Seema Pradhan, Institute of Life Science,

Bhubaneswar, Odissa and Dr. Aditi Agrawal, ICAR-NBAIR, Bengaluru, Karnataka for guiding bioinformatic analysis. The authors profusely thank Dr. David R. Nelson, University of Tennessee, USA for identifying and naming of the CYP genes. The timely release of fund by ICAR-NBFGR and laboratory facilities extended by Director, ICAR-NBAIR, and Director, ICAR-IIHR, Bengaluru is gratefully acknowledged. The authors thank the two anonymous reviewers and Dr. K. Subaharan, ICAR-NBAIR for his critical comments on the manuscript.

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.

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Plant Archives Vol. 20, Supplement 2, 2020 pp. 1255-1261 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

INSECTICIDE USAGE PATTERN AND EVOLUTION OF RESISTANCE IN EGGPLANT SHOOT AND FRUIT BORER, *LEUCINODES ORBONALIS* GUENÉE (LEPIDOPTERA: CRAMBIDAE) IN INDIA

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Abstract

The shoot and fruit borer, *Leucinodes orbonalis* is the major insect pest of eggplant, causes severe yield loss up to 90 per cent. Chemical insecticides remain the sole option for control of *L. orbonalis*, adopted by most of the farmers. Indiscriminate use of various insecticides led to insecticide resistance and subsequent control failure. The pesticide usage history on eggplant revealed that, the farmers from Dharmapuri area sprayed 22.6 times (5.4 insecticides), followed by Raichur and Guntur area with 21.6 times (8.9 insecticies) and 21.4 times with 7.9 insecticides. The *L. orbonalis* collected from the hilly tracts of Almora and Pune received no or negligible amount of insecticides. The dose-mortality bioassays on field-collected populations of *L. orbonalis* indicated, varying levels of resistance development against phosalone (139.6 to 526.6 fold), flubendiamide (31.9 to 363.9 fold), fenvalerate (37.2 to 128.7 fold), emamectin benzoate (9 to 46 fold) and thiodicarb (7.2 to 22.8 fold) as compared to susceptible iso-female colony (Lo-S). Continous and indiscriminate use of the insecticides poses increased selection pressure that leads to development of insect resistance to insecticide.

Keywords **:** Eggplant, *Leucinodes orbonalis*, insecticide resistance, usage pattern.

Introduction

The eggplant, *Solanum melongena* is native to India, widely cultivated in many Asian countries (Doijode, 2001; Tsao and Lo 2006; Anonymous 2014). India stands second in production after China with 0.66 million hectares and a production of 12.4 million tonnes (Anonymous, 2017). West Bengal, Odisha, Gujarat, Bihar, Madhya Pradesh, Chhattisgarh, Karnataka, Maharashtra, Andhra Pradesh, Haryana, Assam, Uttar Pradesh, Jharkhand and Tamil Nadu are the leading states for commercial eggplant production.

Eggplant crop is damaged by more than 53 important insect pests, out of which 8 species are considered as major pests causing severe damage to the crop. The eggplant shoot and fruit borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) is a chronic pest of eggplant that damages the crop throughout the crop season (Biswas *et al.,* 1992; Bhadauria *et al.,* 1999; Muthukrishnan *et al.,* 2005). Yield losses up to 88.7 per cent have been reported in many countries despite best management practices (Haseeb *et al.,* 2009, Mishra *et al.,* 2014). Due to cryptic nature of the larvae, farmers generally adopt calendar-based prophylactic insecticidal sprays to avoid cosmetic damage on the fruits (Chatterjee and Roy, 2004; Sharma *et al.,* 2004; Mishra and Dash, 2007). Out of 13-14 per cent of pesticides used on vegetables in India, eggplant receives the maximum pesticide sprays after chilli (Kodandaram *et al.,* 2013). Due to continous pesticide application evidence of insecticide resistance has been documented in *L. orbonalis* (Kodandaram *et al.,* 2015; Kariyanna *et al*., 2019). The large population size and year-round multiplication capacity of *L. orbonalis* coupled with continuous exposure to various chemicals might further accelerate resistance evolution in this pest. The present study attempted to analyse the insecticide usage pattern on eggplant and level of insecticide resistance in *L. orbonalis* against selected insecticides.

Materials and Methods

Insect collection and maintenance

The field populations of *L. orbonalis* were collected from intensive eggplant growing regions of India *viz*., North India: Varanasi (18.5204° N, 73.8567° E), Delhi (16.2120° N, 77.3439° E), Almora (16.2120° N, 77.3439° E); Central India: Nagpur (16.2120° N, 77.3439° E); Eastern India: Bhubaneshwar (20.2961° N, 85.8245° E); Western India: Pune (18.5204° N, 73.8567° E); South India: Raichur (16.2120° N, 77.3439° E), Guntur (16.2120° N, 77.3439° E), Dharmapuri (12.0933° N, 78.2020° E) and Coimbatore (16.2120° N, 77.3439° E) (Fig. 1) during 2016-2019. Collected field populations were reared under laboratory conditions at 27 ± 2 ºC, 60-70 per cent RH, and a photoperiod of 14:10h (L:D) on a natural diet (Fig. 2) and the F1 individuals were used for bioassay and biochemical studies. The iso-female line (Lo-S) derived from the Bengaluru area (12.9716° N, 77.5946° E) has been maintaining (since October 2012) at insect genomic resources laboratory at ICAR-NBAIR was used as susceptible control and designated as Lo-S.

Data on insecticide usage pattern

The insecticides that are very frequently used on the eggplant to control *L. orbonalis* were recorded from local farmers. The details on types, numbers and frequency of insecticides used along with insecticides rotation pattern and damage percentage were collected from each location (Table 1; Fig. 1).

Insecticide resistance bioassays

The commonly used commercial formulations *viz.,* fenvalerate (20% EC), emamectin benzoate (5% EC), phosalone (35%), thiodicarb (75%) and flubendiamide (20%) were selected based on their usage history on eggplant by farmers for the control of *L. orbonalis*. The filter paper residue assay (Cheng *et al.,* 2010) with crucial adjustments

was used for insecticide resistance bioassays on early second instar larvae of *L. orbonalis* larvae. Based on the preliminary bioassay five to seven appropriate concentrations of each of the selected insecticides were prepared. Filter paper discs of approximately 4.5 cm diameter were immersed in the required dilutions and vertically dried under shade for 1 hour. Then paper discs placed individually in the plastic containers ~5 cm diameter and 10-second instar larvae were allowed on each concentration in three to four repetitions (Fig. 3). After 24 hours of incubation, the larvae were shifted to an untreated natural diet (Fig. 4). The mortality of larvae was recorded from 48 hours. The filter paper residue assay was found simple, precise and consistent in larval mortality for contact insecticides and hence this method was adopted subsequently in all dose-mortality bioassays. The larval mortality data were grouped and used for probit analysis by POLO software (Leora, 1994). For each population, the lethal concentration to kill 50% of the test larvae (LC_{50}) was assessed. The resistance ratios (RR) were analysed by using the formula: LC_{50} of field population/LC_{50} of the Lo-S population.

Fig. 1 : *Leucinodes orbonalis* populations collected from different part of India

Results and Discussion

Insecticide history during crop period

The insecticides used by the farmers in the surveyed location were more diverse and greater numbers than the recommended. Among the eleven collected population (Fig. 1) the Raichur population having highest insecticide uasage history (8.88), followed by Coimbatore (8), Dharmapuri (7.62), Delhi (7.3), Guntur (7.28), Varanasi (7), Nagpur (7) and Bhuvaneshwar (5.33). In contrary, Pune (0.9) (in hilly area) and Almora (0.7) were reported less number of insecticides. The highest number of sprays observed in Coimbatore (22.8) and Dharmapuri (22.62 times), the result was concurrence with 15-time insecticide spray on eggplant and cauliflower, 13 and 12 times spray in chilli and bhendi crop respectively (Jeyanthi and Kombairaju, 2005).

Fig. 2 : Improved rearing method of *L. orbonalis* using natural host

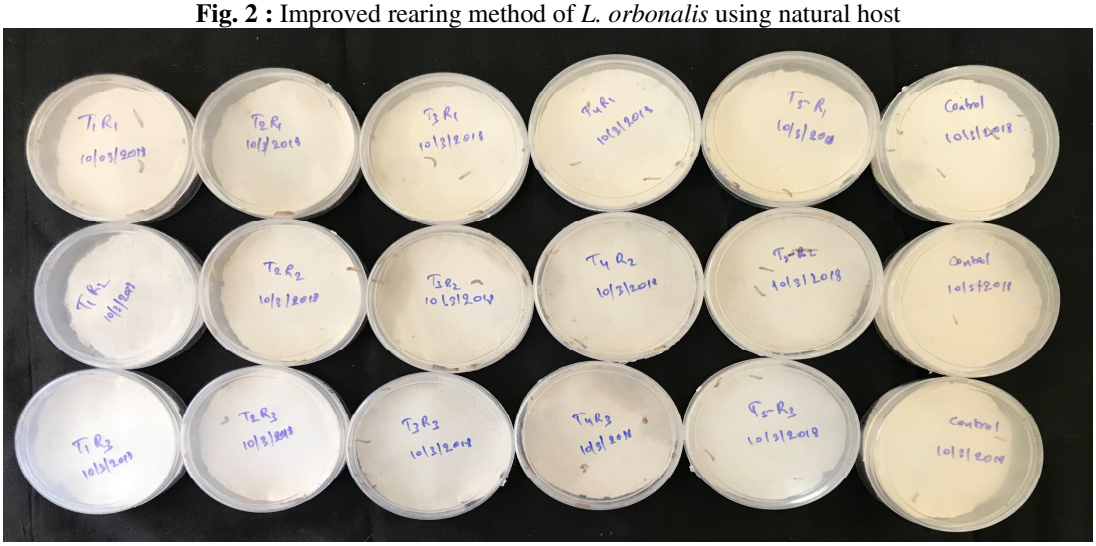

Fig. 3 : Larvae of *L. orbonalis* released on Filter paper discs for 24 hours

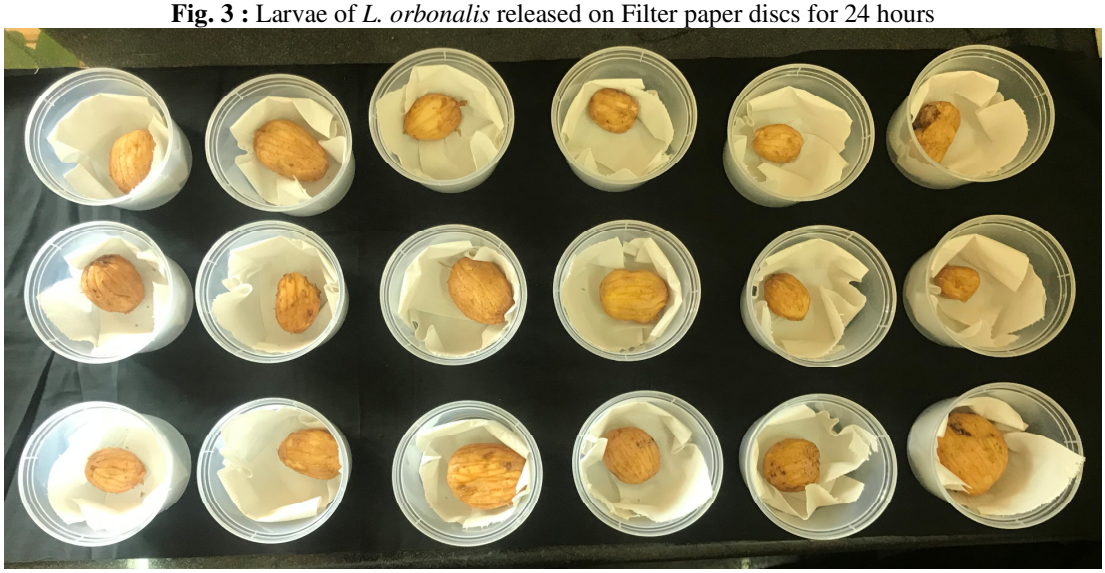

Fig. 4 : Larvae of *L. orbonalis* allowed on natural potato diet for 48-72 hours after filter paper incubation to record the mortality

The overall insecticide usage history in eggplant growing area of India revealed that, the emamectin benzoate is the most commonly used insecticides (12%) in all the locations followed by chlorantraniliprole (10%) (Fig. 5). The spinetoram, fenvalerate, acephate moderately using insecticides (~8%) and corboryl, chlorpyriphos, dimethoate, alphamethrin, triazophos contributing 2 per cent or less in managing *L. orbonalis*. Similarly, highest number of organophosphates and amide group of insecticide to manage insect pests in eggplant and cauliflower were from Himachal Pradesh (Kumar *et al.,* 2017 and Gaganpreet *et al.,* 2018). In bhendi neonicotinoids and combinations of insecticides were largely used to manage sucking pest (Meenambigai *et al.,* 2017) and pyrethroids were the most commonly used insecticides on eggplant followed by organophosphate (Jeyanthi and Kombairaju, 2005).

Table 1 : Insecticides usage pattern against *L. orbonalis* on eggplant

Sl. No.	Place of collection	Commonly used insecticides	Number of farmers interviewed	Number of sprays / crop season (mean ±SE)	Number of insecticides used (mean ±SE)
1.	Almora	Organic by default, rarely fenvalerate	12	0.7 ±0.06	1.0
2.	Bhubaneshwar	Acephate, phosalone, chlorantraniliprole, emamectin benzoate, flubendiamide, acetamaprid	6	18.83 ±2.57	5.33 ±0.33
3.	Coimbatore	Phosalone, acetamaprid, acephate, emamectin benzoate, flubendiamide, spinetoram, fipronil, deltamethrin, fenvalerate	5	22.8 ±1.71	8 ±0.7
4.	Delhi	Chlorantraniliprole, endosulfon, profenophos, corboryl, chlorpyriphos, deltamethrine, dimethoate, emamectin benzoate, endosulfan	3	20 ±1.15	7.3 ±0.8
5.	Dharmapuri	Fipronil, emamectin benzoate, phosalone, fenvalerate, phosalone, chlorantraniliprole, flubendiamide, dimethoate	8	22.62 ±2.18	7.62 ±0.37
6.	Guntur	Acetamaprid, acephate, emamectin benzoate, spinetoram, deltamethrin, cartap-hydrochloride, fipronil, alphamethrin, profenophos	7	21.42 ±1.32	7.28 ±0.52
7.	Nagpur	Emamectin benzoate, acetamaprid, fipronil, acephate, fenvalerate, cartap-hydrochloride, spinetoram, chlrorantraniliprole	6	18.33 ±1.05	7±0.5
8.	Pune	Largely organic, ocaasionally treated with spinetoram and fipronil	10	3.7 ±1.32	0.9 ±0.31
9.	Raichur	Fipronil, acephate, alphamethrin, profenophos, acetamaprid, cartap-hydrochloride, emamectin benzoate, spinetoram, triazophos, monocrotophos, chlorantraniliprole	9	21.55 ±1.38	8.88 ±0.58
10.	Varanasi	Spinosad, fenvalerate, deltamethrin, phosalone, flubendiamide, cartap-hydrochloride, chlorantraniliprole, emamectin benzoate	5	20 ±0.7	7 ±0.54
11.	Bengaluru (Lab S)	Insecticide susceptible (Being maintained since 2012)	-	-	-

Insecticide resistance monitoring

The usage pattern of insecticide to manage *L. orbonalis* indicated that, the insecticides groups *viz.,* organophosphate, carbamate, pyrethroids, synthetic actinomycetes derived chemicals, amide and cartap hydrochloride were commonly used in most of the eggplant growing location. The LC_{50} helps to determine the resistance ratio against the applied insecticides. In present study, the dose-mortality bioassays with insecticides like fenvalerate, phosalone, emamectin benzoate and flubendiamide demonstrated different levels of toxicity against the filed collected *L. orbonalis* compared Lo-S (Table 2).

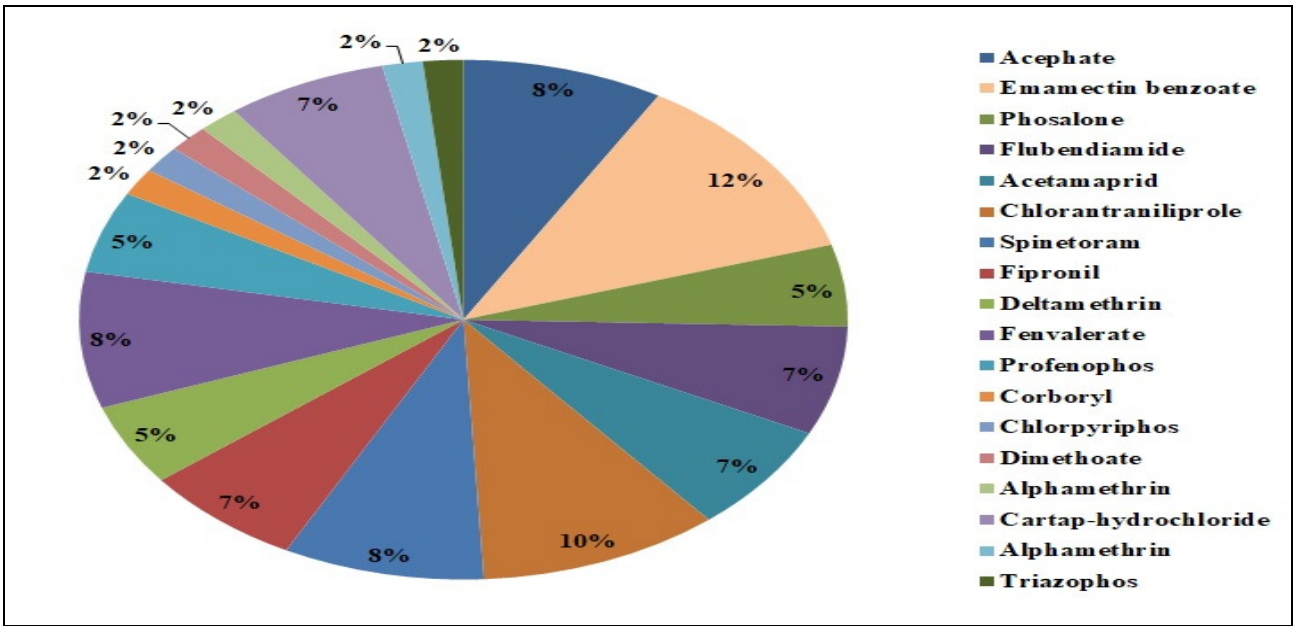

Fig. 5 : Frequency of pesticide used across the eleven eggplant growing regions of India

The bioassay using fenvalerate depicted that, the LC_{50} of Nagpur (128.7 ppm) was highest followed Delhi (114.2 ppm) and least observed in case of Coimbatore (37.2 ppm) populations over Lo-S (table 2) and the range of LC_{50} observed from 1.62 to 259.2 ppm. Similar trend was observed (35.0 ppm to 226.33 ppm) against cypermethrin 10EC on *L. orbonalis* (Ranjith Kumar, 2014).

For phosalone the highest LC_{50} was Nagpure (632.0 ppm) followed by Delhi (483.3 ppm) and least was from Coimbatore (167.5 ppm) population over Lo-S (table 2). Phosalone insecticide has widest LC_{50} range from 1.2 to 632.0 ppm in all the populations. The result was supported with a maximum range of LC_{50} from 140.5 to 1322.5 ppm against monocrotophos on 14 *L. orbonalis* populations across India (Ranjith Kumar, 2014). But the spectrum of LC_{50} was comparatively low when quinolphos used as insecticide against fourteen *L. orbonalis* populations (Ranjith Kumar, 2014).

Thiodicarb displayed the highest LC_{50} in Guntur (264.2 ppm) followed Nagpur (262.8 ppm) and least was observed in Coimbatore (83.2 ppm) population over Lo-S (table 2). The thiodicarb LC_{50} range was 83.2-264.2 ppm in all the tested populations. The data was supported with the LC_{50} of 50-313.53 ppm in 14 population collected across India (Ranjith Kumar, 2014).

The LC_{50} of flubendiamide was highest in Nagpur (83.7 ppm) followed by Delhi (53.0 ppm) and least was observed in Coimbatore (7.34 ppm) over Bengaluru (Lo-S) (table 4). The range of LC_{50} was 7.34-83.7 ppm in all the tested populations. Similalry, for Punjab local populations 11.2 ppm were reported (Kodandaram *et al.,* 2013) and 2.10-28.20 ppm in 14 *L. orbonalis* species tested across India (Ranjith Kumar, 2014).

Emamectin benzoate displayed the least LC_{50} value compared to other insecticides, in which highest was observed in Delhi (0.46 ppm) followed by Nagpur (0.38 ppm) and least was observed in Coimbatore (0.06 ppm) population over Lo-S (table 4). Among all the chemicals use the emamectin benzoate has shown greater mortality with least LC_{50} value (0.06-0.46 ppm) and fold changes (9-46 folds) against all the tested field populations (Table 2). Similar toxicity range of LC_{50} value (302.8 ppm) was reported in neonate *L. orbonalis* larvae (Anil and Sharma, 2010) and from 0.061 to 0.24 ppm in *Leucinodes spp* in Punjab (Kaur *et al.,* 2014).

Table 2 : Insecticide resistance pattern in field populations of *L. orbonalis*

Insecticide	Population	LC_{50} (ppm)	Slope ±SE	F. limits		χ^2 heterogeneity (DF)	Resistance ratio
				Lower	upper		
Fenvalerate	Guntur	93.9	3.2± 0.2	56.8	125.5	3.93 (3)	58.1
	Coimbatore	61.2	2.8± 0.56	30.8	97.5	1.02 (4)	37.2
	Nagpur	208.5	2.2 ± 0.56	103.5	603.2	2.87 (3)	128.7
	Delhi	185.7	3.1 ±0.53	98.05	412.9	6.35 (4)	114.2
	Bengaluru (Lo-S)	1.62	2.2 ± 0.42	0.85	3.57	2.62 (4)	-
Phosalone	Guntur	191.1	1.9± 0.2	109.1	281.1	3.91 (3)	159.3
	Coimbatore	167.5	2.3± 0.34	123.8	245.2	7.0 (4)	139.6
	Nagpur	632.0	2.1 ± 0.09	315.8	2560.5	0.74 (3)	526.6
	Delhi	483.3	1.93± 0.14	243.7	957.5	0.96 (3)	402.8
	Bengaluru (Lo-S)	1.2	1.2± 0.21	0.521	2.42	1.91 (3)	-
Emamectin benzoate	Guntur	0.19	1.1± 0.10	0.07	0.26	1.2 (3)	19
	Coimbatore	0.06	1.3± 0.14	0.03	0.09	0.62 (4)	9
	Nagpur	0.38	2.9 ± 0.21	0.220	1.390	8.62 (4)	38
	Delhi	0.46	1.91 ± 0.19	0.231	0.953	3.77 (3)	46
	Bengaluru (Lo-S)	0.01	1.4 ± 0.07	0.004	0.051	3.17 (3)	-
Thiodicarb	Guntur	264.2	2.2 ± 0.21	122.1	468.1	2.4 (3)	22.8
	Coimbatore	83.2	1.6 ± 0,32	42.78	193.2	6.9 (4)	7.2
	Nagpur	262.8	1.9 ± 0.14	145.9	534.6	7.3 (4)	22.6
	Delhi	106.5	1.82 ± 0.39	55.37	278.4	2.4 (3)	9.17
	Bengaluru (Lo-S)	11.61	1.73±0.32	5.462	23.56	4.7 (3)	-
Flubendiamide	Guntur	42.9	1.72 ± 0.10	22.4	94.02	2.9 (3)	186.5
	Coimbatore	7.34	0.99 ± 0.10	3.23	16.72	6.2 (4)	31.9
	Nagpur	83.7	2.11 ± 0.21	40.00	253.9	3.9 (3)	363.9
	Delhi	53.0	1.92 ± 0.28	26.91	118.0	3.7 (3)	230.4
	Bengaluru (Lo-S)	0.23	1.21 ± 0.26	0.09	1.01	2.1 (3)	-

Conclusion

The present investigation revealed that, the insecticide usage pattern across India with the extent of insecticide resistance in various field collected populations of *L. orbonalis*. Thus, it is important to examine each possible factor that leads to the evolution of insecticide resistance. Lack of resistance in certain populations does not exclude resistance risk in the future. Hence, the insecticide resistance management strategies should essentially include atleast moderately tolerate eggplant cultivar, monitoring of population by pheromone traps, need-based application of effective and softer insecticides in rotation with biopesticides, botanicals and bioagents. Understanding the level of insecticide resistance before a control measure is deployed would help in effective resistance management of *L. orbonalis*.

Acknowledgement

The authors profusely thank the Indian Council of Agricultural Research for generous funding under the CRP Genomics project. The first author acknowledges the award of DST-Inspire fellowship (2016/IF160900). The facilities extended by the Director, ICAR-NBAIR is gratefully acknowledged.

Disclosure statement

The authors have no potential conflict

Reference

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frontiers in Physiology

ORIGINAL RESEARCH
published: 19 November 2020
doi: 10.3389/fphys.2020.594845

Genome Mining and Expression Analysis of Carboxylesterase and Glutathione S-Transferase Genes Involved in Insecticide Resistance in Eggplant Shoot and Fruit Borer, *Leucinodes orbonalis* (Lepidoptera: Crambidae)

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: 14 August 2020
Accepted: 22 October 2020
Published: 19 November 2020

Citation:
Kariyanna B, Prabhuraj A, Asokan R, Agrawal A, Gandhi Gracy R, Jyoti P, Venkatesan T, Bheemanna M, Kalmath B, Diwan JR, Pampanna Y and Mohan M (2020) Genome Mining and Expression Analysis of Carboxylesterase and Glutathione S-Transferase Genes Involved in Insecticide Resistance in Eggplant Shoot and Fruit Borer, Leucinodes orbonalis (Lepidoptera: Crambidae). Front. Physiol. 11:594845. doi: 10.3389/fphys.2020.594845

The shoot and fruit borer, *Leucinodes orbonalis* (Lepidoptera: Crambidae) is the major cause of low productivity in eggplant and insecticides being the mainstay of management of *L. orbonalis*. However, field control failures are widespread due to the evolution of insecticide resistance. Taking advantage of the whole genome sequence information, the present study investigated the level of insecticide resistance and the expression pattern of individual carboxylesterase (CE) and glutathione S-transferases (GSTs) genes in various field collected populations of *L. orbonalis*. Dose-mortality bioassays revealed a very high level of resistance development against fenvalerate (48.2–160-fold), phosalone (94-534.6-fold), emamectin benzoate (7.2–55-fold), thiodicarb (9.64–22.7-fold), flubendiamide (187.4–303.0-fold), and chlorantraniliprole (1.6–8.6-fold) in field populations as compared to laboratory-reared susceptible iso-female colony (Lo-S). Over-production of detoxification enzymes *viz.*, CE and GST were evident upon enzyme assays. Mining of the draft genome of *L. orbonalis* yielded large number of genes potentially belonging to the CE and GST gene families with known history of insecticide resistance in other insects. Subsequent RT-qPCR studies on relative contribution of individual genes revealed over-expression of numerous GSTs and few CEs in field populations, indicating their possible involvement of metabolic enzymes in insecticide resistance. The genomic information will facilitate the development of novel resistance management strategies against this pest.

Keywords: ***Leucinodes orbonalis*, insecticide resistance, genome, arboxylesterase, gene expression, glutathione S-transferase, carboxylesterase**

HIGHLIGHTS

- High levels of multiple insecticide resistance in field collected *Leucinodes orbonalis* populations.
- Enhanced midgut activities of carboxylesterase and glutathione S-transferase enzymes.
- Qualitative changes in esterase banding pattern in resistant populations.
- Mining and retrieval of 94 carboxylesterase and 33 glutathione S-transferase full length gene sequences from draft assembled genome and transcriptome of *L. orbonalis*.
- Over-expression of large number of glutathione S-transferase and few carboxylesterase genes in resistant field collected populations.

INTRODUCTION

Eggplant or aubergine (*Solanum melongena*), also called as brinjal, is one of the most popular vegetable crops in south-east Asia especially India, China, and Bangladesh. The most important limiting factor in brinjal cultivation is the damage caused by shoot and fruit borer, *Leucinodes orbonalis* Guenee (Lepidoptera: Crambidae). It was first described from India and now it is distributed all over Asia, Africa, and in few parts of Europe (Mally et al., 2015). In India and Bangladesh, it causes severe yield losses up to 93% despite best management practices (Kodandaram et al., 2017; Prodhan et al., 2018). Surveys in India and Bangladesh indicated that farmers spray chemical insecticides up to 84 times during a 6–7 month cropping season. A very high level of pesticide load at a concentration 40–450 times higher than the maximum residue limit (MRL) in marketed brinjal fruits poses major health concerns (Srinivasan, 2008; Latif et al., 2010; Kariyanna et al., 2020b). It is also considered as a pest of quarantine significance to the number of countries outside its native range. *L. orbonalis* has high reproductive potential with overlapping generations. Studies reporting high level of insecticide resistance development in *L. orbonalis* might be attributed to the long-term indiscriminate use of various insecticides on eggplant (Kodandaram et al., 2017; Shirale et al., 2017).

Resistant insects overcome the toxic effect of insecticide molecules by adopting one or more mechanisms ranging from cuticular thickening, nerve impenetration, altered production of metabolic enzymes, target site insensitivity, enhanced excretion through ABC transporters as well as by gut symbionts (Mohan et al., 2015). The enhanced detoxification of insecticides by metabolic enzymes *viz.*, carboxylesterases (CE) and glutathione S-transferases (GSTs) are commonly reported in resistant populations of many insect species and mites (Ranson et al., 2002; Enayati et al., 2005; Oakeshott et al., 2005; Perry et al., 2011; Das and Dutta, 2014; Akıner and Ekşi, 2015; Benoit et al., 2015). The resistant individuals metabolize the insecticides faster due to higher catalytic rate or enhanced production of the enzyme as a consequence of increased transcription or gene duplication (Panini et al., 2016; Kariyanna et al., 2020a). The GSTs are phase II metabolic enzymes and are distributed in most of the animals (Hayes and Pulford, 1995). Among several subclasses of GST, the enzymes which are frequently associated with the insecticide resistance are Delta and Epsilon classes (Friedman, 2011; Lumjuan et al., 2011). Similarly, enhanced metabolism of organophosphates, carbamates, and pyrethroids are frequently associated with CEs through gene amplification, upregulation, mutations by coding sequence, or a combination of all these mechanisms (Zhang et al., 2012; Cui et al., 2015).

Considering the economic and social impact of *L. orbonalis*, genetically modified eggplant expressing insecticidal *cry1Ac* gene from *Bacillus thuringiensis* has been developed (Shelton et al., 2018), but, a moratorium on its commercialization was imposed by Indian Government due to public concern. Though *Bt* eggplant is commercialized in Bangladesh way back in 2014, yet the area under cultivation is <2500 Ha (Shelton et al., 2018) due to various reasons. Baring *Bt* brinjal, the insecticides remain the sole method of controlling *L. orbonalis* in all the eggplant growing countries. With the availability of genome sequence, the present study investigated the level of insecticide and the expression pattern of CE and GST genes from field collected insecticide resistant *L. orbonalis* populations, to pinpoint the key metabolic genes involved in insecticide degradation.

MATERIALS AND METHODS

Insect Collection and Maintenance

The field populations of *L. orbonalis* larvae were collected during 2017–2018 from intensive eggplant growing regions of India *viz.*, Raichur (16.2120° N, 77.3439° E), Dharmapuri (12.0933° N, 78.2020° E), Bhubaneshwar (20.2961° N, 85.8245° E), Pune (18.5204° N, 73.8567° E), and Varanasi (25.3176° N, 82.9739° E). All the field populations were reared under laboratory conditions at 27 ± 2°C, 60–70% relative humidity (RH), and a photoperiod of 14:10 h (L:D) on a natural diet and the F_1 individuals were used for bioassay and biochemical studies. The insecticide susceptible iso-female line (National Accession number: NBAIR-IS-CRA-01A), designated as Lo-S (65th generation) was originally derived from *L. orbonalis* collected near Bengaluru (12.9716° N, 77.5946° E) and maintained at insect genomic resources laboratory at ICAR-NBAIR.

Insecticide Resistance Bioassays

Insecticides fenvalarate (20% EC), emamectin benzoate (5% EC), phosalone (35%), thiodicarb (75%), flubendiamide (20%), and chlorantraniliprole (18.5%) were selected for conducting dose-mortality bioassays based on their usage history on brinjal by farmers. Filter paper residue assay (Cheng et al., 2010) with essential modifications was used for insecticide resistance bioassays on early second instar larvae of *L. orbonalis*. Five to seven appropriate concentrations of each of the selected insecticides were prepared based on the dose bracketing technique. The filter paper discs (4.5 cm diameter) were dipped in the appropriate dilutions and dried vertically under shade for 1 h. Then, the discs were placed individually in plastic containers (5 cm diameter) and 10 larvae were released in each container. Moistened filter paper discs without insecticide

TABLE 1 | Primer details for carboxylesterase (CE) genes.

Sl. No	Sequence		Primer sequence 5′→3′	Length	GC (%)	Tm (°C)	Sequence length	Product size	E (%)
1.	Contig8459-0.14	F	GTGTCAAACGTGTTCAGAATC	21	43	54	2796	100	91.43
		R	TACCCAGGAAAGTAGTGTAGT	21	43	54			
2.	Contig12636-0.8	F	CTTGCACTCTGTTCACTAACT	21	43	54	2712	95	88.17
		R	TCTGTCGTCGTTGTTGTATTC	21	43	54			
3.	Contig11486-0.8	F	TCACATGAGACTGGGTAGAA	20	48	55	3936	95	103.22
		R	CTCTGTAGTGTCTGTCGTAGA	21	45	54			
4.	Contig4177-0.23	F	AACCTGTTCTCACAAGCTATC	21	48	55	1146	95	98.05
		R	AGTCTAGTACCTCTCAGGATTG	22	45	55			
5.	Contig5480-0.2	F	TCCCTCACACTAACGAAAGA	20	45	55	6498	95	98.12
		R	TCCAGGTATTCAGGAGTGATAG	22	45	55			
6.	Contig11205-0.1	F	AACGTGGAACCTTACTTCAC	20	45	54	597	100	97.60
		R	CACCTTCGTCTTCGTTGTAA	20	45	54			
7.	Contig4653-0.41	F	TGCAAGTGACTGTGAACGAAG	21	48	58	2417	179	101.88
		R	AGCGTTACCAGGGATGTCTTT	21	48	58			
8.	Contig132-0.69	F	ATCCCTCTGGAACCTCAAAAA	21	43	54	3375	179	88.81
		R	GGGAACATTCTGAAAGGGAAG	21	48	54			
9.	Contig3761-0.1	F	TGTGGGCTAACTTCGCTAAAA	21	43	55	681	233	90.70
		R	TTCGATTTTTCTCACCACACC	21	43	56			
10.	Contig3761-0.2	F	CCTGGCTCTGAAACTGACTTG	21	52	55	1251	150	99.31
		R	TTGATAGGAGGAGCAGCGTAA	21	43	55			
11.	Contig9545-0.25	F	CAAAGAACTTCAACTTCACT	20	35	49	555	193	101.67
		R	TTTTTCGTCAGGTTTGTGAGG	21	43	55			
12.	Contig9545-0.26	F	TCCTTCATTCCTGCAAAGAGA	21	43	55	804	167	100.15
		R	AGTAGTCACCCACCACGTCAG	21	57	60			
13.	Contig11588-0.37	F	CTGGTAACGCTGGTATCAAA	20	45	54	1269	111	97.11
		R	CAGCTGATTGACCGAAGATAG	21	48	55			
14.	Contig5480-0.52	F	ACCAATCAGACGACTCACACC	21	52	59	1863	157	90.48
		R	GCGATGTTCACCAGTTTTGAT	21	43	55			
15.	Contig5480-0.3	F	GGTCCTGTGAAAGGTTACAA	20	45	55	663	94	92.15
		R	TGTATTTGTCAGCACCAGTAG	21	43	55			
16.	Contig11205-0.4	F	GAAGGTGAAATCGTGAACAAC	21	43	54	225	165	97.11
		R	TTGGTATGATGATGAACCGTGT	22	41	56			

were used in control. After 24 h, the larvae were transferred to untreated natural diet. All the assays were replicated and repeated at least thrice on alternate days. The mortality of the larvae was assessed after 48 h. The pooled larval mortality data were subjected to probit analysis using the software POLO (LeOra, 1987) and the lethal concentration to kill 50% of the test larvae (LC_{50}) was calculated for each population. Resistance ratios (RRs) were calculated with the following formula: LC_{50} of field population/LC_{50} of insecticide susceptible Lo-S population.

Preparation of Midgut Homogenate

The starved late second instar larvae were used for the preparation of midgut homogenate. Midguts were dissected and homogenized with homogenization buffer (0.1 M sodium phosphate buffer pH 7.8 containing 1 mM each of DTT, EDTA, PTU, and PMSF). The content was centrifuged at 12,000 rpm for 20 min and the clear supernatant was used as a enzyme source for estimating the titers of carboxylesterase and GST. The total protein content of the preparations was assessed by Coomassie brilliant blue G-250 dye-binding method using bovine serum albumin (BSA) as the standard (Bradford, 1976).

Assays on Carboxylesterase and Glutathione S-Transferase

The activity of carboxylesterase was determined using the method described by van Asperen (1962) with essential modifications and α-naphthyl acetate as a substrate. The assay mixture in a 96 well microplate (iMark, Biorad microplate reader) consisted of an appropriate amount of midgut homogenate, 0.1 M sodium phosphate buffer pH 7.8 and 800 µl of 3 mM α-naphthyl acetate containing 0.3 mM eserine. The mixture was incubated at 30°C for 30 min. Finally, 200 µl of 0.1% tetrazotized o-dianisidine (Fast blue B) in 3.5% sodium dodecyl sulfate was added and incubated for 20 min at room temperature in dark. The α-naphthol formation was measured at 590 nm and the enzyme activity was computed from α-naphthol standard curve.

Glutathione S-transferase activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Kao et al.,

TABLE 2 | Primer details for glutathione S-transferase (GST) genes.

Sl. No	Sequence		Primer sequence 5′→3′	Length	GC%	Tm (°C)	Sequence length	Product size	E (%)
1.	Contig7206-0.56	F	GCCTCAAGACTGCATGAAAAG	21	48	56	600	223	98.59
		R	GTCAGCCAGAGTGATTTCGTC	21	52	58			
2.	Contig2323-0.22	F	TTCACCTCCTTGCAGATCAGT	21	48	58	375	160	87.80
		R	GAAGTCACCGTCTTTCAGCAG	21	52	58			
3.	Contig8855-0.81	F	ACGACATCCTGAACACTCTGC	21	52	59	657	165	98.93
		R	TTCTCCATTGTCTCACCCAAG	21	48	57			
4.	Contig2841-0.35	F	CGCTATGAGATTCTGCCCTTAC	22	50	57	762	120	104.58
		R	GAGAAGTCGAACAGCCATTCA	21	48	57			
5.	Contig2596-0	F	TGCTACCTGGTGGACAAATTC	21	48	57	639	236	92.92
		R	CCCATTTAGTGTCGTTCAGGA	21	48	56			
6.	Contig14202-0.24	F	TATCCTGGCTCTGAACGCTAA	21	48	58	717	171	105.55
		R	TCGTCCAGGTATTCCACAGTC	21	52	59			
7.	Contig1970-0.34	F	GACGCTCTGAGACTGTTCGAC	21	57	60	564	190	96.96
		R	CTTCGTAACCAGGAGCAGTTG	21	52	58			
8.	Contig1510-0.48	F	ATCGGTTGGCTGAACACTATG	21	48	57	726	227	95.00
		R	AAAGTAGCCAGCATTTGAGCA	21	43	57			
9.	Contig2478-0.25	F	TCTGGGACTCACACGCTATCT	21	52	56	654	208	100.59
		R	ATTTGAGCCAGGTTTTCAGGT	21	43	60			
10.	Contig3755-0.39	F	CGCTATCATGCAATACGTGTG	21	48	56	654	183	102.61
		R	AGACCCAGAGGAGTTCTTTCG	21	52	59			
11.	Contig3537-0.3	F	ACAAATTCAACCTGCTGCCTA	21	43	56	423	227	96.58
		R	CAGTTTACCAGCGAAGTGACC	21	52	58			
12.	Contig10769-0.3	F	TCACTGGCTATCGCTAGATACA	22	45	57	621	116	98.89
		R	CGTTTGACCAGAAGTCGAAGAT	22	45	57			
13.	Contig1010-1.18	F	GACGCTCTGAGACTGTTCGAC	21	57	60	648	190	98.00
		R	CTTCGTAACCAGGAGCAGTTG	21	52	58			
14.	Contig9166-0.22	F	ACAAATCAGTGGCTGCTAAA	20	40	54	345	97	95.31
		R	GGCAGGTAGTACAGTTTGATAG	22	45	54			
15.	Contig6423-0.33	F	TGCTACCTGGTGGACAAATTC	21	48	57	645	236	89.22
		R	CCCATTTAGTGTCGTTCAGGA	21	48	56			
16.	Contig199-0.91	F	TGTGAAAGCTCTGGGTGAATC	21	48	57	567	233	99.14
		R	GAAGTCCACGTTCATGTCGAT	21	48	57			
17.	Contig8048-0.1	F	TCAATCGAAGAAGACCTGGAA	21	43	55	486	150	98.83
		R	CAGCAGTTGAGGGTACACGTT	21	52	60			
18.	Contig11878-0.11	F	TGCTACCTGGTGGACAAATTC	21	48	57	540	236	98.55
		R	CCCATTTAGTGTCGTTCAGGA	21	48	56			
19.	Contig7006-0	F	CGCTAGATACCTGGCTAACAAA	22	45	56	606	98	92.55
		R	CCCAGAAGTCGTAGATGTTCAG	22	50	57			
20.	Contig3023-0.16	F	GCTGCTTACGAAGGTAGAATG	21	48	55	618	173	99.14
		R	GCAGGGAAGTGTTCTTTAGGG	21	52	58			
21.	Contig2596-0	F	TGCTACCTGGTGGACAAATTC	21	48	57	639	235	101.31
		R	CCCATTTAGTGTCGTTCAGGA	21	48	56			
22.	Contig2478-0.15	F	CATCACACGCTATCACTACTT	21	43	54	456	102	97.25
		R	GCAGTCTTTGGTCGATTCT	19	47	54			
23.	Contig3291-0.7	F	TATCGTGTCAGGTATCCAAC	20	45	53	402	179	92.16
		R	GTCAGCCAGAGTCAGTTGGTC	21	57	60			
24.	Contig4841-1.1	F	TGGACGACACTAGACCTAAA	20	45	54	327	81	93.78
		R	GTTGGATACCTGACACGATAG	21	48	54			
25.	Contig2323-0.23	F	CTCACACGCTATCACTACTTAC	22	45	54	645	101	95.56
		R	GCAGTCTTTGGTCGATTCT	19	47	54			

1989). The assay mixture composed of 50 mM each of CDNB and L-glutathione reduced and 10 µl gut homogenate. The change in absorbance was measured at 340 nm for 5 min and the enzyme activity in terms of nmoles of CDNB conjugated per minute per mg protein using the molar extinction coefficient of 5.3 mM^{-1} (optimized for the path length: 0.55 cm).

TABLE 3 | Resistance pattern of field populations of *L. orbonalis* against various insecticides.

Insecticide	Population	LC50 (ppm)	Slope ± SE	Fiducial limits		χ^2 heterogeneity (DF)	Resistance ratio
				Lower	Upper		
Fenvalerate	Bhubaneswar	259.2	2.6 ± 0.52	125.61	623.8	7.01 (3)	160
	Raichur	93.2	3.1 ± 0.2 2	56.1	125.8	3.92 (3)	57.5
	Dharmapuri	78.4	2.5 ± 0.23	53.2	145	2.81 (3)	48.3
	Pune	5.9	2.1 ± 0.11	2.51	16.32	2.91 (3)	3.6
	Varanasi	153.2	1.6 ± 0.23	75.2	432.4	3.37 (4)	94.6
	Bengaluru (Lo-S)	1.62	2.2 ± 0.42	0.85	3.57	2.62 (4)	–
Phosalone	Bhubaneswar	641.5	2.61 ± 0.22	283.51	2564.6	6.90 (3)	534.6
	Raichur	190.2	1.9 ± 0.2 1	110.1	280.5	3.90(3)	158.5
	Dharmapuri	113	3.3 ± 0.56	56.4	203.2	0.89 (3)	94.2
	Pune	8.81	1.7 ± 0.08	4.34	24.81	3.48 (3)	7.3
	Varanasi	435.8	1.9 ± 0.21	210.5	1202.8	1.37 (3)	363.2
	Bengaluru (Lo-S)	1.2	1.2 ± 0.21	0.521	2.42	1.91 (3)	–
Emamectin benzoate	Bhubaneswar	0.072	1.4 ± 0.09	0.291	0.204	3.4 (3)	7.2
	Raichur	0.1	1.1 ± 0.10	0.07	0.2	1.19 (3)	10
	Dharmapuri	0.07	1.5 ± 0.31	0.03	0.1	2.66 (3)	7
	Pune	0.012	1.8 ± 0.09	0.005	0.045	1.07 (3)	1.2
	Varanasi	0.55	1.2 ± 0.28	0.231	1.821	6.89 (4)	55
	Bengaluru (Lo-S)	0.01	1.4 ± 0.07	0.004	0.051	3.17 (3)	–
Thiodicarb	Bhubaneswar	211.6	1.71 ± 0.83	116.23	621.78	1.91 (3)	18.2
	Raichur	263.2	2.1 ± 0.21	121.3	467.9	2.3 (3)	22.7
	Dharmapuri	161.9	2.05 ± 0.30	83.82	341.7	7.2 (3)	13.9
	Pune	23.6	2.0 ± 0.12	32.01	184.8	1.3 (3)	2.03
	Varanasi	112	2.2 ± 0.18	56.82	456.8	1.7 (4)	9.6
	Bengaluru (Lo-S)	11.61	1.73 ± 0.32	5.462	23.56	4.7 (3)	–
Flubendiamide	Bhubaneswar	69.7	1.20 ± 0.08	36.34	173.35	3.7 (3)	303.0
	Raichur	43.1	1.72 ± 0.10	22.39	93.72	2.9 (3)	187.4
	Dharmapuri	61.5	1.3 ± 0.13	35.65	129.39	7.2 (3)	267.4
	Pune	6.79	1.10 ± 01.0	3.02	17.93	14.6 (4)	29.5
	Varanasi	61.3	1.79 ± 0.08	20.32	173.8	17.5 (4)	266.5
	Bengaluru (Lo-S)	0.23	–	–	–	–	–
Chlorantraniliprole	Bhubaneswar	2.29	0.66 ± 0.16	0.28	6.4	4.08 (3)	6.9
	Raichur	0.80	0.59 ± 0.16	0.02	2.9	2.80 (3)	2.4
	Dharmapuri	2.84	0.63 ± 0.15	0.41	7.94	4.92 (3)	8.6
	Pune	1.76	0.6 ± 0.16	0.12	5.59	3.08 (3)	5.3
	Varanasi	0.60	0.47 ± 0.15	0.002	3.2	5.68 (3)	1.6
	Bengaluru (Lo-S)	0.33	0.81 ± 0.26	0.003	1.24	1.55 (3)	–

TABLE 4 | Metabolic enzymes activities in the midgut of *L. orbonalis* populations.

L. orbonalis population	Glutathione -S- transferase		Carboxylesterase	
	Specific activity (nMoles/min/mg protein)	Fold variation as compared to Lo-S	Specific activity (μMoles/mg/min)	Fold variation as compared to Lo-S
Bhubaneswar	70.5 ± 3.6*	5.6	6.02 ± 0.21*	2.8
Raichur	73.0 ± 3.6*	5.8	6.83 ± 0.21*	3.2
Dharmapuri	112.3 ± 11.2*	8.9	5.78 ± 0.33*	2.7
Varanasi	51.8 ± 2.8*	4.1	3.52 ± 3.4	1.7
Pune	103.4 ± 9.5*	8.2	3.28 ± 0.33	1.5
Bengaluru (Lo-S)	12.62 ± 1.3	–	2.12 ± 0.13	–

**Indicates significant differences by Mann–Whiteney U-test ($p < 0.05$) as compared to Lo-S population.*

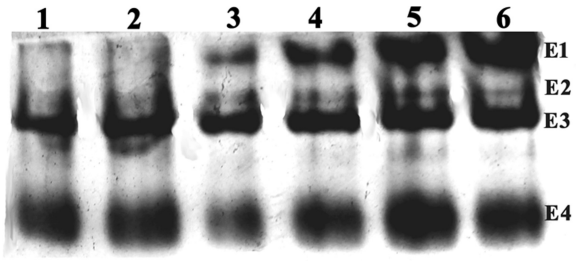

FIGURE 1 | The Native PAGE of the esterase isozyme pattern of *L. orbonalis* (1. Lo-S, 2. Pune, 3. Varanasi, 4. Raichur, 5. Bhubaneswar, 6. Dharmapuri).

Esterase Isozyme Studies

The native polyacrylamide gel electrophoresis (PAGE) with 10% resolving gel was performed as per Davis (1964) to visualize the esterase isozymes from the 2nd instar midgut larval homogenates of the *L. orbonalis* populations tested. Midgut homogenates with 20 μg protein were loaded onto the native PAGE and run at a constant voltage of 70 for 1.5 h. Gel was briefly stained with freshly prepared 0.05% (w/v) α-naphthyl acetate and 0.1% (w/v) fast blue B in 50 mM phosphate buffer pH 7.8.

Genome Mining and Phylogenetic Analysis

The draft assembled genome (NCBI Bioproject ID: PRJNA377400) of *L. orbonalis* was used as query for extracting the gene sequences of glutathione S-transferases (GSTs) and carboxylesterases (CEs). The hmm model for carboxylesterase and GST (CE-PF00135, PF02230, and GST-C PF00043, GST-N PF02798, GST-PF13417, PF14497, PF17171, PF17172)

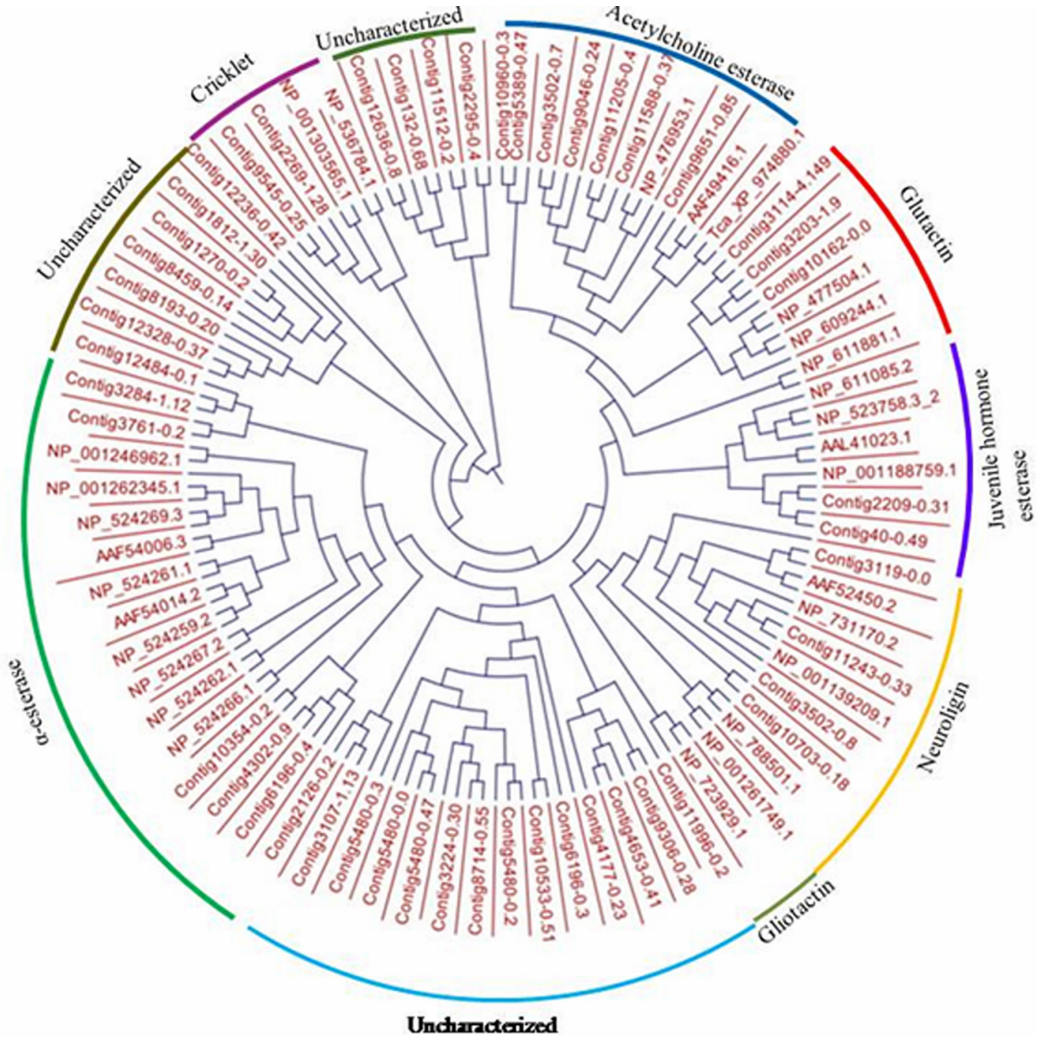

FIGURE 2 | Phylogenetic relationships of 94 CE proteins of *L. orbonalis* with selected *D. melanogaster* protein representative of seven characterized carboxylesterase classes. Putative CE proteins in *L. orbonalis* were identified using hmmsearch against PFAM CE families (CE PF00135). The Maximum likelihood tree was constructed using CLC workbench.

were obtained from PFAM database[1]. A total of 94 and 33 putative CE and GST sequences were mined using hmm search with HMMER3 software package[2]. Among them, the genes with known history of involvement in insecticide resistance in other insects were selected and further confirmed with NCBI BLASTX. The GST and CE protein sequences of *Drosophila melanogaster* was downloaded from its genome database (Marygold et al., 2013), GenBank[3] and Uniprot[4]. We combined these putative GST protein sequences with *D. melanogaster*'s annotated protein sequences for each of the six subclasses of GST (Epsilon, Sigma, Omega, Delta, Zeta, and Theta). The sequences were multiple aligned using CLUSTALW and a maximum likelihood tree with 1000 bootstrap value was constructed using MEGA7[5] and TreeDyn online server[6] was used to visualize the tree. Similarly, 94 CE protein sequences under seven subclasses (α-esterases, gliotactin, glutactin, neurolignin, juvenile hormone esterases, acetylcholinesterases, and cricklet) were combined, multiple aligned using MAFFT online server using conserved domain feature and phylogenetic tree of CE genes from *L. orbonalis* using *D. melanogaster* as a reference were constructed with the help of CLC workbench (QIAGEN Bioinformatics[7]) using maximum likelihood approach.

[1]http://pfam.xfam.org

[2]http://hmmer.janelia.org

[3]http://www.ncbi.nlm.nih.gov/

[4]http://www.uniprot.org/

[5]http://www.megasoftware.net

[6]www.treedyn.org

[7]https://www.qiagenbioinformatics.comproductsclc-main-workbench

Quantitative Real-Time PCR

The late 2nd instar larvae were used for RNA extraction (ISOLATE II RNA mini kit) by following the manufacturer's guidelines (Bioline). Purity and concentration of total RNA were measured in a spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific) and denatured agarose gel (Masek et al., 2005). RNA samples with an A260/A280 ratio ranging from 1.8 to 2.0 and A260/A230 ratio >2.0 were used for cDNA preparation. First strand of complementary DNA was synthesized from 4 μg of total RNA using Revert AID first strand cDNA synthesis kit (Thermo Fisher Scientific™, Lithuania) following the suppliers guidelines and stored at −80°C till further use.

qPCR primers for 25 GST and 16 CE gene sequences were designed using Primer 3.0 software. The detailed information of the primers used in the current study is listed in **Tables 1**, **2**. To remove the primer dimer and confirm the efficiency, OligoEvaluator™ sequence analysis tool (accessed on July 2018[8]) was used. Primer specificity analysis was observed by single peak in the melting curve and single band from the agarose gel of the all candidate genes of CE and GST ranged from 90.36 to 104.58% (**Tables 1**, **2**). Quantitative real-time PCR (RT-qPCR) amplifications were performed in 20 μL reaction consisted of 10 μL 2×SYBR® Premix EX TaqTM II (Tli RNaseH Plus, TAKARA®, Japan), 1 μl cDNA and gene specific primer pair. The parameters used for RT-qPCR are: One cycle of 95°C for 3 min; 35 cycles of 95°C for 30 s, 53°C for 45 s, and 72°C for 1 min; a final cycle of 72°C for 10 min using Roche 480II machine. All the samples including control (no template)

[8]http://www.sigmaaldrich.com/life-science/custom-oligos/custom-dna/learning-center/calculator.html

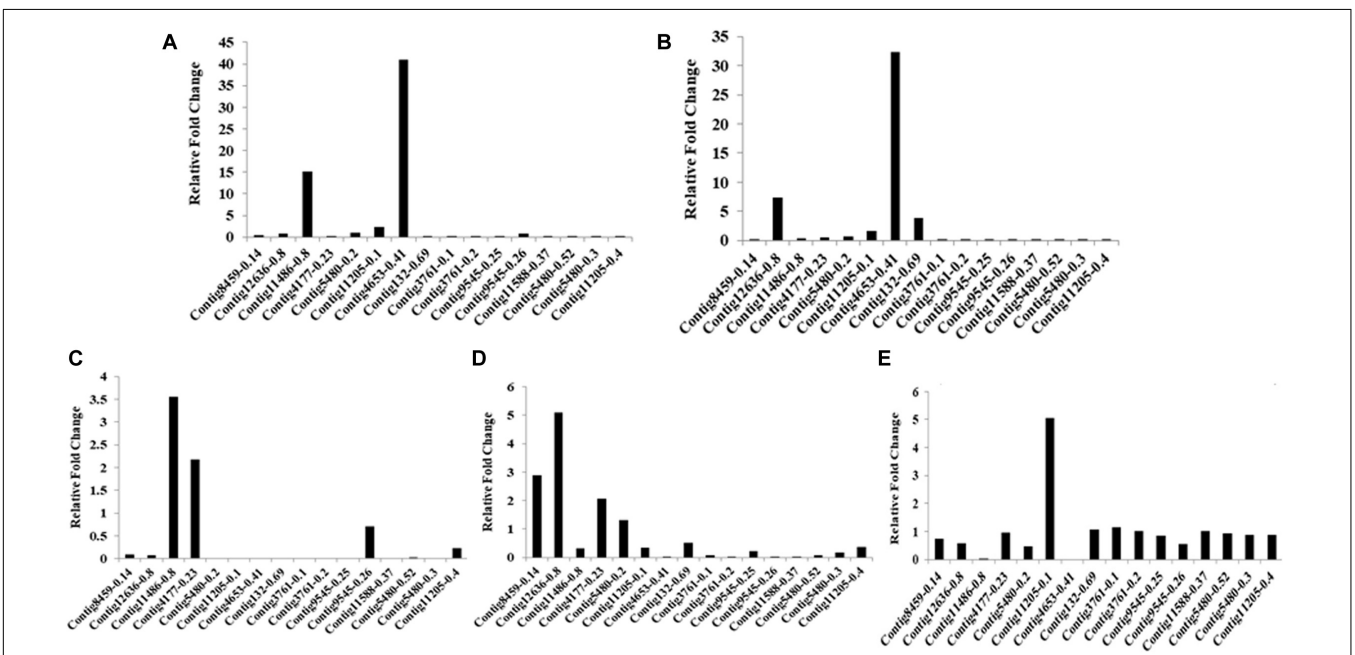

FIGURE 3 | Transcription profiling of carboxylesterase genes in field collected populations of *L. orbonalis* depicted as fold change over the susceptible Lo-S colony. **(A)** Bhubaneswar, **(B)** Dharmapuri, **(C)** Pune, **(D)** Raichur, **(E)** Varanasi.

and internal control were performed in triplicates. The PCR products were electrophoresed on 2.0% agarose gel in a 1.0×TAE buffer. Relative expression levels for the CE and GST genes were calculated by $2^{-\Delta\Delta CT}$ method. The 28SR3 (28S ribosomal protein S3 mitochondrial) (Kariyanna et al., 2019) gene was used as an internal control to normalize the expression of target genes.

RESULTS

Insecticide Resistance Monitoring

The LC_{50} values against fenvalerate, phosalone, thiodicarb, emamectin benzoate, flubendiamide and chlorantraniliprole indicated that there is a large shift in susceptibility of field-populations of *L. orbonalis* as compared to the laboratory reared susceptible iso-female colony (Lo-S). The field collected populations exhibited 3.6–160-fold resistance against fenvalerate, 7.3–534.6-fold resistance against phosalone, 7.0–55.0-fold resistance against emamectin benzoate, 2.0–22.7-fold resistance against thiodicarb, 29.5–303.0-fold resistance against flubendiamide, and 1.6–8.6-fold resistance against chlorantraniliprole. However, the susceptibility level of all the field populations and the Lo-S did not vary significantly against chlorantraniliprole based on the overlapping fiducial limit values. High level of resistance development against phosalone and flubendiamide was observed in *L. orbonalis* collected from Varanasi whereas other field populations, the RRs were non-significant as compared to Lo-S. *L. orbonalis* populations from Bhubaneswar showed a significantly very high levels of resistance against fenvalerate, phosalone and flubendiamide as compared to Lo-S. The population of *L. orbonalis* collected from high hills near Pune recorded less RR and the susceptibility status was almost on par with Lo-S population (**Table 3**).

Activities of Detoxification Enzymes

Quantitative differences in the titer of GST and CE were determined using α-naphthyl acetate and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates, respectively. Significant level of elevated GST activities (4.1–8.9-fold) was observed in field collected *L. orbonalis* as compared to susceptible Lo-S population. However, the overproduction of CE has not pronounced much (1.5–3.2-fold), but there was a significant level of overproduction in two field populations based on Mann–Whiteney U-test. The results indicated that GST activity was altered more profoundly than the CE (**Table 4**).

The qualitative differences in carboxylesterase isozymes were visualized under native PAGE after incubation of the gels in the α-naphthyl acetate as a substrate. Among the four major esterase activity bands (E_1 to E_4), the high molecular weight E_1 and E_2 bands were absent in Lo-S and Pune populations and faint in case of Varanasi population. However, the E_1 band was very prominent and intense in other field populations indicating its over-production nature (**Figure 1**).

Differential Expression of CE Genes

Ninety-four CE genes were identified from the genome and transcriptome sequences of *L. orbonalis*. They were classified under seven subfamilies *viz.*, α-esterases, gliotactin, glutactin, neurolignin, juvenile hormone esterases, acetylcholinesterases, and cricklet like orthologs (**Figure 2**). The length of the gene sequences ranged from 225 to 6498 bp. Expression profiling of 16 genes (homologous to resistant genes in other insects with ≥90% query coverage) was examined from the mRNA samples derived from late second instar larvae of five field-collected *L. orbonalis* populations by RT-qPCR. The CE genes contig11486-0.8 and contig4653-0.41 were over-expressed more than 10-fold as compared to susceptible Lo-S population (**Figures 3**, **4**).

Differential Expression of Glutathione S-Transferase Genes

Mining of genome and transcriptome data of *L. orbonalis* yielded 33 unigenes codings for GSTs. Lengths of the identified GST unigene sequences ranged from 370 to 5,048 bp. The phylogenetic analysis revealed that the predicted GST genes are represented under all six classes of GST *viz.,* Epsilon (10 genes), Sigma (6 genes), Omega (5 genes), Delta (8 genes), Zeta (3 genes), and Theta (1 gene) (**Figure 5**). RT-qPCR analysis of 25 GST genes (homologous to other insects resistant genes) revealed that many of them were over-expressed in larvae collected from the field. The GST genes named contig2323-0.22, contig14202-0.24, contig3755-0.39, contig3537-0.3, contig199-0.91, contig3023-0.16, contig3291-0.7, contig4841-1.1, and contig2323-0.23 were

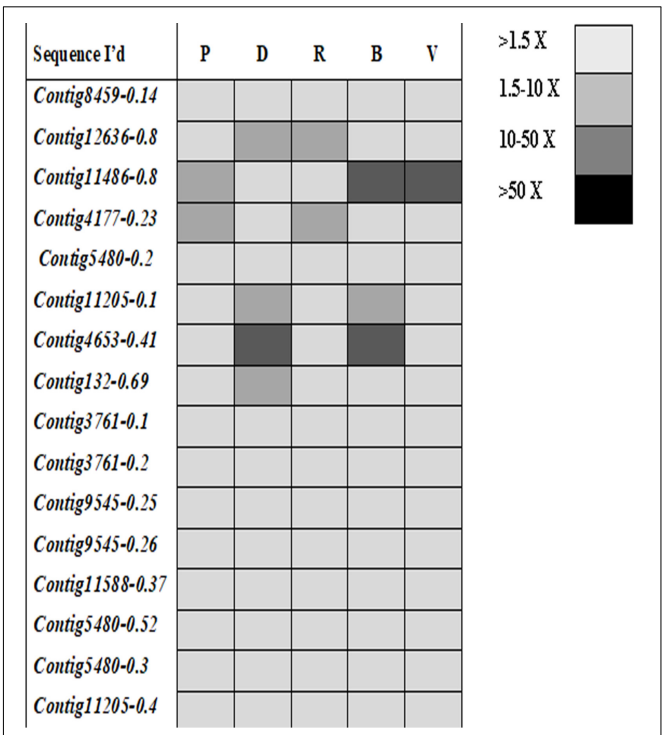

FIGURE 4 | Expression profiles (fold changes over Lo-S population) of carboxylesterase genes across field collected *L. orbonalis* populations (P, Pune; D, Dharmapuri; R, Raichur; B, Bhubaneswar; V, Varanasi). The fold changes are indicated in different shades indicate significant difference as per Mann–Whiteney U-test p-value < 0.05.

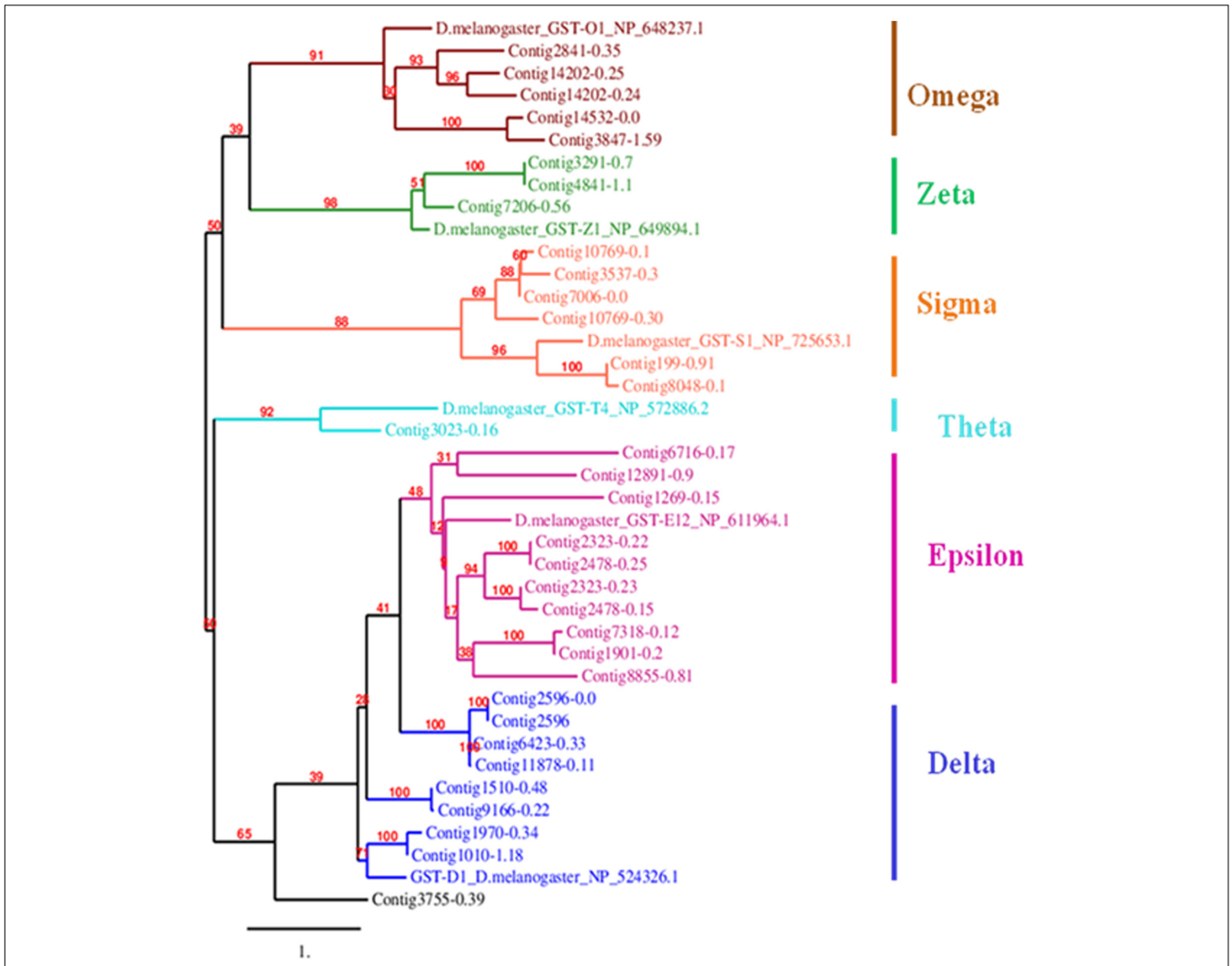

FIGURE 5 | Phylogenetic tree of 34 predicted *L. orbonalis* glutathione S-transferase proteins with *D. melanogaster* genes representative of the six characterized GST classes. Putative GST proteins in *L. orbonalis* were identified using hmmsearch against PFAM GST families (GST-C PF00043, GST-N PF02798), the Maximum Likelihood tree with 1000 bootstraps was constructed using MEGA7 and TreeDyn online server was used to visualize the tree. Number on each node is bootstrap values in percent. Scale = 1 amino acid substitution per site.

very highly expressed (>50-fold) in one or more resistant field-collected *L. orbonalis* populations (**Figures 6**, **7**) over the susceptible Lo-S population.

DISCUSSION

Damage caused by *L. orbonalis* is the major limiting factor in realizing maximum productivity of eggplant in many Asian countries including India, China, and Bangladesh. The high reproductive potential and shorter life cycle of the pest coupled with the evolution of multiple insecticide resistance pose a major challenge in profitable cultivation of eggplant (Prodhan et al., 2018). The insecticide resistance levels detected in the field collected populations ranged from low (>10-fold) to moderate (10–100-fold) and high (>100-fold) against fenvalerate (48.2–160-fold), phosalone (94–534.58-fold), emamectin benzoate (7–55-fold), thiodicarb (9.64–22.67-fold), and flubendiamide (29.51–363.91-fold). The resistance levels reflect the occurrence of differential selection pressure associated with long term history of insecticide use against *L. orbonalis*. However, the response to chlorantraniliprole was similar among the resistant field collected populations with RRs below 10-fold. The efficacy of chlorantraniliprole has been reported earlier (Kodandaram et al., 2013; Munje et al., 2015). The chlorantraniliprole and flubendiamide are anthranilic and phthalic diamides, respectively, are the newer insecticides with little or no cross-resistance among them and also with other classes of insecticides. They are the activators of the ryanodine receptor.

Insect employs various mechanisms to nullify the toxic effect of xenobiotic compounds. Insect metabolic enzymes play a major

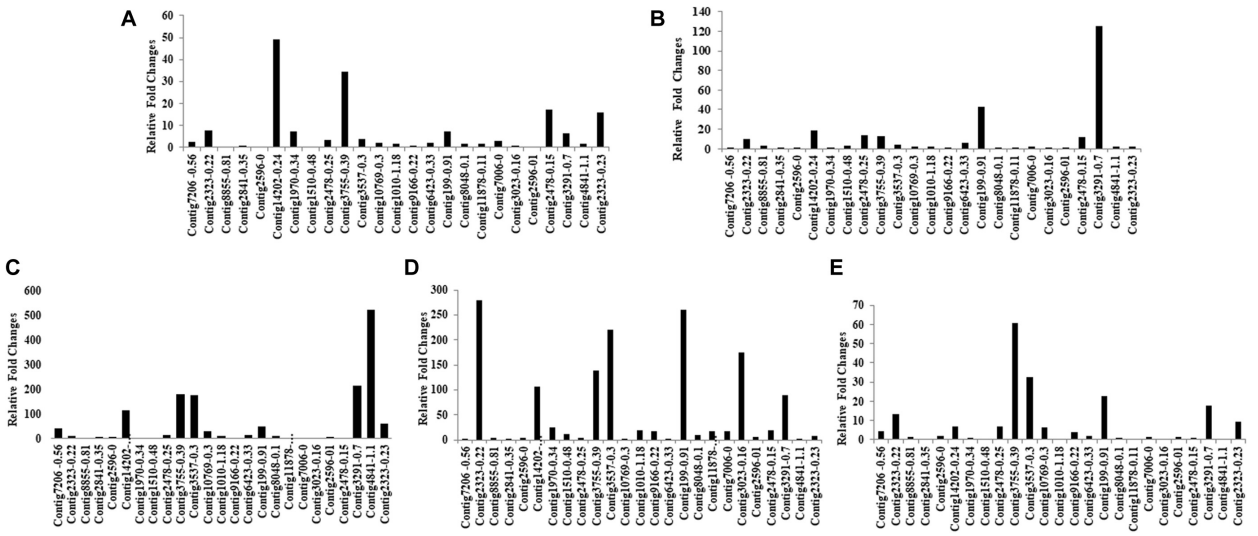

FIGURE 6 | Transcription profiling of glutathione -S- transferase genes in field collected populations of *L. orbonalis* depicted as fold change over the susceptible Lo-S colony. **(A)** Bhubaneswar, **(B)** Dharmapuri, **(C)** Pune, **(D)** Raichur, **(E)** Varanasi.

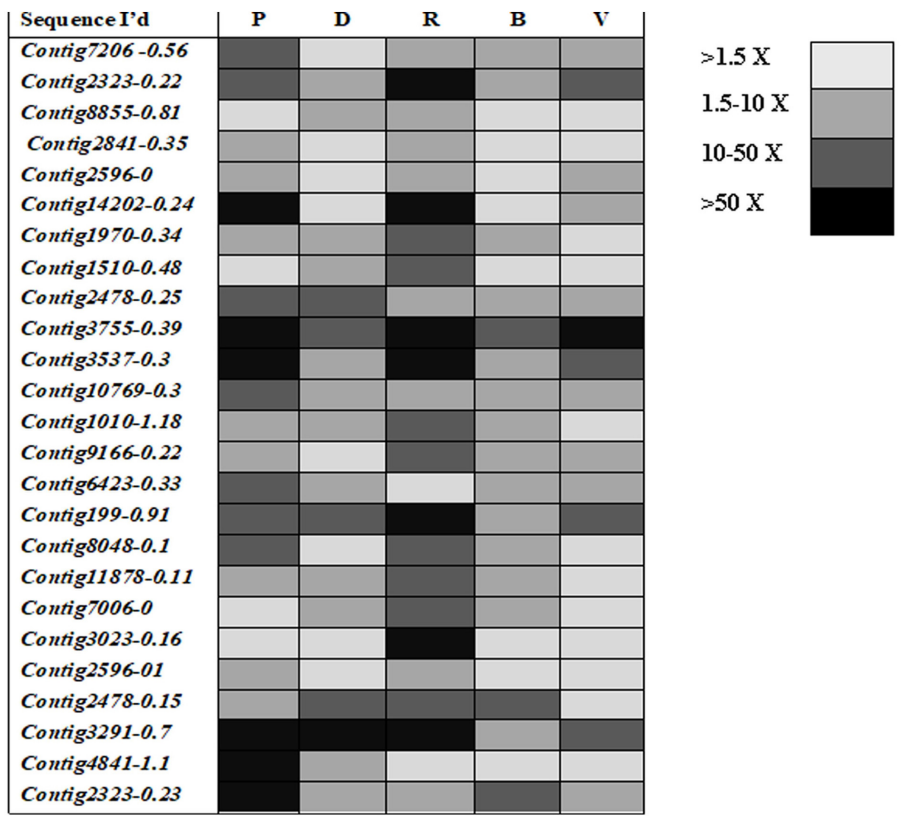

FIGURE 7 | Expression profiles (fold changes over Lo-S population) of glutathione -S- transferase genes across field collected *L. orbonalis* populations (P, Pune; D, Dharmapuri; R, Raichur; B, Bhubaneswar; V, Varanasi). The fold changes are indicated in different shades indicate significant difference as per Mann–Whiteney U-test p-value < 0.05.

role in detoxification of plant allelochemicals and pesticidal compounds in addition to other physiological roles. Insect pests have evolved large reservoirs of various detoxification enzymes. Glutathione S-transferases mediated detoxification can be direct or by the metabolism of secondary products generated from other detoxification enzymes. The insecticides are metabolized rapidly into readily excretable water-soluble metabolites by reductive dehydrochlorination or by conjugation with reduced glutathione (Pavlidi et al., 2018). The carboxylesterase is a phase-1 detoxification enzyme that mainly hydrolyses organophosphates, carbamates, and synthetic pyrethroids.

The quantitative mechanisms underlying metabolic resistance is characterized by over-production due to gene amplification or transcriptional upregulation (Mohan et al., 2015; Feyereisen, 2015; Pavlidi et al., 2018). A qualitative mechanism occurs as a result of changes in the enzymatic characteristics. The present investigation on the enhanced metabolism of insecticides in resistant populations of *L. orbonalis* resistance is very prominent as inferred by elevated GST (1.2–2.6-fold) and carboxylesterases (6.3–13.1-fold) titers. The involvement of CEs and GSTs in insecticide resistance is commonly reported in many other insect species (Mohan et al., 2015; Pavlidi et al., 2018).

With the advent of genome and transcriptome information, multiple GSTs and carboxylesterases encoding genes have been characterized from insects and mites such as *Plutella xylostella, Culex* spp., *Tribolium castaneum, Bombyx mori, Leptinotarsa decemlineata, Tetranychus cinnabarinus, Musca domestica* and in many other insect species (Yang and Liu, 2011; Wang et al., 2014; Cui et al., 2015; Meisel and Scott, 2018; Pavlidi et al., 2018). *L. orbonalis* genome has large expansion of CE and GST genes which is comparable to many other insects. However, the GSTs have prominent role in insecticide resistance as compared to CEs. mRNA level of at least nine GSTs were over-expressed >50-fold where as none of the CEs showed very high levels of expression in the field collected resistant populations. The over-expression of few CE genes in field populations, especially from Varanasi, Raichur, and Bhubaneswar, also aligned with additional high molecular weight esterase band (E1) probably contributed to overall resistance. A similar high molecular weight esterase band associated with B-esterase type was reported from diamondback moth (Mohan and Gujar, 2003). In addition to qualitative changes in esterase banding pattern, there is a significant increase in the production of the CEs in at least three field populations. The over-production and appearance of new esterase isozymes might be the consequence of increased transcription or gene duplication events (Malathi et al., 2015; Panini et al., 2016).

Most of the GST gene expansions are from epsilon and delta subclasses together accounting for 18 genes (54.5%) out of 33 genes identified from the draft genome of *L. orbonalis*. These two GST subclasses are insect-specific, widely expanded in many other insect species whereas other classes have wider taxonomic distribution (Friedman, 2011). There were at least nine GSTs over-expressed >50-fold in one or more of the field-collected resistant populations of *L. orbonalis*. In *Anopheles gambiae, Apis mellifera, B. mori, D. melanogaster, Nasonia vitripennis,* and *T. castaneum* respectively 28, 8, 23, 37, 19, 35 GSTs and 51, 24, 76, 35, 41, 49 CEs were identified (Yu et al., 2008, 2009; Oakeshott et al., 2010). Similarly, the gene expansion of cytochromoe p450 monooxygenases (CYP) and their role in insecticide resistance in *L. orbonalis* were recently documented (Kariyanna, 2019; Kariyanna et al., 2020a). Together with the results of present and earlier studies it is very much evident that multiple metabolic enzymes contribute toward overall insecticide resistance in *L. orbonalis*. However, in many insect species including *L. orbonalis*, it is difficult to distinguish whether the expansion of these metabolic genes and their involvement in insecticide detoxification are driven from ancestry or adaptation to stress or both.

CONCLUSION

In conclusion, the present study provides the first genomic level information on GST and CE gene families of *L. orbonalis* and their role in insecticide resistance. The information further supports insect resistance management through use of reduced risk pesticides including biologicals.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

BKr: conducted the experiment, analysis, manuscript writing, materials and methods, and hypothesis. AP: analysis, material methods, and corrections. RA: analysis and technical suggestion, manuscript correction, and resource supply. AA and PJ: bioinformatics and analysis. RG and BKl: resources supply and, technical guidance and suggestions. TV: resources supply, technical guidance and suggestions, and reviews and literatures. MB and JD: analysis, technical guidance and suggestions, and reviews and literatures. MM: designing experiment, resources supply, manuscript corrections, analysis, material and methods, technical guiding, and suggestions. YP: technical guidance and suggestions, and reviews and literatures. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors profusely thank Indian Council of Agricultural Research for generous funding under ICAR-CRP Genomics project. The first author acknowledges the award of DST-Inspire fellowship (2016/IF160900) and UAS Raichur for

necessary support. Authors are thankful to Director, ICAR-NBFGR for timely release of fund. The facilities extended by Director, ICAR-NBAIR, and Director, ICAR-IIHR, Bengaluru are gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.594845/full#supplementary-material

Supplementary Table 1 | Highlights of the research.

Supplementary Table 2 | Sequence information GST and CE from *L. orbonalis*.

Supplementary Table 3 | Resistance genes of GST and CE from *L. orbonalis*.

Supplementary Table 4 | Detailed sequence information of CE from *L. orbonalis*.

Supplementary Table 5 | Detailed sequence information of GST from *L. orbonalis*.

Supplementary Table 6 | NCBI ID and description for the Reference protein taken in GST and CE phylogeny.

Supplementary Table 7 | Comparative characterization *L. orbonalis* CE genes with other group based on the nr hit.

Supplementary Table 8 | Descriptive information of the *L. orbonalis* CE sequences.

Supplementary Presentation 1 | Graphical representation of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Indian Journal of Entomology 83(2021) Online published Ref. No. e20223 DoI No.: 10.5958/0974-8172.2021.00043.2

INFLUENCE OF CULTIVARS ON POPULATION DYNAMICS OF BRINJAL SHOOT AND FRUIT BORER *LEUCINODES ORBONALIS*

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ABSTRACT

Shoot and fruit borer *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) is the key pest of brinjal. The present study on its population dynamics in relation to brinjal cultivars revealed significant variation in fruit damage (6.73% to 60.26%). The least damage was observed on Eeranagere (23.02%) and maximum on Mullubadane (27.92%). The infestation varied at different growth stages; least (<22.00%) between 8-11th and 35-38th standard meteorological weeks (SMW) in rabi and kharif seasons, respectively. The maximum damage (35.14%) was observed at 50th SMW during rabi 2017 and 43rd and 44th SMW (~35.60%) during kharif 2018; the least damage was in rabi (24.83%) compared to kharif (26.39%). The wind velocity and maximum temperature had a significant negative relation with fruit damage during the rabi season; but in kharif morning relative humidity and minimum temperature had a significant negative association.

Key words: *Leucinodes orbonalis*, brinjal, cultivars, Eeranagere, Mullubadane, population dynamics, seasons, correlation coefficient, wind velocity, temperature, relative humidity

Brinjal is the second important solanaceous vegetable after tomato (FAO, 2017) and India is the largest producer of it in entire South Asia (Anonymous, 2017). The brinjal is attacked by 53 insect pests, and among these, the most destructive and regular pest is the shoot and fruit borer *Leucinodes orbonalis* Guenée (Biswas et al., 1992; Bhadauria et al., 1999). Its damage results in wilting of the shoot and withering of leaves (Haseeb, et al., 2009; Mishra et al., 2014); and causes severe yield loss to the extent up to 90% (Kodandaram et al., 2017; Prodhan et al., 2018), with severity being in the autumn season (Atwal, 1976). The pest distribution and abundance vary with weather factors determining the population density (Indirakumar et al., 2016; Kassi et al., 2019). Without observing the level of incidence farmers use mixture of conventional insecticides to manage the *L. orbonalis*, that leads to resistance development against many insecticide (Chatterjee and Roy, 2004, Sharma et al., 2004; Mishra Dash, 2007; Kariyanna et al., 2019; 2020a; 2020b). Hence the ecofriendly approach is to adopt resistant cultivars along with other strategies (Lit, 2009), but no proper resistant varieties are available for the shoot and fruit borer (Srinivasan, 2008; Yousafi et al., 2013). This study evaluates seven commonly grown brinjal cultivars against *L. orbonalis* under the changing weather conditions.

MATERIALS AND METHODS

The field trial was conducted for two seasons from September-2017 to March-2018 (rabi) and July to November-2018 (kharif), at the research farm of ICAR-National Bureau of Agricultural Insect Resources, Yelahanka, Bengaluru (13°03″N,77°34″E, 924 masl). Seven cultivars viz., Gourav and Lalitha (hybrids in Karnataka), Manjarigota (improved cultivar from UAS, Dharwad), Mullubadane, Madhugiri Mulla, Eeranagere and Mattugulla (local cultivars of Karnataka) were raised by adopting the randomized complete block design (RCBD) tool. The plot size was 4x 5 m with 60x 60 cm spacing under unprotected condition, replicated thrice using RCBD design. The first crop was taken during rabi (2017-18) and the second one in the subsequent kharif (2018). Observation on fruit damage was recorded from the 9th week after the transplantation and continued till 22nd week. Fruits (both damaged and healthy) were harvested at weekly interval and the % fruit damage (based on bore holes and fecal pellets) observed. The weekly data of the weather parameters viz., maximum and minimum temperature, morning and afternoon RH, wind velocity, sunshine hours, total rainfall were obtained from the Department of Agrometeorology, University of Agricultural Sciences, Bengaluru. The data on damage were subjected to angular transformation before ANOVA (Snedecor and

Cochran, 1956; Sokal and Rohlf. 1995); and means separated by CD values (Sokal and Rholf, 1981) using SAS software (SAS Institute Inc., 2015). The influence of weather parameters on damage was analysed through Karl Pearson's correlation analysis and step-down correlation and coefficient of determination using the IBM-SPSS 24.0 Software Package (IBM-CROP, 2016).

RESULTS AND DISCUSSION

During rabi 2017-18, the fruit damage by *L. orbonalis* on the evaluated cultivars varied from 21.76 to 27.8%, with the least being in Madhugiri Mulla; during kharif, 2018, Eeranagere was the least damaged (22.43%). Such variations in cultivers was also reported by Javed et al. (2017). During rabi, the least damage (20.13%) was observed at 11th standard meteorological week (SMW) and it was on par with 9th (20.37%) and 8th (20.51%) SMW; while the maximum of 35.14% was observed in the 50th SMW. During kharif maximum damage (~35.60%) occurred during 43rd and 44th SMW (Fig. 1). These observations on the peak infestation in October is known (Oomen and Kumar, 2004; Jat et al., 2002); also from October to December (Shukla and Khatri, 2010; Kumar and Sing, 2012). During rabi, incidence revealed a decreasing trend with increase in temperature, and decreased rainfall irrespective of cultivars; while during kharif, it was due to decrease in temperature and increased rainfall.

During rabi, the wind and temperature influenced the incidence more. In cultivars Manjarigota, Mattugulla and Mullubadane, the incidence was observed to show significant negative correlation with the wind velocity (r= -0.69, -0.57 and -0.63, respectively); while, Gourav (r= -0.56) and Lalitha (r= -0.62) registered a significant negative relation with maximum temperature. The multiple regression revealed that weather factors influenced 33-58% of the fruit damage (Table 1). Earlier work (Kumar et al., 2004; Mannan et al., 2015; Kassi et al., 2019) observed that the weather parameters influenced the dynamics of *L. orbonalis*. During kharif, morning RH and minimum temperature exhibited a negative effect on damage in all the cultivars; it was significant in Eeranagere (r= -0.71) and Mattugulla (r= -0.74) with morning RH. Multiple regression revealed that, the weather factors contributed to 48-88% of *L. orbonalis* fruit damage (Table 1); during rabi, wind velocity showed a significant negative correlation in most of the cultivars (r= -0.55−-0.69), also with maximum temperature (r= -0.41−-0.62). But, no such relations were observed in case of Eeranagere and Madhugiri Mulla (Table 1). Similar trends with rainfall, wind velocity, maximum and minimum temperature are known (Singh et al., 2011; Anjali et al., 2012; Indirakumar et al., 2016). Rainfall had no significant effect, as observed by Kassi et al. (2019) and contrary to the observations of others (Naik et al. 2011; Singh et al. 2009; Shukla and Khatri, 2010).

The cultivar Eeranagere was the best with least fruit damage (23.02%). Likewise, Pusa Purple Long-74 and Navkiran and accessions like EG058, BL009, and hybrid Turbo were found superior (Mathur et al., 2012; Srinivasan, 2008). The fruit damage was more in Mullubadane (27.92%) followed by Mattugulla (27.50%) and the results are in line with the varieties like Krishna and Kanahya (Choudhary et al., 2018); also Ft Long and FI Round hybrids (Tapa et al., 2009). Among the two seasons, the rabi-winter had lesser fruit damage (24.83%) compared to kharif (26.39%). In the kharif, the crop was found more vulnerable (Malshe et al., 2016).

ACKNOWLEDGEMENTS

The study was supported by the Department of Science & Technology, Ministry of Science &

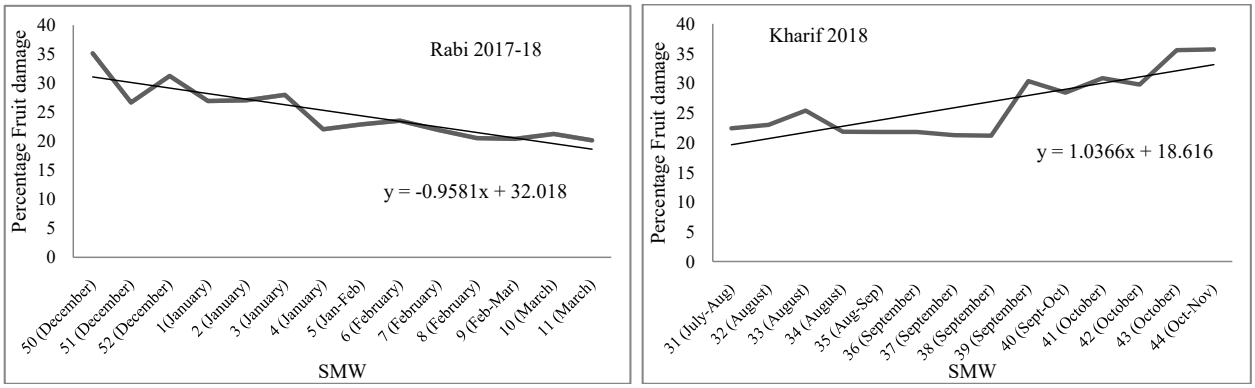

Fig. 1. Fruit damage by *L. orbonalis* in cultivars over standard meteorological weeks

Table 1. Correlation coefficients- *L. orbonalis* fruit damage vs. weather factors on cultivars of brinjal

Sl. No.	Season / Factors	Rabi 2017-18						Kharif 2018-19					
		Eeranagere	Madhugiri Mulla	Manjari-gota	Mattu-gulla	Mullu-badane	Gourav	Lalitha	Eeranagere	Madhugiri Mulla	Manjari-gota	Mattu-gulla	Mullu-badane	Gourav	Lalitha
1	Minimum Temperature	-0.2	-0.22	-0.06	-0.21	-0.20	-0.15	-0.16	-0.55*	-0.70**	-0.18	-0.79**	-0.81**	-0.70**	-0.07
2	Maximum Temperature	-0.38	-0.25	-0.42	-0.41	-0.58*	-0.56*	-0.62**	0.32	0.29	0.11	*0.50*	0.26	0.23	0.18
3	RH morning	0.11	-0.11	0.24	0.33	0.37	0.33	0.38	-0.71**	-0.63*	-0.37	-0.74**	-0.65*	-0.56*	-0.34
4	RH afternoon	0.3	0.31	0.3	0.28	0.43	0.43	0.51	-0.055	-0.11	-0.15	-0.21	-0.064	0.06	0.08
5	Wind velocity	-0.43	-0.07	-0.69**	-0.57*	-0.63*	-0.55*	-0.41	-0.16	-0.12	-0.23	-0.22	0.001	-0.2	-0.4
6	Rainfall	-0.31	-0.23	-0.2	0.24	-0.29	-0.21	-0.25	-0.26	-0.29	0.21	-0.28	-0.41	-0.22	-0.03
7	Sunshine	-0.23	-0.8	-0.49	-0.34	-0.47	-0.4	0.36	0.53*	0.52	0.28	0.71**	0.49	0.47	0.34
8	R^2	0.33	0.38	0.58	0.42	0.58	0.44	0.57	0.84	0.83	0.48	0.84	0.88	0.85	0.77
9	Regression equation	y= -0.6892x+ 21.607	y= -0.2807x+ 16.191	y= -1.0718x+ 30.105	y= -0.2951x+ 21.351	y= -1.674x+ 31.445	y= -2.0306x+ 33.374	y= -3.681x+ 48.105	y= -0.8215x+ 8.8414	y= -1.8795x+ 7.3042	y= 0.7103x+ 14.199	y= 2.317x+ 7.0326	y= -1.8271x+ 13.235	y= 1.504x+ 7.1275	y= -1.526x+ 6.774

*Significant at p=0.05; **Significant at p=0.01

Technology, Government of India, DST/INSPIRE Fellowship/2016/IF160900. Field facilities extended by the Director, ICAR-NBAIR, Bengaluru are acknowledged.

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(Manuscript Received: July, 2020; Revised: September, 2020;
Accepted: September, 2020; Online Published: February, 2021)
Online published (Preview) in www.entosocindia.org Ref. No. 20222

Biologia
https://doi.org/10.2478/s11756-019-00346-4

ORIGINAL ARTICLE

Identification of suitable reference genes for normalization of RT-qPCR data in eggplant fruit and shoot borer (*Leucinodes orbonalis* Guenée)

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Received: 19 January 2019 / Accepted: 15 August 2019

Abstract

The eggplant shoot and fruit borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) is a monophagous and destructive insect pest of eggplant. *L. orbonalis* developed multiple insecticide resistance that led to frequent field control failures in many countries. The possible reversal of resistance in *L. orbonalis* requires an understanding of the expression dynamics of individual genes conferring resistance to insecticides through gene expression analysis. The availability of reference genes (RG) to normalize transcript data of target genes, achieved by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), is mandatory. Hence, the present study investigated the stable expression pattern of nine putative reference genes viz., *actin, 28S ribosomal protein S18c, mitochondrial isoform X2 (28SM), calnexin, β-tubulin, 28S ribosomal protein S3 mitochondrial (28SR3), TATA box binding protein (TATA), elongation factor-1α (EF-1α), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH),* and *ubiquitin 60S ribosomal protein (Ubiquitin)* to select the most stable genes that could be used as reference genes for target transcript gene normalization. Different algorithms such as ΔCt, geNorm, NormFinder and BestKeeper were used to assess gene expression stability. By integrating these data into online RefFinder tool, *28SR3 and GAPDH,* were chosen as highly suitable RGs based on their stable expression pattern.

Keywords *Leucinodes orbonalis* · Reference genes · RT-qPCR · Normalization · *28SR3* · *GAPDH*

Abbreviations

28SM	*28S ribosomal protein S18c, mitochondrial isoform X2*
28SR3	*28S ribosomal protein S3 mitochondrial*
TATA	*TATA box binding protein*
EF-1α	*Elongation factor-1α*
GAPDH	*Glyceraldehyde-3-phosphate-dehydrogenase*
Ubiquitin	*Ubiquitin 60S ribosomal protein*
RG	Reference gene
M value	Expression stability value
Cq	Qquantification cycle
RT-qPCR	Quantitative reverse-transcription polymerase chain reaction
GST	Glutathione S-transferase
NTC	No template control

Electronic supplementary material The online version of this article (https://doi.org/10.2478/s11756-019-00346-4) contains supplementary material, which is available to authorized users.

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Introduction

Eggplant, *Solanum melongena* L. popularly known as brinjal or aubergine is a popular vegetable crop cultivated across the world. The damage caused by shoot and fruit borer, *L. orbonalis* (Lepidoptera: Crambidae) is a major limiting factor in realising maximum productivity of eggplant. The larva bores into the petiole and midrib

Published online: 26 August 2019

of leaves and also the tender shoots resulting in drooping and withering of stems. The tunnelling and larval excrement make the fruits unfit for human consumption. Losses due to feeding damage by *L. orbonalis* ranged from 20.7 to 88.7% in South-Asian countries (Haseeb et al. 2009; Mishra et al. 2014).

Since 1980's, *L. orbonalis* became resistant to many classes of insecticides viz., organophosphates, pyrethroids, carbamates and amides (Shirale et al. 2017). One of the causes for rapid development of insecticide resistance in *L. orbonalis* is the existence of stronger innate defence system to detoxify the toxic glycoalkaloids produced by eggplant. Although *L. orbonalis* has remained a devastating pest of eggplant for more than four decades, no genomic information on this pest was available till the present experiment.

At the molecular level insecticide resistance is generally conferred by one or more point mutations in genes governing allelochemical metabolism like cytochrome P450 monooxygenase, glutathione S-transferese, carboxylesterase and at the target sites (Mohan et al. 2015). In light of the availability of genome information now, it is possible for genome wide identification and characterization of genes governing insecticide resistance. This can be achieved in a quick and accurate manner by employing quantitative real-time PCR (RT-qPCR) and subsequent gene knockdown studies (Wong and Medrano 2005; Espy et al. 2006; Bustin et al. 2010). For a reliable estimate of target gene transcription using RT-qPCR, validation of an appropriate reference genes (RGs) is a precondition (Vandesompele et al. 2002). Reference genes must be constitutively expressed, not changing across experimental conditions considered under study, which could simply means across cell types, tissues or organs.

Multiple algorithms such as geNorm, BestKeeper, and NormFinder are being employed to study the stability of reference genes in diverse systems (Shakeel et al. 2017). The NormFinder model assesses the standard deviation for individual gene and correlates it with target gene expression (Andersen et al. 2004). The BestKeeper shows highest stability index for the suitable reference genes and compare the genes across all treatments based on the analysis of a stability index (Pfaffl et al. 2004). Similarly, the geNorm model evaluates the best pair of suitable reference genes by assessing the gene expression stability value (M) for each gene. This model works on the principle of mean pairwise variation between genes across all samples. The genes with least M value (<0.15) are considered as more stable (Vandesompele et al. 2002). The ΔCt algorithm compares the individual sample basic Cq values and the relative expression of gene pairs (Silver et al. 2006). The overall stability of the RGs could be ranked using comprehensive RefFinder tool. To assess the most stable reference genes suitable for normalization of target gene expression in *L. orbonalis*, nine candidate reference genes were selected and transcript stability evaluated.

Materials and methods

Insect culture

The susceptible laboratory iso-female strain of *L. orbonalis* used in this investigation was originally collected during 2012 from fields of eggplant in Bengaluru, India (12.97° N and 77.58° E), and being maintained on potato-based diet system at ICAR-National Bureau of Agricultural Insect Resources, Bengaluru. The rearing conditions were 27 ± 2 °C, 60–70% RH, and a photoperiod of 14:10 h (L:D). The insecticide resistant *L. orbonalis* population used in this study was collected from eggplant field of Bhubaneshwar district (20.29″ and 85.82″) where the crop usually receives a high level of pesticides sprays every season because of extensive eggplant cultivation compered to other regions and crop present throughout the season. We have used two experimental conditions to assess gene stability: 1) developmental stage (3rd and 4th instar larvae of the susceptible population) and 2) susceptible versus resistant population (3rd instar larvae of susceptible laboratory and resistant field population)..

RNA extraction and cDNA synthesis

The total RNA was extracted by using 50 mg of third instar larva by Isolate II RNA mini kit (Bioline, USA) by following manufacturers guidelines. Three biological replicates were used for each experimental conditions and the experiment was repeated twice. The quality and purity of RNA were assessed by using NanoDrop (NanoDrop Lite, Thermo scientific, USA) and denatured agarose gel (Masek et al. 2005). The RNA samples (1806–2105 ng/μL) with an A260/A280 ratio ranging from 1.8 to 2.0 and A260/A230 ratio > 2.0. DNA contamination was removed from the RNA by using DNase I, RNase-free (Thermo Scientific, USA) by following manufacturers instructions. cDNA synthesis was carried out by using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, US). Briefly, for 20 μL reaction contains the following reagents: 4 μg of total RNA, 0.5 μM of Oligo $(dT)_{18}$ primer, 1x Reaction buffer, RiboLock RNase Inhibitor (20 U), 1 μM dNTPs and RevertAid M-MuLV RT (200 U). The reaction was incubated at 42 °C for 60 min followed by 70 °C for

5 min. The synthesized cDNA was diluted to 1:10 with nuclease-free water and stored at −80 °C till further use.

Quantitative real-time PCR

Nine candidate RGs were chosen to study their expression stability: *actin, 28S ribosomal protein S18c mitochondrial isoform X2 (28SM), calnexin, β-tubulin, 28S ribosomal protein S3 mitochondrial (28SR3), TATA box binding protein, elongation factor-1a (EF1a), glyceraldehyde 3 phosphate dehydrogenase (GAPDH)* and *ubiquitin 60s ribosomal protein (Ubiquitin)* (Table 1).

Primer 3.0 software was used to design the gene-specific primers. OligoEvaluator™ sequence analysis tool was used to study the primer dimer, secondary structure as well as efficiency (OligoEvaluator™: accessed on July 2018; http://www.sigmaaldrich.com/life-science/custom-oligos/custom-dna/learning-center/calculator.html). The PCR amplifications were executed in 20 μL reactions containing 10 μL 2 × SYBR® Premix EX Taq™ II (Tli RNaseH Plus, TAKARA®, Japan), 0.2 μM of each forward and reverse primers, 2 μL of diluted cDNA (40 ng/reaction) and 5.5 μL of water. Three technical replicates, including no-template controls (NTCs) used to assess contaminations and primer dimers, were considered per samples. Three Five-fold serial dilutions of cDNA template were considered to perform a standard curve using, $10^{(-1/\text{slope})-1)*100}$ to calculate primer efficiency (Pfaffl 2001) by measuring the quantification cycle (Cq), using Light Cycler 480II (Roche Applied Science, Switzerland) equipment by following PCR parameters: one cycle of 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min; a final cycle of 72 °C for 10 min. The subsequent PCR products were electrophoresed on 2.0% agarose gel in a 1.0 × TAE buffer with 0.5 μg/mL of ethidium bromide..

Analysis of gene expression stability

To select the most stable genes to be used as RGs, four algorithms viz., NormFinder (Andersen et al. 2004), BestKeeper (Michael 2003; Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002) and ΔCt (Silver et al. 2006)) were employed and the web-based RefFinder tool was used to incorporate all these algorithms for comparison.

Results

Selection of suitable reference genes and source

Nine candidate reference genes belonging to five functional groups, that includes two genes encoding structural proteins (*Actin* and *β-tubulin*), three genes encoding ribosomal proteins (*28SM, 28SR3* and *ubiquitin 60s*), one encoding a metabolic enzyme (*GAPDH*), a chaperone protein (Calnexin), a transcription factor (TATA) and a protein factor (*EF-1α*) were selected (Tables 1 and 2) from de novo genome and transcriptome sequence assemblies of *L. orbonalis* previously published (BioProject ID: PRJNA377400; BioSample: SAMN06858697 and GenBank accession number PQWD00000000).

Primers efficiency and specificity

For all candidate reference genes, PCR amplification was carried out to assess primer specificity and efficiency. For primer specificity analysis, the presence of a single peak in melting curve was investigated followed by an agarose gel electrophoresis to confirm the presence of a single band. Primer efficiency of all the nine candidate genes ranged from 91.11 to 100.74% (Table 1). The regression coefficient (R^2) values ranged between 0.96 and 0.998 indicated there were no inhibitory contaminants present in the cDNA. The melting curves displayed that a single product generated from the qPCR.

Stability of reference genes

The Cq values in the present investigation ranged from 17 to 33 cycles (Table 2). Highly expressed gene was actin that showed a Cq average of 17.5, with a minimum value of 15.6 and a maximum of 21.02 followed by *GAPDH* (20.16) and *β-tubulin* (20.49). *Ubiquitin* was the most abundantly expressed gene with an average Cq value of 21.9 cycles (Fig. 1a & 1b).

The expression level of the nine candidate RGs under two different experimental conditions was analyzed by using four algorithms (geNorm, NormFinder, BestKeeper and ΔCt). The stability values were obtained by employing integrated RefFinder tool (Tables 3 and 4).

geNorm

Based on this model, *GAPDH* genes was the most stably expressed genes followed by *28SR3* and *β-tubulin* with M value below 0.9, under both the experimental conditions (Table 3; Fig. 2a, b). The pairwise variation value was calculated by the geNorm algorithm described that the pairwise variation V6/7 value is more than V5/6 (Fig. 3a, b). It clearly depicts that an increasing trend of variation in relation to decreased stability after adding moderately unstable sixth gene (Fig. 3). Hence, five candidate RGs viz., (*Actin, β-tubulin, 28SM, 28SR3* and *TATA*) are pre-requisite for perfect normalization. Adding a sixth RG gene has no impact on the normalization factor (Fig. 3a, b).

Table 1 Primers used in the normalization of reference genes for *L. orbonalis*

Reference genes	Function	Homologous species and their accession number	Primer Sequence 5′ –> 3’	Sequence Length (bp)	Amplicon size (bp)	E (%)	R^2	Tm (°C)
Actin	Cell division	*Plutella xylostella* & NP_001296030.1	F: CACCGAGAGGGGTT ACTCTTT	1149	73	98.42	0.984	66
			R: GCACAGCTTCTCCT TGATGTC					66
28S Ribosomal Protien S_{18c} Mitochondrial Isoform X_2 (28SM)	Protein synthesis	*Ostrinia furnacalis* & XP_028178958.1	F: CCCATAGACATCGA AAATCCA	380	115	92.62	0.990	62
			R: AAGGGCTTTGGAAC TGAGAAA					64
β-tubulin	Cellular process and also mitosis	*Agrotis ipsilon* & AEJ84083.1	F: AGGCTTTCTTGCAT TGGTACA	1287	102	91.11	0.960	64
			R: GGTACTGCTGGTAC TCGGACA					69
Calnexin	Protein binding and quality control	*Helicoverpa armigera* & XP_ 021187739.1	F: CTACGACCAGACGC CTTACAC	176	103	96.46	0.984	67
			R: AGTGCCATTCTTTG GGTTTTT					63
28S Ribosomal Protein S3 Mitochondrial (28SR3)	Protein synthesis	*Helicoverpa armigera* & XP_ 021185601.1	F: CAAAACTGGATGTG TGGGAGT	224	71	100.11	0.996	65
			R: GTTAGCTGGTGGTT GCAGTGT					68
TATA box binding protein (TATA)	Transcription	*Papilio xuthus* & XP_013182806.1	F: AAAGCGAGAAACAA TCACGAA	597	88	92.74	0.995	62
			R: TTGAAGTCCCCAAA GTTAGCA					64
Elongation factor-1a (EF-1)	Translation	*Spodoptera litura* & XP_022819255.1	F: ATGGATATTGGCAG AGCACAG	1013	88	92.01	0.998	65
			R: TGTTTGGGATGAAA TCAGCAT					62
Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH)	Cell function and apoptosis	*Corcyra cephalonica* & AHL24709.1	F: AAGTCATTCCCGCT CTTAACG	561	89	100.74	0.995	65
			R: GCGGACAGTAAGGT CAACAAC					66
Ubiquitin	Membrane protein and transcriptional regulation	*Helicoverpa armigera* & XP_ 021183635.1	F: CCTGACCAGCAGCGATTG	231	98	91.81	0.993	67
			R: ACCAAGTGCAGTGT TGATTCC					66

Table 2 The stability values of the reference gene by ΔCt-Method

Reference genes	Experimental conditions	
	Developmental Stages (±SE)	Populations (±SE)
Actin	17.65 ± 0.51	17.65 ± 2.14
28SM	29.53 ± 1.12	29.53 ± 1.27
Calnexin	28.17 ± 1.24	28.16 ± 0.22
β-tubulin	20.49 ± 1.37	20.49 ± 1.04
28SR3	25.15 ± 1.16	25.15 ± 0.74
TATA	33.12 ± 1.96	33.12 ± 2.65
EF-1	29.90 ± 2.09	29.9 ± 0.82
GAPDH	20.19 ± 1.07	20.19 ± 1.2
Ubiquitin	20.31 ± 1.86	20.31 ± 0.25

NormFinder

Under this algorithm, the most stably expressed gene is *GAPDH* followed by *28SR3* across the experimental conditions (Table 3). The gene expression stability value of *GAPDH* is 0.290 for developmental stages and 0.487 for populations tested.

BestKeeper

In the present study, all candidate RGs showed expression level less than the cut-off value (<0.3). The expression cut-off of *β-tubulin* was the least, followed by *ubiquitin* and *28SR3* across the experimental conditions (Table 3). *β-tubulin, ubiquitin* and *28SR3* were the most stably expressed genes having a cut-off value less than one (SD < 1) as compare to other RGs.

RefFinder

It is the web-based comprehensive online program that integrates results obtained from all other algorithms viz., BestKeeper, Normfinder, geNorm and the ΔCt. It ranks the RGs on the basis of their geo-mean constancy. The RGs rankings recorded in order of descending constancy across developmental stages and populations are depicted (Tables 3 and 4). For *L. orbonalis* growth stages, the cumulative ranking achieved with RefFinder was *28SR3, GAPDH, β-tubulin, ubiquitin, calnexin, EF-1α, actin, TATA* and *28SSM* (Fig. 4a). The comprehensive stability ranking across the populations was *28SR3, GAPDH, β-tubulin*, *ubiquitin, calnexin, EF-1α, actin, TATA* and *28SSM,* (Fig. 4b). This integrated tool indicates that for *L. orbonalis, 28SR3* and *GAPDH* genes could be adopted as appropriate RGs for normalization of target gene expression.

Discussion

A stable and highly expressed single RG would fulfill the essentiality of computing mRNA transcript level for the given gene of interest. However, for accurate normalization of gene expression data, it is suggested to use at least two or three RGs (Vandesompele et al. 2002). The present study employed four different algorithms viz., BestKeeper, geNorm, NormFinder, ΔCt and integrated these data into the RefFinder tool for comprehensive analysis. The expression pattern of the RGs was unique but a similar trend was noticed when analysed with different methods. Among the nine candidate RGs, expression of *28SR3* and *GAPDH* were highly stable and corroborate with earlier reports (Kim et al. 2003; Zhang et al. 2009; Chen et al. 2016). However, with the ΔCt method, the *GAPDH* was found to express more stable than *28SR3*. Since the ΔCt uses raw Cq values as an input data source, might have resulted in false positives as also reported earlier (Livak and Schmittgen 2001; Mehdi-Khanlou and Van-Bockstaele 2012; Chen et al. 2016). In eukaryotes, the ribosomal RNA (rRNA) genes are highly conserved and

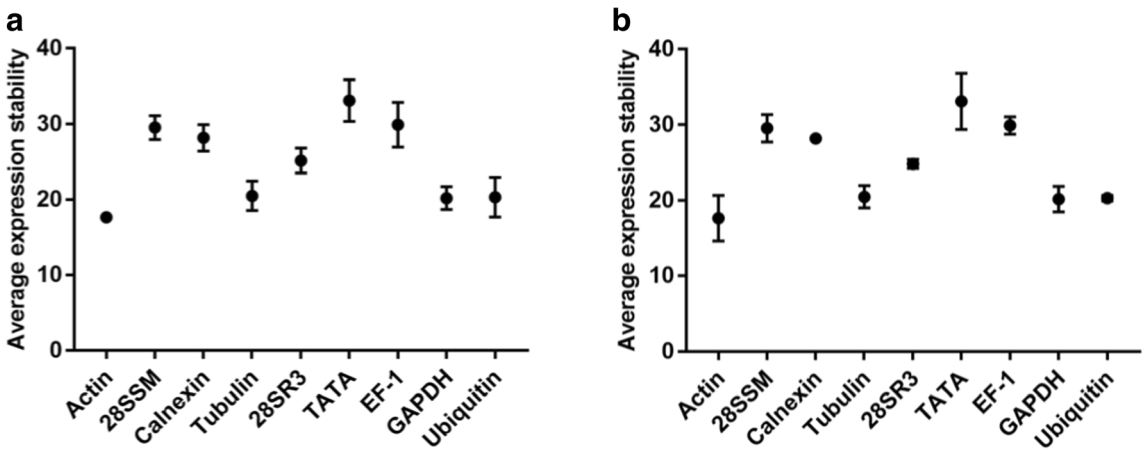

Fig. 1 Cq values of reference genes by ΔCt algorithm under different conditions in *L. orbonalis*. **a**) developmental stages and **b**) Populations

Table 3 Analysis of gene expression stability values and rankings of the reference gene

Reference genes	NormFinder		BestKeeper		geNorm		RefFinder Comprehensive	
	Developmental stage	Populations	Developmental stage	Populations	Developmental stage	Populations	Developmental stage	Populations
28SR3	0.461 (2)	0.939 (3)	0.32 (3)	0.74 (3)	0.54 (2)	0.95 (3)	1.19 (1)	1.19 (1)
GAPDH	0.290 (1)	0.487 (1)	0.43625 (6)	1.2 (6)	0.54 (1)	0.72 (1)	1.57 (2)	1.57 (2)
Calnexin	1.149 (6)	0.976 (4)	0.5625 (8)	1.04 (5)	0.76 (3)	0.72 (2)	3.66 (3)	3.66 (3)
β-tubulin	1.391 (7)	1.773 (8)	0.023 (1)	0.23 (1)	1.26 (6)	1.78 (7)	4.56 (5)	4.56 (5)
Ubiquitin	0.771 (4)	0.764 (2)	0.07 (2)	0.26 (2)	1.13 (5)	1.41 (6)	4.16 (4)	4.16 (4)
EF-1	0.667 (3)	1.098 (5)	0.425 (5)	0.82 (4)	0.94 (4)	1.09 (4)	4.68 (6)	4.68 (6)
Actin	1.106 (5)	1.191 (6)	0.7125 (9)	2.15 (8)	1.51 (7)	1.25 (5)	7 (7)	7 (7)
28SM	3.306 (9)	2.315 (9)	0.40125 (4)	1.28 (7)	2.3 (9)	2.43 (9)	8 (8)	9 (9)
TATA	1.651 (8)	1.490 (7)	0.54625 (7)	2.65 (9)	1.8 (8)	2.05 (8)	9 (9)	8 (8)

commonly used reference genes (Eickbush and Eickbush 2007). The ribosomal protein, *28SR3*, involved in the translation process and protein production revealed the maximum stability amongst studied genes. RT-qPCR studies with *Apis mellifera* and *Cimex lectularius* used *RPL18* as the reference gene gene due to its stable expression (Scharlaken et al. 2008; Mamidala et al. 2011). The ribosmal protein genes, *RP4* and *RP18* were found suitable for the gene expression studies in *Leptinotarsa decemlineata* (Shi et al. 2013). The *18S* and *28S* are commonly suggested as reference genes for human liver, lung, spleen and small intestine because of their stable expression pattern across mammalian tissues (Goidin et al. 2001; Thellin et al. 1999). Though the expression of *28SR3* gene is highly stable and to be best used as an reference gene in experiments with *L. orbonalis,* another ribosomal protein *28SM* could not qualify the criteria.

GAPDH, a metabolic enzyme with considerable roles in diverse physiological activities (Sirover 1997), revealed to be stably expressed as the 28SR3. *GAPDH* was used as reference gene for gene expression in many insects and vertebrates (Van-Hiel et al. 2009, Hornakova et al. 2010, Jiang et al. 2010, Lord et al. 2010, Mamidala et al. 2011, Rajarapu et al. 2011; Cardoso et al. 2014: Chapman and Waldernstrom 2015, Reim et al., 2013). In contrary, the *GAPDH* was found as one of the most unstable reference gene in other groups of insects (Bagnall and Kotze 2010; Hornakova et al. 2010; Chapuis et al. 2011). The differences in ranking of reference genes could also be due to the algorithms used and also with respect to the co-regulated reference genes. Studies on insects with optimal selection of RGs for gene expression experiments are species-specific and highly dependent on the experimental conditions and require assessment of reference genes before

Table 4 Analysis of gene expression stability rankings of the reference gene using RefFinder tool

Algorithm	Ranking* / Treatment details	1	2	3	4	5	6	7	8	9
ΔCt-Method	Developmental stage (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Calnexin*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Calnexin*	*Actin*	*TATA*	*28SM*
BestKeeper	Developmental stage (combined)	*28SR3*	*Calnexin*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3*	*Calnexin*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Actin*	*TATA*	*28SM*
Normfinder	Developmental stage (combined)	*GAPDH*	*28SR3*	*Ubiquitin*	*EF-1*	*β-tubulin*	*Calnexin*	*Actin*	*TATA*	*28SM*
	Population (combined)	*GAPDH*	*28SR3*	*Ubiquitin*	*EF-1*	*β-tubulin*	*Calnexin*	*Actin*	*TATA*	*28SM*
geNorm	Developmental stage (combined)	*28SR3* | *GAPDH*		*β-tubulin*	*EF-1*	*Ubiquitin*	*Calnexin*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3* | *GAPDH*		*β-tubulin*	*EF-1*	*Ubiquitin*	*Calnexin*	*Actin*	*TATA*	*28SM*
RefFinder integrative ranking	Developmental stage (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*Calnexin*	*EF-1*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*Calnexin*	*EF-1*	*Actin*	*TATA*	*28SM*

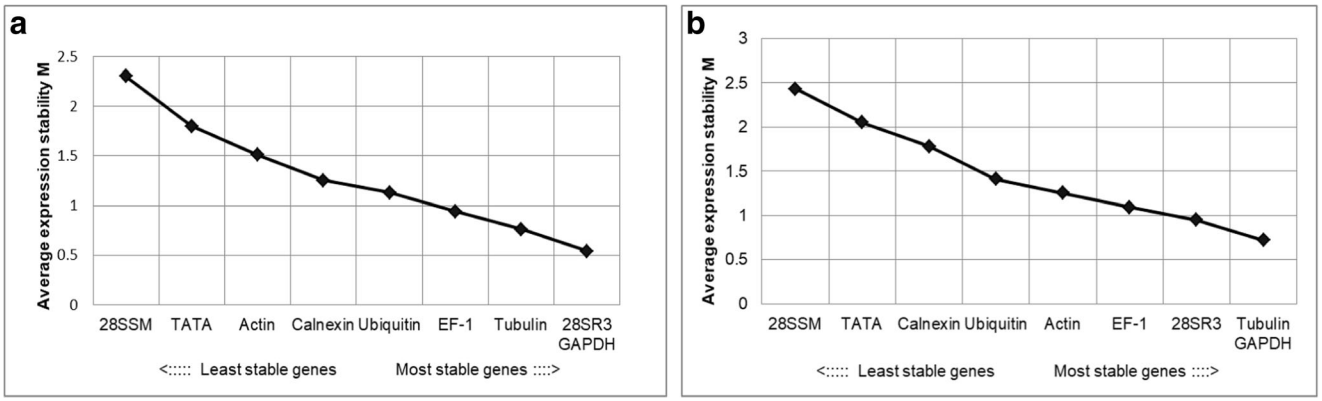

Fig. 2 Analysis of gene expression stability (M) and ranking of reference genes using geNorm algorithm in *L. orbonalis*. **a**) developmental stages and **b**) Populations

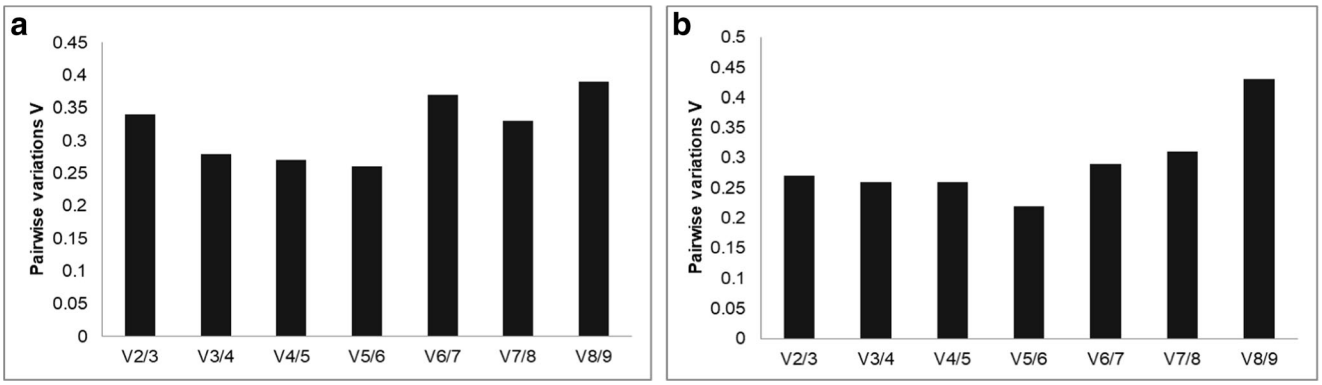

Fig. 3 The Pairwise variation (V) analysis of the RGs by geNorm in *L. orbonalis* developmental stages (**a**) and populations (**b**). The pairwise variation (Vn/n1) among the normalization factors NFn and NFn1 (along x-axis) was analysed. Every bar depicts the deviation in normalization when adding reference genes

undertaking gene expression studies on a new insect (Zhu et al. 2014; Zhai et al. 2015; Velada et al. 2014; Petriccione et al. 2015; Koramutla et al. 2016). In desert locust *Schistocerca gregaria,* the gene expression stability of nine reference genes in the brain of nymphs and adults were assessed. Multiple genes viz., *Actin, Ef1α100E, GAPDH,* and *Rp49* were considered as highly suitable to be used as reference genes (Van-Hiel et al. 2009). The use of multiple reference genes would permit more precise and consistent normalization of gene expression data (Vandesompele et al. 2002).

L. orbonalis is a devastating insect pest of eggplant in Asia and other continents. With the availability of de novo genome, transcriptome information, the *28SR3* and *GAPDH* could be used as appropriate candidate RGs for normalizing gene expression and gene knockdown experiments.

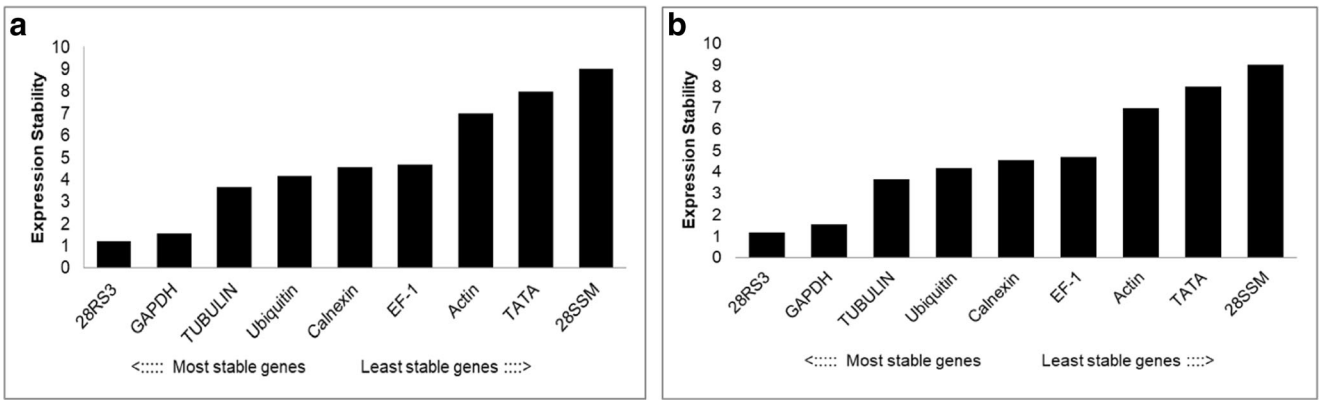

Fig. 4 Analysis of gene expression stability values and ranking of RGs using RefFinder algorithm in *L. orbonalis*. **a**) developmental stages (**b**) populations

Acknowledgements The authors profusely thank Indian Council of Agricultural Research for generous funding under CRP Genomics project. The first author acknowledges the award of **DST-Inspire fellowship (2016/IF160900).** The facilities extended by Director, ICAR-NBAIR, and Director, ICAR-IIHR, Bengaluru are gratefully acknowledged.

Authors contributions Kariyanna B. Conducted experiment, Analysis, Manuscript writing, Material and methods, Hypothesis. Prabhuraj A. Constructing experiment, Analysis, Material methods, corrections. Asokan, R. Analysis and Technical suggestion, Manuscript Correction, Resource supply. Prasad Babu Materials and Methods, Analysis, Reviews and literatures. Jalali S. K. Resources supply, technical guiding and suggestions, primary preview of manuscript. Venkatesan T. Resources supply, technical guiding and suggestions, Reviews and literatures. Gandhi Gracy R. Analysis, technical guiding and suggestions, Reviews and literatures. Mohan M. Designing experiment, Resources supply, Manuscript corrections, analysis, Material and methods, technical guiding and suggestions.

Funding information The present research was supported by Indian Council of Agricultural Research- CRP Genomics project and Department of Science & Technology, Ministry of Science & Technology, Government of India, **DST/INSPIRE Fellowship/2016/IF160900.**

Compliance with ethical standards

Disclosure statement The authors have no potential conflict.

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Biocatalysis and Agricultural Biotechnology 20 (2019) 101188

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

The relationship between genome size, morphological parameters and diet breadth in insect species

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ARTICLE INFO

Keywords:
Insect species
Genome size
Correlation
Morphology
Host range

ABSTRACT

Genome size estimation is the first step involved in the complete genome sequencing project. Knowledge on genome size is also useful in research work related to genetics, molecular biology, and systematics. Though there are many methods of genome estimation, the Propidium Iodide (PI) based flow cytometry is simple and gives an accurate estimation. In the present investigation, the diploid genome size of four insect species *viz.,* cotton leafhopper, *Amrasca biguttula biguttula* (392.47 Mb) (Cicadellidae: Hemiptera), bean pod borer, *Maruca vitrata* (1489.63 Mb) (Crambidae: Lepidoptera), tomato pinworm, *Tuta absoluta* (1128.61 Mb) (Gelechiidae: Lepidoptera) and eggplant shoot and fruit borer, *Leucinodes orbonalis* (944.52 Mb) (Crambidae: Lepidoptera) were estimated using chicken RBC (2.33 pg) as a reference standard. The relationship of genome size with the insect morphological traits and host plant range showed no evident correlation between them.

1. Introduction

An organism cell nucleus being a core element that carries a hereditary blueprint called genetic material. The obvious essentiality of genetic material is the role in cell cycle duration, cell size, and adaptivity under different selection pressure and so on (Leitch and Bennett, 2007). These make the genome size information, a critical for many fields of research that deals with taxonomy, evolutionary changes (Kron et al., 2007) and genome sequencing projects (Rabinowicz and Bennetzen, 2006). The C-value or genome size refers to the amount of DNA present in a cell which is constant for each organism and varies across eukaryotes over 64,000-fold (Pellicer et al., 2018). Eukaryotic genomes not only contain genetic information but also act as structural components that determine nuclear properties and influence various biological features such as cell size, developmental rate, and developmental complexity (Gregory and Hebert, 1999; Koshikawa et al., 2008). Genome size is described by either mass (pg) or the number of base pairs (bp) (Gregory, 2005). So far, the genome sizes of 8005 animal species have been recorded in the animal genome size database (Accessed 15 December 2018) (Gregory, 2018). Compared to those of mammals and birds, the genome sizes of invertebrates remain poorly studied. Of the nearly 1000000 described insect species, the genome sizes of 1345 (0.134%) insects are known with the coverage of species under Lepidoptera and Hemiptera are only around 6.5% each (Gregory, 2018).

Flow cytometry is a convenient and reliable method that has been used to estimate nuclear genome size in many organisms, including plants, animals, and insects (Waring, 1965; Bennett et al., 2003). The variation in genome size in extant species is on the level of ten to hundred thousand folds (Gregory, 2018). DNA flow cytometry explains the use of flow cytometry for the estimation of DNA quantity in cell nucleus. Flow cytometry analyses microscopic particles in suspension that are compelled to move a single flow file within a fluid stream through the focus of intense light. Scattered light pulses and fluorescence are collected and converted to electric pulses by optical sensors and classified.

The cotton leafhopper, *Amrasca biguttula biguttula* (Cicadellidae: Hemiptera), eggplant shoot and fruit borer, *Leucinodes orbonalis* (Crambidae: Lepidoptera), bean borer, *Maruca vitrata* (Crambidae: Lepidoptera) are all originated from India but invaded many other countries across the globe. Whereas the tomato pinworm, *Tuta absoluta* (Gelichiidae: Lepidoptera) have originated from Peru in South America and invaded many other countries including India. All these insects cause severe yield losses in cotton, eggplant, legumes, and tomato

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https://doi.org/10.1016/j.bcab.2019.101188
Received 20 March 2019; Received in revised form 28 May 2019; Accepted 30 May 2019
Available online 31 May 2019
1878-8181/

respectively not only in their country of origin but in all the invaded countries so far (Tonnang et al., 2015).

One of the major rationales for genome size estimation of these insect species is due to their economic importance as dreaded pests of crops and for further work on their genome as well as transcriptome sequencing. Using flow cytometry, it is possible to create a precise and accurate estimate of the diploid genome size of an insect that is useful for genomics, genetics, molecular or cell biology and systematics. Genome size estimation is a basic component in complete genome sequencing and also approximate estimators of the cost and difficulty of genome sequencing programs for the non-model organisms. Bennett et al. (2000) said that the knowledge on the prior estimation of genome size is also essential for gene cloning.

In this study, the diploid genome size of the four economically important insect species was estimated by Propidium Iodide (PI)-based flow cytometry. To our knowledge, we present the first data set that relates the diploid genome size of all the four insect pests and their relation with morphological parameters and host plant range.

2. Materials and methods

2.1. Test insects collection and maintenance

The cotton leafhopper, *A. biguttula biguttula*, and tomato pinworm, *T. absoluta* were and maintained on cotton and tomato plants respectively under net-house conditions at the experimental farm of ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. *L. orbonalis* larvae were reared on potato tuber-based artificial diet. The larvae of *M. vitrata* were reared on soybean-cowpea-wheat-germ based diet developed by Wang et al. (2013) with little modifications. The insect obtained from isogenic populations were used in flow cytometry analysis.

The moths of *T. absoluta*, *L. orbonalis*, *M. vitrata*, and the leafhopper, *A. biguttula biguttula* derived from single isogenic line were released (1:1 sex ratio) in separate oviposition cages provided with fresh host plants/paper towel for mating and egg laying. The cotton wads soaked in sugar solution (0.2%) and honey (0.5%) served as food for the moths. The plant parts/paper towel containing the freshly laid eggs were removed daily and kept for hatching. Newly hatched larvae or the nymphs (leafhopper) were transferred to specimen jars with respective host plants diet. Rearing was continued until the emergence of the adults. The information on the host range of the test insects was obtained from various literature sources.

2.2. Morphometric measurements

The body measurements of the insect species were made for all the stages using stereoscopic microscope (Carl-ZEISS (Zoom 1:10), Model: Discovery. V8, Camera: Axiocam.105 colour) supported with ZEN software. Antennal length, eye width, length and breadth of 20 adults were measured. Correlation between the morphological parameters, number of host plants and the mean genome size were estimated with the Spearman rank correlation using the program SPSS 16.0 (IBM Corp, 2016).

2.3. Preparation of samples for flow cytometry

Intact nuclei suspension for each insect species was prepared by complete homogenization of dissected head samples in 400 µl of Galbraith buffer (45 mM $MgCl_2$; 30 mM sodium citrate; 20 mM MOPS; 0.1% (w/v) Triton X 100; pH 7.0) (Galbraith et al., 1983). The homogenate was passed through a 20µ nylon filter to obtain the cells free from debris. The final volume of the suspension was made to 1.5 ml using the Galbraith buffer. For further enrichment of the nuclei population, cells were centrifuged at 1500 rpm for 2 min and the pelleted cells were resuspended in 1.5 ml of hypotonic solution (Distilled water containing 0.1% trisodium citrate, 40 µg/ml RNAse, 25 µg/ml propidium iodide and 0.03% NP40/igepal) for 15 min.

2.4. Flow cytometry analysis

The samples were subjected to flow cytometry analysis (BD FACS™, USA) (Li et al., 2015) for estimating the genome size. The measurements of relative fluorescence intensity of stained nuclei were performed on a linear scale and 5000–20000 nuclei were analysed for each sample (Galbraith et al., 1983). Nuclei from chicken (*Gallus domesticus*) red blood cells (2.3 pg/2C) were used as an internal reference standard. The estimates of three replicate samples of each insect species were subjected to statistical analysis using simple mean variance analysis in SPSS 16.0 (IBM Corp, 2016).

2.5. Data analysis

Results were obtained in the form of a histogram of relative fluorescence correlating the relative DNA content after comparison with nuclei of a reference standard, whose genome size is known (Galbraith et al., 1983). The absolute DNA content of a sample was calculated on the values of the G_1 peak means

$$\text{Sample 2C DNA content} = \frac{(\text{sample G1 peak mean})}{(\text{standard G1 peak mean})} \times \text{standard 2C DNA content (pg DNA)}$$

3. Results

3.1. Genome size estimation

The genome sizes of four economically important insect species were estimated by flow cytometry. The chicken erythrocyte cells were the reference standard (Fig. 1). All the samples were replicated thrice and showed good reproducibility. The estimated genome sizes of the lepidopteran insects *viz., L. orbonalis, T. absoluta* and *M. vitratta* were 944.52, 1128.61 and 1489.63 Mb respectively. The genome size of hemipteran insect, *A. biguttula biguttula* was estimated at 392.47 Mb (Table 1). The fluorescence histograms of genome size assessments were depicted in the form of G1 peak mean (Figs. 1–3). These data indicate that the lepidopteran insects have relatively bigger genome size than the hemipteran bug.

Table 1
Genome size estimates of economically important insect species.

Insect species	Family/Order	Principal host plant(s)	2C- value (pg)	n	Diploid genome size (Mb)
T. absoluta	Gelechiidae: Lepidoptera	Tomato	1.165919	3	1128.61 ± 2.98
L. orbonalis	Crambidae: Lepidoptera	Brinjal	0.975744	3	944.52 ± 10.07
A. biguttula biguttula	Cicadellidae: Hemiptera	Cotton, ladies finger	0.405444	3	392.47 ± 3.11
M. vitrata	Crambidae: Lepidoptera	Cowpea, lima bean, common bean, pigeon pea	1.538874	3	1489.63 ± 5.64

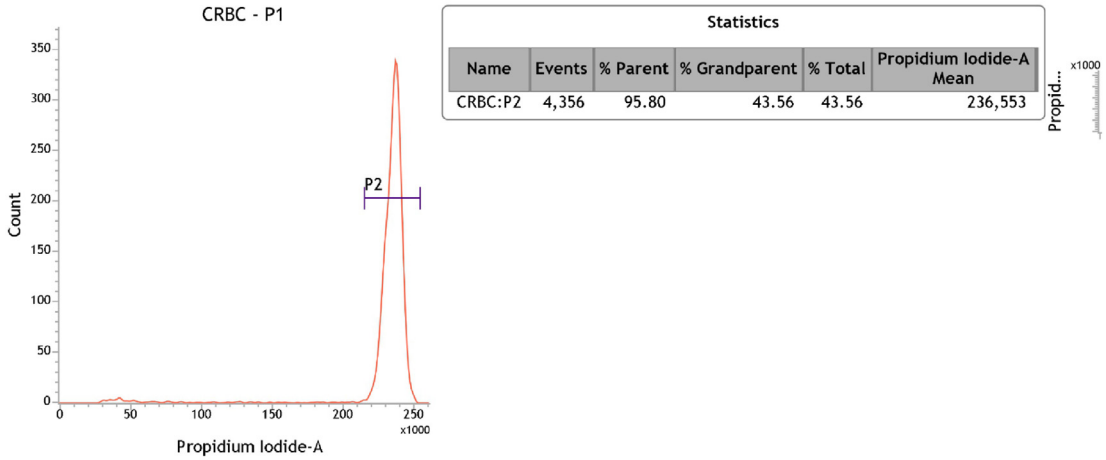

Fig. 1. Flow cytometric fluorescence histogram of reference chicken RBC cells.

3.2. Correlation of genome size with morphology and host range

The morphological data were obtained based on the adult size measurement, which includes eye width (EW), antennal length (AL), body length (BL), and body width (BW) of all insects. Correlation between the insect morphology and genome size depict that, there was no influence of morphology on insect genome size. Because, the small insect like *T. absoluta* has greater genome size compared to *L. orbonalis,* which belong to the same order Lepidoptera with different morphology. Similarly, the diploid genome sizes of all four insects did not correlate with their host plant range *viz.,* 22, 21, 25 and 13 of *T. absoluta*, *L. orbonalis*, *A. biguttula biguttula* and *M. vitrata* (Table 2) respectively (https://www.plantwise.org).

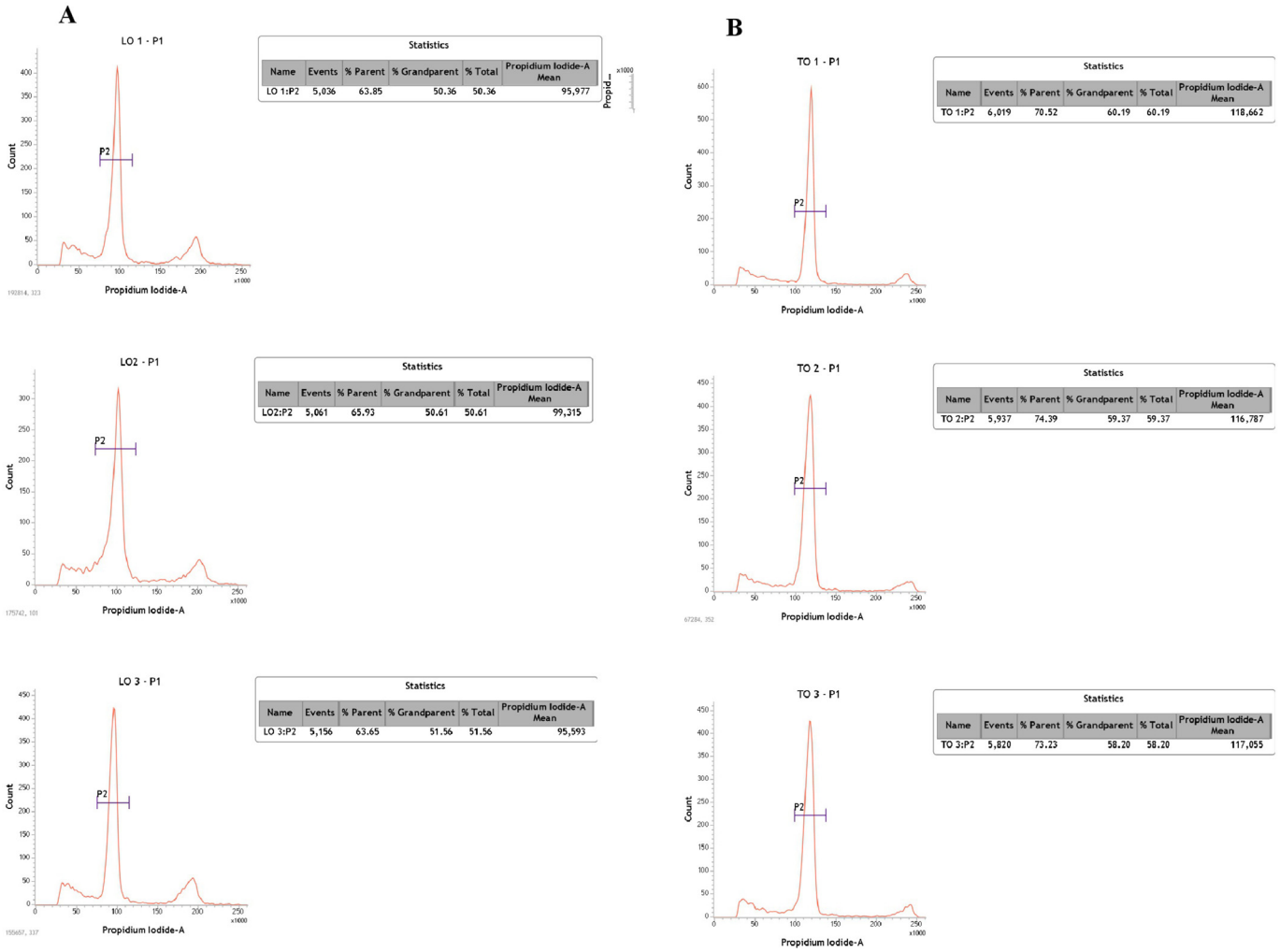

Fig. 2. Flow cytometric fluorescence histograms of *Leucinodes orbonalis* (A) and *Tuta absoluta* (B) cells.

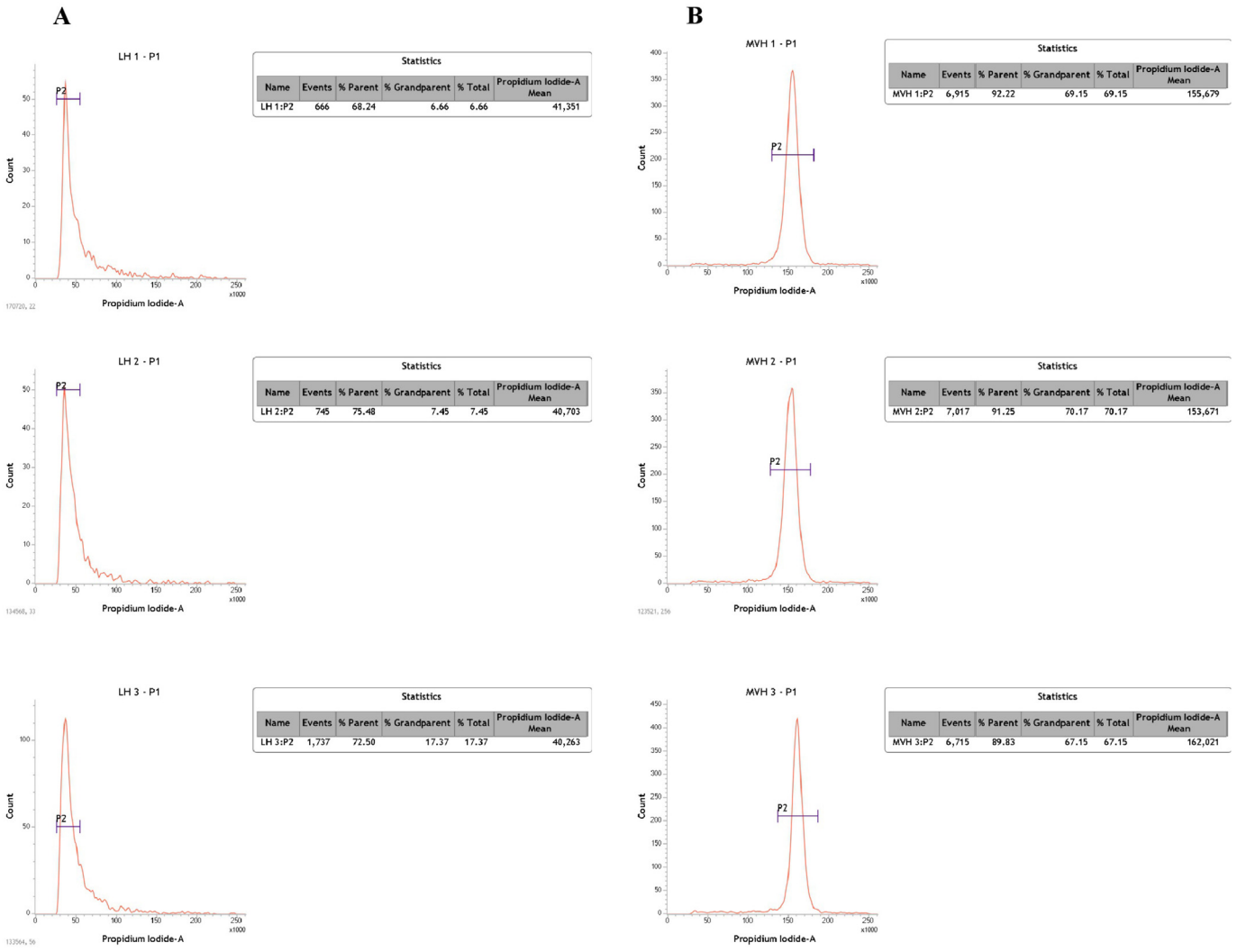

Fig. 3. Flow cytometric fluorescence histograms of *Amrasca biguttula biguttula* (A) and *Maruca vitratta* (B) cells.

4. Discussion

To explore the genome size, we performed C-value measurements for four important insect pest species (three from Lepidoptera and one from Hemiptera) using flow cytometry. Flow cytometry, a mere comparative analysis depends on the selection of the standard references. Galbraith et al. (1983) observed differential staining intensity of PI to a type of DNA. In their observation on the binding capacity of PI among plants and animals, they observed that PI binds more to methylated DNA than unmethylated DNA. PI being a non-base pair specific fluorochrome irrespective of species, its binding saturates in 1hr and the stain remains constant for 1–24 h (Bennett et al., 2003).

Rapid analyses of flow cytometry for estimating the size of haploid genome with known standard facilitated many researchers to predict the genome size of unknown organisms. Loureiro et al. (2007) confirmed the genome size of *B. tabaci* (640–680 Mb) with *D. melanogaster* (176 Mb) as a standard. Similar findings were obtained on other important insects like the honey bee, *Aphis melliferra* (234.7 Mb) (Ardila-Gracia et al., 2010), mosquito *Anopheles gambiae* (264 Mb) (Holt et al., 2002) and *Bombyx mori* (508 Mb) (Rasch, 1974).

The genome size of two different orders *viz.,* Lepidoptera and Hemiptera displayed greater variation in this study. Alfsnes et al. (2017) depicted the genome size varies both within and between taxonomic levels in animals as well as plants. Over the evolution, duplication of the whole genome or accumulation of noncoding elements like transposable and repetitive elements would influence the small or large genome size. The lack of correlation between phylogeny and host plant range is questionable, as it has been reported that phylogenetically related insects are associated with phylogenetically related host plants and this association is ancient (Kergoat et al., 2005, 2012). Similarly, Insecta and Crustacea a weaker association between genome size and phylogeny, suggesting life cycle strategies and habitat as more

Table 2
Correlation analysis of genome size with respect to morphology and host range.

Species	Eye Circumference	Antennal Length	Body Width	Body Length	Host range	R^2
T. absoluta	0.87 ± 0.41 (r = 0.55)	3.84 ± 0.36 (r = −0.55)	1.01 ± 0.04 (r = −0.58)	2.77 ± 0.03 (r = −0.50)	22 ± 0.04 (r = −0.25)	0.36
L. orbonalis	3.84 ± 0.04 (r = 0.26)	18.3 ± 0.08 (r = 0.25)	10.3 ± 0.03 (r = 0.22)	25.52 ± 0.05 (r = −0.25)	21 ± 0.04 (r = −0.52)	0.61
A. biguttula biguttula	1.01 ± 0.36 (r = −0.23)	1.27 ± 0.41 (r = −0.23)	7.5 ± 0.02 (r = −0.23)	3.74 ± 0.04 (r = 0.22)	25 ± 0.21 (r = −0.25)	−0.24
M. vitrata	2.77 ± 0.04 (r = −0.34)	17.43 ± 0.02 (r = 0.45)	10.95 ± 0.21 (r = 0.37)	23.76 ± 0.03 (r = −0.20)	13 ± 0.04 (r = 0.09)	0.51

important determinants (Alfsnes et al., 2017).

Considering all the obvious variations, differences in the DNA references standard and dyeing time explains the probable distortions of genome sizes from their accurate values. Hence, we suggest a closely related species as a standard reference and staining time of PI for 15 min. In our unpublished data on whole-genome sequencing of *L. orbonalis*, we discovered more than 35.4% of the genome is repeat-rich, non-coding DNA sequence. Determining the complete genome sequence of these important pests will fetch informative data. Many factors affects genome size, such as polyploidy, accessory chromosomes, endoduplications (Uozu et al., 1997; John and Miklos, 1988; Ullmann et al., 2005), intron size (Moriyama et al., 1998), presence of microsatellites (Warner and Noor, 2000) and transposons (Vieira et al., 2002).

No correlation between the genome size and the insect's host plant range could be derived in the present study. In contrast, Calatayud et al. (2016) reported that the genome size was influenced not only by host plant range but also by climatic from 21 distantly related lepidopteran insects. On the contrary, a negative correlation was reported between host plant range and genome size in *Helicoverpa* species (Zhang et al., 2019). The evolution of solanaceous specialist *H. assulta* (430 Mb) from the generalists *H. virescens* (408 Mb), *H. armigera* (394 or 337 Mb) and *H. zea* (363 Mb) indicates that the expansion of host plant range is inversely correlated with the genome size in this closely related terminal lineage (Kogan et al., 1978; Fitt, 1989; Mitter et al., 1993; Jallow et al., 2004; Cho et al., 2008; Zhang et al., 2019).

Hence, the main mechanisms contributing to genome size variations are polyploidy and accumulation of DNA sequence repeats (Pellicer et al., 2018) rather than the insects' morphological features or host plant range. The causes of expansion and shrinkage of genome size will require a closer look on their genetic make-up that includes the number and length of repeats comprising transposons and microsatellites and also the size of introns between the coding regions. Further, whole genome sequencing projects would focus on these elements for the reason for genome size variations between and among species to draw a conclusion on genome evolution and parameters that influence their variations.

Conflicts of interest

The authors in this manuscript have no conflict of interest.

Declarations of interest

None.

Acknowledgment

This work was supported by funding from Indian Council of Agricultural Research through CRP on Genomics project. The guidance and timely release of fund from ICAR-NBFGR are greatly acknowledged. K.B. and A.C.G are grateful to financial support received from DST-INSPIRE (2016/IF160900 and 2017/IF170435). Thanks to Prof. Krishnamurthy, National Centre for Biological Sciences for providing flow cytometry facility. The technical suggestion rendered by Dr. Ramkumar G. NPDF, ICAR-IIHR is greatly acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101188.

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