GMR
The Original

COMPARATIVE TRANSCRIPTOMIC ANALYSIS REVEALS DIVERGENT RESPONSES TO BROFLANILIDE EXPOSURE IN DIAPHANIA PULVERULENTALIS AND BOMBYX MORI, HIGHLIGHTING MECHANISMS OF TOLERANCE AND SUSCEPTIBILITY

Senguttuvan K[1*], KA Murugesh[2], R. Aruna[3], M Jayakanthan[4]

[1.] Assistant Professor (Entomology), Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
[2.] Professor and Head, Department of Sericulture, Forest College and Research Institute, Tamil Nadu Agricultural University, Mettupalayam, India
[3.] Teaching Assistant (Entomology), Department of Sericulture, Forest College and Research Institute, Tamil Nadu Agricultural University, Mettupalayam, India
[4.] Assistant Professor (Bioinformatics), Department of Plant Molecular Biology and Bioinformatics, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India
* Corresponding Author's mail: senguttuvan.k@tnau.ac.in

ABSTRACT
Insecticide treatment in sericulture endangers the delicate silkworm, Bombyx mori, while seeking to suppress pests like the mulberry leaf webber, Diaphania pulverulentalis. Understanding the molecular responses of both species is necessary to develop safer pest management strategies. In this work, RNA sequencing (RNA-Seq) was used to evaluate the transcriptome profiles of D. pulverulentalis and B. mori larvae exposed to a sublethal amount (0.01 ppm) of the more modern insecticide Broflanilide. High-quality transcriptome data were generated from scratch for D. pulverulentalis and linked to a reference genome for B. mori. Studies on differential gene expression (DEG) revealed conflicting responses in D. pulverulentalis showed a robust response with 3,194 DEGs, primarily increased (2,992 genes), including detoxification-related genes (e.g., serine protease like, gelsolin). On the other hand, B. mori displayed extensive transcriptional repression, with 797 downregulated DEGs out of 802. Significantly, B. mori exhibited substantial repression of important silk protein genes (fibroin heavy chain, light chain, P25) ($log_2FC < -12$). In B. mori, functional enrichment analysis revealed disruptions in glycan biosynthesis, glutathione metabolism, DNA repair, and metabolic pathways (carbohydrate, lipid). In contrast to considerable physiological disruption and vulnerability in B. mori, these divergent transcriptome patterns indicate a better tolerance and adaptive detoxifying capacity in D. pulverulentalis. In addition to identifying possible gene markers for risk assessment and the development of silkworm strains with increased resistance to insecticide residues, the results offer genetic insights into insecticide selectivity.

KEYWORD: Transcriptomics (RNA-Seq), Broflanilide, Differential Gene Expression (DEG), Detoxification, Silk Protein Genes, Insecticide Selectivity

1. INTRODUCTION

Sericulture, an agro-based industry focused on producing silkworms (Bombyx mori), is a vital source of income for rural people [1]. B. mori, which mostly feeds on mulberry leaves (Morus sp.), produces silk, the "Queen of Textiles," which is valued for its unique properties [2, 3]. India buys a lot of silk to meet demand even though it generates a lot of raw silk [1]. Mulberry farming is threatened by a number of pests [4] including the leaf webber, Diaphania pulverulentalis which can cause yield losses of up to 30% [5, 6].

Insecticides are frequently used to control mulberry pests [7], but their application is limited by B. mori's great sensitivity to chemical residues [8]. Significant yearly losses in silk production are caused by insecticide toxicity [9, 10]. Although there are newer insecticides with unique modes of action, including the meta diamide Broflanilide [11] and their safety profile for silkworms is frequently unclear, requiring customized pest management techniques [12, 13]. For sustained sericulture it is essential to comprehend the tolerance levels and resistance mechanisms in both the target pest [14] and the non-target silkworm [15].
Even in the absence of a completely annotated genome, recent developments in transcriptomics, especially RNA sequencing, provide potent tools to examine changes in gene expression in response to stressors like pesticides [16, 17]. DEGs related to detoxification [18], stress response, immunology, and metabolic pathways can be found by

transcriptome profiling, providing insight into the molecular underpinnings of susceptibility or resistance [19, 20]. Because of its sequenced genome and position as a model lepidopteran, Bombyx mori is especially suitable for these kinds of investigations [21, 22].

The purpose of this study was to use comparative transcriptomics to examine the molecular reactions of D. pulverulentalis and B. mori to sublethal exposure (0.01 ppm) of the insecticide broflanilide, which was discovered through initial toxicity tests [12]. The goals were to: (1) compile the transcriptome of D. pulverulentalis [23] and map reads for B. mori [24], (2) find DEGs linked to exposure to broflanilide in both species [25] and (3) use enrichment analyses to functionally characterize these DEGs in order to shed light on the molecular mechanisms underlying susceptibility or tolerance [26, 27]. Pesticide selectivity and potential genetic markers for creating silkworm strains more resistant to residual pesticide exposure are anticipated to be better understood as a result of the findings [28, 29].

2. MATERIALS AND METHODS

2.1 Insect Rearing and Insecticide Exposure

Diaphania pulverulentalis larvae were collected and grown in the lab on G4 mulberry branches in compliance with established protocols for managing populations in South India [5, 6]. Bombyx mori eggs (Kolar Gold crossbreed, PM × CSR2) were collected and raised on G4 mulberry leaves in standard conditions (24–27°C, 75–80% humidity) [1]. During the rearing phase, proper cleaning and disinfection protocols were followed to safeguard the health of the bivoltine hybrids [30]. Based on initial toxicity and sublethal effect investigations, fifth instar larvae of both species were treated to Broflanilide 20% SC at a sublethal concentration of 0.01 ppm by leaf dip method for transcriptome profiling [12, 31]. Untreated leaves were given to the control groups. For RNA extraction, samples were taken at a predetermined point after exposure [25, 32].

2.2 RNA Extraction and Quality Control

TRI Reagent® (Sigma Aldrich, USA) was used in RNase-free conditions to extract total RNA from homogenized tissue samples (30–50 mg) of control and Broflanilide treated larvae [33]. The procedure included homogenization, ethanol washes, isopropanol precipitation, phase separation with chloroform and resuspension in water devoid of RNase [34, 35]. The QubitTM 4 Fluorometer [36] and NanodropOneTM [37] were used to measure the quantity and purity of RNA. The Agilent 5400 Fragment Analyzer System was used to assess RNA integrity, library construction requires an RNA Integrity Number equivalent (RINe) > 6.0 [36, 37]. Samples were kept at -80°C until further processing after quality evaluation [33].

2.3 Library Preparation and Sequencing

The TruSeq RNA Library Prep Kit v2 (Illumina) was used to prepare the mRNA library [34]. Oligo dT magnetic beads were used to enrich polyadenylated mRNA, which was then thermally fragmented [16]. End-repair, A-tailing, and ligation of Illumina-compatible sequencing adapters came after first and second-strand cDNA synthesis [37]. QubitTM and QuantStudioTM Real time PCR System were used to amplify, purify, and quantify libraries [16]. Agilent Fragment Analyzer was used to evaluate the library size distribution (goal 300–350 bp), and libraries with concentrations greater than 10 nM were combined [37]. A 2 × 150 bp paired end method was used for sequencing on the Illumina NovaSeq 6000 platform [22, 36, 38].

2.4 Bioinformatics Analysis

2.4.1 Quality Control and Preprocessing

Raw sequencing reads (FASTQ) were assessed using FastQC (v0.12.0) [16]. Adapters and low quality bases (Phred score < 30) were removed using Trim Galore (v0.6.10), and reads shorter than 20 bp were discarded [36].

2.4.2 De novo Assembly (D. pulverulentalis)

Clean reads were assembled using Trinity (v2.15.2) [23]. Open reading frames (ORFs) were predicted using Transdecoder (v5.7.0). Redundancy was reduced using CDHIT-est (v4.8.1). Functional annotation was performed against the NCBI NR database. The assembled transcripts were indexed using HISAT2 (v2.1.0) [38].

2.4.3 Reference-Based Mapping (B. mori)

The B. mori reference genome (ENA: GCA_014905235.2) was indexed using HISAT2 (v2.2.1) [22]. Clean reads were aligned to this indexed genome using HISAT2 with default parameters [23].

2.4.4 Gene Quantification and Differential Expression

For both species, aligned reads were quantified at the gene level using HTSeq-count (v0.13.5 for B. mori, v2.0.3 for D. pulverulentalis) with the 'union' mode [36]. Differential expression analysis between treated and control groups was performed using the EdgeR package (v3.42.0) in RStudio, following normalization (e.g., using limma v3.56.0 for D. pulverulentalis) [26]. Genes with an adjusted P value (FDR) < 0.05 and an absolute $\log_2$ fold change ($|\log_2 FC|$) > 2

were considered significantly differentially expressed (DEGs) [19]. Visualizations included heatmaps, volcano plots, and boxplots using ggplot2.

2.4.5 Functional Enrichment Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on the DEGs (primarily focusing on B. mori due to reference availability) to identify over-represented biological processes, molecular functions, cellular components, and metabolic/signaling pathways [39, 27]. Adjusted P-values were used to determine significance [34].

3. RESULTS

3.1 Sequencing, Assembly, and Mapping Statistics

High quality RNA Seq data were obtained for both species. For D. pulverulentalis, after quality filtering, 18.3 million clean reads (Control, CLW) and 27.3 million clean reads (Treated, TLW) were generated, with Q30 scores > 98.4% [34, 37]. The de novo assembly generated 34,145 transcripts with a N50 of 2,362 bp [34]. This assembly had good clean read mapping rates (81.87% for CLW and 83.75% for TLW) [38, 23]. For B. mori, 70.7 million clean reads (Control, CSW) and 27.2 million clean reads (Treated, TSW) were generated with Q30 > 98.4% [22, 24]. Reads were mapped to the reference genome (460.3 Mb, N50 16.8 Mb) with mapping rates of 85.73% (CSW) and 73.63% (TSW) [2, 22]. Overall data quality supported reliable downstream analysis [16].

3.2 Differential Gene Expression (DEG) Analysis

Comparative analysis revealed stark differences in transcriptomic responses between the two species upon Broflanilide exposure [12]. In D. pulverulentalis, 3,194 genes were significantly differentially expressed ($|\log_2FC| > 2$, FDR < 0.05), with a vast majority (2,992 genes, ~94%) being upregulated and only 202 downregulated [40, 25]. This indicates a strong transcriptional activation response [41]. In contrast, B. mori showed a predominantly suppressive response, with 802 significant DEGs identified, of which 797 (~99%) were downregulated and only 5 were upregulated [26, 42].

3.3 Characterization of Top DEGs

3.3.1 Diaphania pulverulentalis

The top upregulated genes included modular serine protease-like ($\log_2FC$: 6.04), protein yellow-like ($\log_2FC$: 5.01), gelsolin ($\log_2FC$: 4.81), and antichymotrypsin-2-like ($\log_2FC$: 4.68), suggesting roles in detoxification, immunity, and stress response, these expression levels are detailed in Figure 1 [40, 25, 18, 43]. The most down regulated genes included attacin-like ($\log_2FC$: -3.59) and glycine-rich protein DOT1-like ($\log_2FC$: -3.01), potentially related to immune function and structural components [44, 6]. The distinct expression patterns between control and treated groups are clearly illustrated through the visualizations provided in Figure 2 and Figure 3. Specifically, the heatmaps in Figure 2 and the volcano plots in Figures 3a and 3b confirmed the significant transcriptional shifts across the experimental groups [19].

3.3.2 Bombyx mori

Protein retinal degeneration B (LOC101742166, $\log_2FC$: 2.09) and 26S proteasome non-ATPase regulatory subunit 1 (LOC101746253, $\log_2FC$: 2.37) were two of the few elevated genes that may be implicated in stress signaling and protein degradation [27, 36, 20]. The differential expression data displayed in Figure 4 [45, 46, 47] highlights the significant downregulation of three important silk production genes: silk fibroin heavy chain (GeneID 693030, $\log_2FC$: -14.14), silk fibroin light chain (GeneID 693047, $\log_2FC$: -13.34), and silk protein P25 (GeneID 100146105, $\log_2FC$: -12.45]. Metabolic enzymes and cuticular proteins were among the other genes that were significantly downregulated [48]. The sample distance maps and heat maps shown in Figure 5 provide additional proof of the treatment-induced transcriptional changes. Control and treatment samples were clearly separated by sample distance maps and heatmaps, and Figures 6a and 6b further supported the different expression profiles across the experimental groups [26].

3.4 Functional Enrichment Analysis (Bombyx mori)

Significant enrichment was found across 25 Biological Process (BP), 10 Molecular Function (MF), and 1 Cellular Component (CC) categories in GO enrichment analysis of the 802 DEGs in B. mori [39, 48, 47]. Chitin binding, catalytic activity, hydrolase activity, and oxidoreductase activity were among the enriched MF keywords; Table 1 provides a thorough distribution of these functional categories [25, 20]. Amino acid metabolism, defense responses, small molecule metabolic activities, and carbohydrate metabolism were among the enriched BP keywords [39, 48]. "Extracellular region" was the enhanced CC phrase [46]. Figure 7 further demonstrates the overall functional distribution and importance of these enhanced pathways, confirming the extensive metabolic and defensive changes brought about by the therapy [26].

A thorough statistical analysis of these enhanced terms may be seen in Table 2 [34]. Significant alterations in several pathways were found by the KEGG pathway analysis (FDR significant). Top enriched pathways included Metabolic

pathways (bmor01100), Glutathione metabolism (bmor00480), Folate biosynthesis (bmor00790), Galactose metabolism (bmor00052), Pentose and glucuronate interconversions (bmor00040), and Fructose and mannose metabolism (bmor00051) [26, 27, 34]. Pathways linked to detoxification (xenobiotic metabolism by cytochrome P450), lipid metabolism (metabolism of arachidonic acid), glycan biosynthesis and DNA repair (base excision repair, non homologous end-joining) were also significantly enriched [49, 27]. These pervasive disruptions of basic metabolic, detoxifying, and cellular maintenance processes are further demonstrated by Figure 8, which shows the interdependence of the affected biological systems [34].

4. DISCUSSION

The comparative transcriptome analysis provided molecular insights into the tolerance and susceptibility of D. pulverulentalis and B. mori to sublethal Broflanilide exposure, revealing fundamentally distinct responses [12, 15]. A powerful, adaptive transcriptional response targeted at reducing pesticide toxicity is suggested by the significant overexpression of several genes (2,992 out of 3,194 DEGs) in D. pulverulentalis [41, 40]. Increased metabolic resistance mechanisms, which are frequently seen in pest insects adjusting to chemical pressure, are suggested by the particular overexpression of genes such as serine proteases, antichymotrypsin, and possibly other detoxifying enzymes (though additional annotation is needed) [43, 50]. This is consistent with the enzymatic studies demonstrating that under sublethal stress, D. pulverulentalis induces detoxifying enzymes such as CaE and GST [43]. A trade-off between detoxification and some components of immunity under stress may be indicated by the downregulation of immune genes such as attain [44, 51].

In contrast, 99% of B. mori's DEGs were downregulated, indicating a transcriptome profile that was predominantly suppressive [12, 47]. Particularly when it comes to highly expressed genes necessary for its primary commercial product, silk (fibroin heavy chain, light chain, P25), this widespread regulation is suggestive of significant physiological disruption rather than an adaptive response [52, 45]. This molecular data is strongly associated with the negative effects of Broflanilide (as well as other insecticides like Fipronil and Imidacloprid) on silkworm growth, development, silk gland weight, cocoon quality, and biochemical parameters reported in the larger thesis results [53, 54, 55]. The downregulation most likely results from energy diversion, metabolic stress, and possible cellular damage (shown by enhanced DNA repair pathways), overwhelming the silkworm's comparatively weaker detoxification and stress response systems in comparison to the pest [10, 48, 56, 57].

This hypothesis is further supported by the GO and KEGG enrichment analysis in B. mori. Systemic metabolic distress is characterized by disruptions in fundamental metabolic pathways (carbohydrate, lipid, amino acid), detoxification pathways (glutathione metabolism, cytochrome P450) and stress markers (DNA repair, folate biosynthesis) [26, 27, 34]. Certain genes, such as the proteasome subunit, may be upregulated in an attempt to control degraded proteins under stress [27]. Because farmed B. mori has less genetic diversity and possibly less effective detoxifying systems than its wild counterparts or field-adapted pests, the observed transcriptome vulnerability is consistent with the known sensitivity of domesticated B. mori to xenobiotics [56, 58].

Sericulture will be significantly impacted by these discoveries. Although physiological results indicated that Broflanilide was comparatively safer for B. mori compared to other pesticides examined, the differential response highlighted Broflanilide's potential selectivity, being more harmful to B. mori than D. pulverulentalis at the transcriptome level [12]. This emphasizes the difficulty of converting transcriptome alterations into consequences on the entire organism and the necessity of combining various biological analysis levels. But a serious worry is the dramatic inhibition of silk genes [45]. The identified DEGs, especially the stress/silk-related genes in B. mori and the detoxification-related genes in D. pulverulentalis are promising candidates for future functional validation (e.g., via RNAi) and could be developed into biomarkers for tracking insecticide exposure, evaluating pest resistance development, or searching for silkworm strains with enhanced tolerance [59, 28, 29]. Developing sustainable pest management strategies that safeguard the economically significant silkworm industry requires an understanding of these molecular pathways [15, 60, 61].

5. CONCLUSION

The transcriptome responses of the silkworm B. mori and the mulberry pest D. pulverulentalis to sublethal exposure to broflanilide were successfully characterized in this study using RNA Seq. The findings showed several molecular approaches such as D. pulverulentalis exhibited widespread transcriptional activation perhaps due to increased detoxification and stress reactions, suggesting a capacity for adaptability or tolerance. However, B. mori demonstrated widespread transcriptional suppression, including robust silk protein gene repression, highlighting its vulnerability to chemical stress and severe physiological disruption. These discoveries provide crucial molecular level insights on pesticide selectivity and sensitivity by identifying significant genes and pathways involved. By using this information

to direct efforts to breed or design silkworm strains that are more resistant to unavoidable insecticide residues and to create safer pest management strategies in sericulture, the sustainability of the silk industry can be maintained.

REFERENCES

1. Dandin SB, Kumar NS, Basavaraja HK, Reddy NM. Effect of high temperature and high humidity on the quantitative traits of parents, foundation crosses, single and double hybrids of bivoltine silkworm, Bombyx mori L. Int J Indust Entomol Biomater. 2003;6(2):197–202.
2. Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T, Kanamori H, et al. The genome sequence of silkworm, Bombyx mori. DNA Res. 2004;11(1):27–35.
3. Nagaraju J, Goldsmith MR. Silkworm genomics progress and prospects. Curr Sci. 2002;83(4):415–425.
4. Prety Rekha N, Manimegalai S, Vinothkumar B, Suganthi A, Radha P, Shanmugam R, et al. Bioefficacy and persistent toxicity of newer insecticide against thrips, Pseudodendrothrips mori and leaf webber, Diaphania pulverulentalis in mulberry cultivation. Plant Sci Today. 2024;11:5754.
5. Ali SMA. Leaf roller infestation in mulberry. Indian Silk. 1995;33:15–16.
6. Seelan V. Biology and management of leaf webber, Glyphodes pulverulentalis (Hampson) on mulberry [Master's thesis]. Coimbatore: Tamil Nadu Agricultural University; 1999.
7. Sathish R, Ramakrishnan K, Kumar S. Evaluation of toxicity of chlorfenapyr on leaf eating caterpillars and silkworms. J Agric Res. 2014;38(1):79–83.
8. Arakawa T, Inoue T, Nishigaki T. Oral exposure to a fungicide containing iminoctadine triacetate affects the growth and development of silkworm larvae, Bombyx mori. Appl Entomol Zool. 2013;48(3):285–291.
9. Wu JX, Zhang YL, Chen DZ. Pesticide poisoning and sericulture in China. China Seric. 2006;2(1):21–23.
10. Naseema Begum A, Shivanandappa T. Toxicity and neurochemical effects of some insecticides on silkworm, Bombyx mori. J Appl Toxicol. 2003;23(6):381–385.
11. Nakao T, Banba S. Broflanilide: A meta-diamide insecticide with a novel mode of action. Bioorg Med Chem. 2016;24(3):372–377.
12. Huang J, Zhou X, Liu X, Zhou H, Li R, He Y, et al. Sublethal effects of the novel insecticide broflanilide on the silkworm Bombyx mori. Ecotoxicol Environ Saf. 2023;248:114378.
13. Mahesh M, Sunitha V, Satyanarayana J, Singh TVK, Jagadeeshwar R. Relative toxicity of new insecticide molecules against field population of Spodoptera litura. J Entomol Zool Stud. 2020;8(1):791–794.
14. Bhat SK. Studies on insecticide resistance in Plutella xylostella (L.) in Karnataka [PhD thesis]. Bangalore: University of Agricultural Sciences; 1999.
15. Stanley J, Preetha G. Pesticide toxicity to non-target organisms: Exposure, toxicity and risk assessment methodologies. In: Pesticide Toxicity to Non Target Organisms. 2016. p. 229–275.
16. Wang Z, Gerstein M, Snyder M. RNA-Seq: A revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10(1):57–63.
17. Vitiello A. Transcriptome characterization and transcriptional reprogramming of zucchini plant in response to Aphis gossypii feeding [Doctoral dissertation]. Naples: University of Naples Federico II; 2018.
18. Mamidala P, Wijeratne AJ, Wijeratne S, Kornacker K, Sudhamalla B, Rivera-Vega LJ, et al. RNA-Seq and molecular docking reveal multi-level pesticide resistance in the bed bug. BMC Genomics. 2012;13:6.
19. Zhang H, Liu Y, Wang X, Li J. RNA-Seq-based analysis of differentially expressed genes in response to insecticide exposure in lepidopteran pests. Front Genet. 2022;13:867543.
20. Xin S, Zhang W. Construction and analysis of the protein–protein interaction network for the detoxification enzymes of the silkworm, Bombyx mori. Arch Insect Biochem Physiol. 2021;108(4):e21850.
21. Xia Q, Cheng D, Duan J, Wang G, Cheng T, Zha X, et al. A draft sequence for the genome of the domesticated silkworm (Bombyx mori). Science. 2004;306(5703):1937–1940.
22. Kawamoto M, Jouraku A, Toyoda A, Yokoi K, Minakuchi Y, Katsuma S, et al. High-quality genome assembly of Bombyx mori. GigaScience. 2019;8(12):139.
23. Duan J, Li R, Cheng D, Fan W, Zha X, Cheng T, et al. SilkDB v2.0: A platform for silkworm genome biology. Nucleic Acids Res. 2010;38:D453–D456.
24. Tong X, Li Z, Guo Y, Cheng T, Xia Q. A high-resolution silkworm pan-genome provides insights into artificial selection and ecological adaptation. Nat Commun. 2022;13:6584.
25. Shao Y, Xin XD, Liu ZX, Wang J, Zhang R, Gui ZZ. Transcriptional response of detoxifying enzyme genes in Bombyx mori under chlorfenapyr exposure. Pestic Biochem Physiol. 2021;177:104899.
26. Zheng X, Liu F, Shi M, Li S, Xie X, Li G, et al. Transcriptome analysis under dimethoate stress. Pestic Biochem Physiol. 2022;183:105081.
27. Ye Y, Shi YX, Jiang Q, Jin Y, Chen FX, Tang WH, et al. Transcriptome analysis reveals antioxidant defense mechanisms. Animals. 2024;14(12):1822.
28. Li X, Dong Z, Pan M. Advances in silkworm breeding for disease resistance. Seric Sci Technol. 2023;45(2):89–102.

29. Fan X, Li Y, Zhang W, Chen K. Advances in Bombyx mori genomics. Insect Sci. 2023;30(1):1–15.
30. Sunil Kumar N, Ranganathaiah KG, Nataraju B. Effect of pesticide residues on silkworm performance. J Appl Nat Sci. 2016;8(2):833–838.
31. Sunil Kumar T, Ramakrishna Naika, Kumar KP. Studies on toxicological effect of new generation insecticides, chlorantraniliprole, flubendiamide and imidacloprid on rearing performance of mulberry silkworm, Bombyx mori L. Int J Pure Appl Biol. 2017;4(3):5513.
32. Wang H, Lu Z, Li M, Fang Y, Qu J, Mao T, et al. Responses of detoxification enzymes in the midgut of Bombyx mori after exposure to low-dose of acetamiprid. Chemosphere. 2020;251:126438.
33. Cai Y, Yu T, Liang Y, Li C, Hou N, Zheng X, et al. RNA-seq analysis of transcriptome in Bombyx mori. PLOS ONE. 2013;8(3):e58557.
34. Li W, Liu L, Yang J, Wu Y, Zhang H, Xiang Z. Comprehensive transcriptome analysis of Bombyx mori. DNA Res. 2012;19(1):1–9.
35. Wang Y, Cao L, Xu Y, Zhao X. Discovery and expression profile analysis of microRNAs from Bombyx mori. Mol Biol Rep. 2012;39(1):261–268.
36. Kakeru Y, Shibata T, Hara S, Tsubota T, Maeyama K, Okamoto K, et al. A comprehensive reference transcriptome for Bombyx mori. Insect Biochem Mol Biol. 2021;131:103547.
37. Yinu C, Wu Z, Xie W, Zhang C, Xia Q. Transcriptome analysis of silkworm by paired-end RNA sequencing. BMC Genomics. 2012;13:116.
38. Shimomura M, Minami H, Suetsugu Y, Ohyanagi H, Sato R, Mita K. KAIKObase: An integrated silkworm genome database. BMC Genomics. 2009;10:486.
39. Hoda HA. Effect of some insecticides on economic characters and enzyme activities in Bombyx mori. Egypt J Agric Res. 2011;89(1):185–197.
40. Murugan K. Biochemical and physiological mechanisms of insecticide resistance in insects. J Entomol Res. 2004;28:1–11.
41. Balabaskaran S, Mathavan S, Nair KK. Biochemical response of insecticide-resistant populations. Indian J Exp Biol. 1989;27:730–733.
42. Wang Q, Liu S, Yang K, Zhang Y, Xia Q. Sublethal effects of tolfenpyrad on silkworm development. Environ Pollut. 2023;318:120891.
43. Mohan M, Gujar GT. Local variation in susceptibility of Plutella xylostella to insecticides. Pestic Biochem Physiol. 2003;77:145–154.
44. Brown TM, Brogdon WG. Enhanced detoxification and insecticide resistance. Annu Rev Entomol. 1987;32:145–170.
45. Wang S, You Z, Feng M, Che J, Zhang Y, Qian Q, et al. Molecular mechanisms associated with silk production. J Proteome Res. 2016;15(1):15–28.
46. Juan Z, Sheng L, Wenwen Z, Xiang Z, Xia Q. Transcriptomic analysis of silk gland genes. Insect Sci. 2016;23(4):511–519.
47. Wang SH, You ZY, Ye LP, Che J, Qian Q, Nanjo Y, et al. Proteomic and transcriptomic analyses of silk production. J Proteome Res. 2014;13(2):735–751.
48. Nath BS, Suresh A, Varma BM, Kumar RP. Changes in protein metabolism in silkworm. Ecotoxicol Environ Saf. 1997;36(2):169–173.
49. Bin S, Jiang G, Xia Q, Li S, Xiang Z. Analysis of cytochrome P450 gene content. Sci China Ser C. 2005;48(5):414–418.
50. Yu SJ, Nguyen SN. Detection and characterization of insecticide resistance. Pestic Biochem Physiol. 1992;44:74–81.
51. Ranson H, Claudianos C, Ortelli F, et al. Evolution of supergene families. Science. 2003;298:179–181.
52. Xiao R, Yuan Y, Xia H, Ge Q, Chen L, Zhu F, et al. Transcriptomics and proteomics-based analysis of heterosis. J Proteomics. 2020;229:103941.
53. Abhay Kumar Singh AK, Gautam RD, Nair KS. Toxic effects of insecticides on Bombyx mori. J Environ Biol. 2008;29(4):593–596.
54. Munhoz REF, Bignotto TS, Pereira NC, et al. Toxic effect of chlorantraniliprole on silkworm. Open J Anim Sci. 2013;3(4):343–353.
55. Lu XM, Wu YJ. Toxic effects of imidacloprid on silkworm. Sericologia. 2000;40(3):371–376.
56. Li S, Wang Y, Zhang J, Li F. Insecticide resistance in silkworm populations. Pestic Biochem Physiol. 2010;98(2):174–178.
57. Zou H, Gu H, Cheng J, Tian C, Shu Q, Peng P, et al. Midgut damage mechanism in Bombyx mori. Insects. 2024;15(12):911.
58. Xia Q, Li S, Feng Q. Advances in silkworm studies. Annu Rev Entomol. 2014;59:513–536.
59. Xu HJ, O'Brochta DA. Genetic manipulation technologies in Bombyx mori. Insect Biotechnol. 2015;8:93–105.

60. Ma Y, Li S, Wei C, Zhang Z, Ge W, Tang M, et al. Spatial transcriptomics of silk glands. Cell Rep. 2024;43(2):113654.
61. Ma Y, Wei C, Xu H, Tang M, Zhang Z, Ge W, et al. Single-cell transcriptomic atlas of silk gland. Nat Commun. 2022;13:1245.

Table 1. GO enrichment analysis of differentially expressed genes in Bombyx mori

Source	GO Term Name	GO ID	Adjusted P-value	$Log_{10}(P)$	Term Size	Query Size	Intersection Size
GO:MF	Chitin binding	GO:0008061	0.00	3.69	55	503	15
GO:MF	Catalytic activity	GO:0003824	0.00	3.37	3652	503	283
GO:MF	Hydrolase activity	GO:0016798	0.00	3.00	102	503	20
GO:MF	Hydrolase activity, O-glycosyl	GO:0004553	0.00	2.59	91	503	18
GO:MF	Oxidoreductase, CH-OH group (NAD/NADP)	GO:0016616	0.01	1.91	51	503	12
GO:MF	Ligase activity (C-N bonds)	GO:0016879	0.01	1.83	24	503	8
GO:MF	D-threo-aldose dehydrogenase activity	GO:0047834	0.02	1.82	5	503	4
GO:MF	Intramolecular oxidoreductase	GO:0016860	0.02	1.61	14	503	6
GO:MF	Structural constituent of cuticle	GO:0042302	0.03	1.49	169	503	24
GO:MF	Oxidoreductase activity	GO:0016491	0.03	1.48	617	503	62
GO:BP	small molecule metabolic process	GO:0044281	7.19	8.14	371	325	56
GO:BP	oxoacid metabolic process	GO:0043436	5.79	5.24	164	325	30
GO:BP	organic acid metabolic process	GO:0006082	5.79	5.24	164	325	30
GO:BP	carboxylic acid metabolic process	GO:0019752	5.79	5.24	164	325	30
GO:BP	small molecule catabolic process	GO:0044282	0.00	3.14	55	325	14
GO:BP	alpha-amino acid metabolic process	GO:1901605	0.00	2.65	60	325	14
GO:BP	carboxylic acid catabolic process	GO:0046395	0.00	2.40	40	325	11
GO:BP	organic acid catabolic process	GO:0016054	0.00	2.40	40	325	11
GO:BP	defense response to other organism	GO:0098542	0.01	2.23	49	325	12

GO:BP	response to other organism	GO:0051707	0.01	2.04	51	325	12
GO:BP	biological process involved in interspecies interaction between organisms	GO:0044419	0.01	2.04	51	325	12
GO:BP	response to external biotic stimulus	GO:0043207	0.01	2.04	51	325	12
GO:BP	response to biotic stimulus	GO:0009607	0.009180132	2.037151083	51	325	12
GO:BP	amino acid metabolic process	GO:0006520	0.011636423	1.93418051	125	325	20
GO:BP	innate immune response	GO:0045087	0.013490981	1.869956473	45	325	11
GO:BP	defense response to symbiont	GO:0140546	0.013490981	1.869956473	45	325	11
GO:BP	alpha-amino acid catabolic process	GO:1901606	0.013569668	1.867430764	24	325	8
GO:BP	defense response	GO:0006952	0.013850102	1.858547015	53	325	12
GO:BP	carbohydrate metabolic process	GO:0005975	0.014013932	1.853439984	190	325	26
GO:BP	immune response	GO:0006955	0.020793917	1.682063698	47	325	11
GO:BP	response to external stimulus	GO:0009605	0.024662226	1.607967728	56	325	12
GO:BP	immune system process	GO:0002376	0.025567736	1.592307733	48	325	11
GO:BP	amino acid catabolic process	GO:0009063	0.025953096	1.585810822	26	325	8
GO:BP	amide metabolic process	GO:0043603	0.026717302	1.573207398	65	325	13
GO:BP	response to bacterium	GO:0009617	0.034997397	1.455964259	27	325	8
GO:CC	extracellular region	GO:0005576	4.42E-08	7.355055967	317	212	45

Table 2. KEGG enrichment analysis of differentially expressed genes in Bombyx mori

Rank	Pathway Name	KEGG ID	Input Genes	Background Genes	P-Value	FDR (Corrected P-Value)
1.	Metabolic pathways	bmor01100	48	1142	1.73	1.36
2.	Glutathione metabolism	bmor00480	8	54	1.68	0.00
3.	Folate biosynthesis	bmor00790	7	41	2.52	0.00
4.	Galactose metabolism	bmor00052	5	28	0.00	0.01
5.	Pentose and glucuronate interconversions	bmor00040	7	64	0.00	0.01

6.	Fructose and mannose metabolism	bmor00051	5	33	0.00	0.01
7.	Arachidonic acid metabolism	bmor00590	4	31	0.00	0.04
8.	Protein processing in endoplasmic reticulum	bmor04141	8	132	0.00	0.04
9.	Tyrosine metabolism	bmor00350	4	34	0.01	0.04
10.	Metabolism of xenobiotics by cytochrome P450	bmor00980	5	64	0.01	0.06
11.	Chemical carcinogenesis - reactive oxygen species	bmor05208	4	38	0.01	0.09
12.	Histidine metabolism	bmor00340	3	23	0.01	0.13
13.	Ascorbate and aldarate metabolism	bmor00053	3	24	0.03	0.18
14.	Ribosome	bmor03010	13	232	0.03	0.18
15.	Ascorbate and aldarate metabolism	bmor00053	3	25	0.03	0.18
16.	Arginine and proline metabolism	bmor00330	4	50	0.04	0.18
17.	Glycolysis / Gluconeogenesis	bmor00010	6	108	0.04	0.19
18.	Biosynthesis of amino acids	bmor01230	6	113	0.04	0.19
19.	Drug metabolism - cytochrome P450	bmor00982	4	59	0.04	0.19
20.	Lysosome	bmor04142	5	93	0.05	0.23
21.	Alanine, aspartate and glutamate metabolism	bmor00250	3	41	0.08	0.30
22.	Pyruvate metabolism	bmor00620	3	44	0.08	0.30
23.	Carbon metabolism	bmor01200	6	130	0.10	0.35
24.	Fatty acid degradation	bmor00071	3	48	0.11	0.36
25.	Amino sugar and nucleotide sugar metabolism	bmor00520	4	75	0.12	0.38
26.	Glycine, serine and threonine metabolism	bmor00260	3	49	0.13	0.39
27.	Peroxisome	bmor04146	3	52	0.14	0.40
28.	Phagosome	bmor04145	4	84	0.15	0.41
29.	Protein export	bmor03060	2	14	0.15	0.41
30.	Insect hormone biosynthesis	bmor00981	2	14	0.15	0.41
31.	ABC transporters	bmor02010	4	91	0.16	0.41
32.	Antigen processing and presentation	bmor04612	2	18	0.17	0.43
33.	Cyanoamino acid metabolism	bmor00460	2	19	0.19	0.45
34.	Galactose metabolism	bmor00052	3	33	0.20	0.45
35.	Tryptophan metabolism	bmor00380	3	37	0.20	0.45
36.	RNA transport	bmor03013	5	107	0.21	0.47
37.	Glycerolipid metabolism	bmor00561	3	40	0.22	0.47
38.	Chemical carcinogenesis - DNA adducts	bmor05204	2	24	0.24	0.50
39.	Glutamatergic synapse	bmor04724	3	44	0.25	0.50
40.	Neuroactive ligand-receptor interaction	bmor04080	5	136	0.28	0.56
41.	Retinol metabolism	bmor00830	2	29	0.29	0.56

42.	Wntsignaling pathway	bmor04310	2	30	0.32	0.58
43.	ECM-receptor interaction	bmor04512	2	30	0.32	0.58
44.	Spliceosome	bmor03040	4	95	0.32	0.58
45.	Oocyte meiosis	bmor04114	3	62	0.34	0.58
46.	Regulation of actin cytoskeleton	bmor04810	4	107	0.34	0.58
47.	Glycerophospholipid metabolism	bmor00564	3	56	0.36	0.60
48.	Endocytosis	bmor04144	5	131	0.39	0.61
49.	Longevity regulating pathway	bmor04211	2	41	0.40	0.61
50.	Thiamine metabolism	bmor00730	2	21	0.41	0.61
51.	Notch signaling pathway	bmor04330	1	14	0.41	0.61
52.	Axon regeneration	bmor04360	1	14	0.41	0.61
53.	Oxidative phosphorylation	bmor00190	4	118	0.42	0.61
54.	Apoptosis	bmor04210	2	46	0.42	0.61
55.	MAPK signaling pathway	bmor04010	6	198	0.42	0.61
56.	Endocrine and other factor-regulated calcium reabs.	bmor04961	1	17	0.43	0.61
57.	Peroxisome proliferator-activated receptor (PPAR)	bmor03320	1	18	0.46	0.61
58.	SNARE interactions in vesicular transport	bmor04130	1	19	0.46	0.61
59.	Cardiac muscle contraction	bmor04260	1	20	0.47	0.61
60.	Antifolate resistance	bmor01523	1	21	0.48	0.61
61.	Vasopressin-regulated water reabsorption	bmor04962	1	22	0.48	0.61
62.	mRNA surveillance pathway	bmor03015	2	58	0.48	0.61
63.	Cell cycle	bmor04110	3	90	0.52	0.64
64.	Calcium signaling pathway	bmor04020	3	98	0.52	0.64
65.	Apoptosis - multiple species	bmor04215	1	26	0.55	0.66
66.	Ubiquitin mediated proteolysis	bmor04120	4	139	0.55	0.66
67.	Adherens junction	bmor04520	2	68	0.58	0.69
68.	Spliceosome (repeated pathway ID, likely split)	bmor03040	3	93	0.59	0.69
69.	Fatty acid metabolism	bmor01212	2	63	0.61	0.70
70.	DNA replication	bmor03030	3	85	0.63	0.71
71.	Basal transcription factors	bmor03022	2	67	0.66	0.72
72.	FoxOsignaling pathway	bmor04068	2	73	0.66	0.72
73.	Glucagon signaling pathway	bmor04922	1	31	0.72	0.78
74.	Toll-like receptor signaling pathway	bmor04620	1	33	0.74	0.79
75.	NOD-like receptor signaling pathway	bmor04621	1	34	0.75	0.79

76.	Fat digestion and absorption	bmor04975	1	35	0.81	0.84
77.	Melanogenesis	bmor04916	2	88	0.84	0.86
78.	Oocyte meiosis (repeated pathway)	bmor04114	2	85	0.88	0.90
79.	ErbBsignaling pathway	bmor04012	2	89	0.90	0.90

Figure 1. Sample distance map - Diaphania pulverulentalis

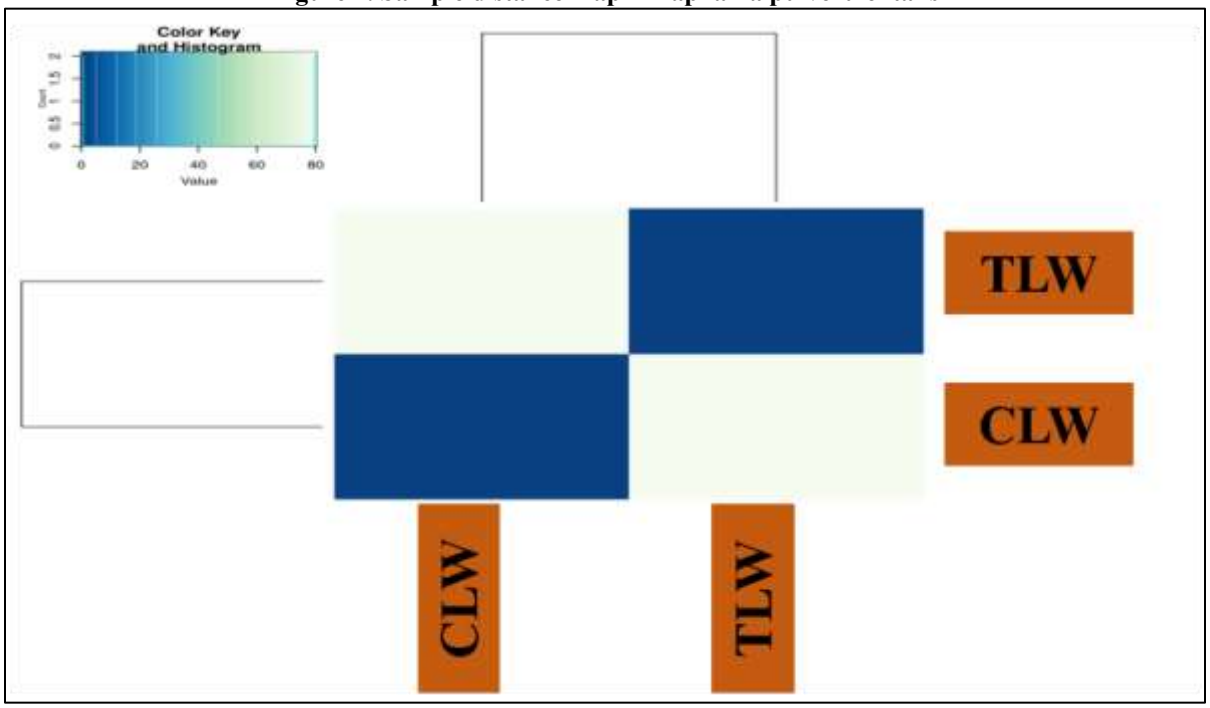

Figure 2. Box plot – Diaphania pulverulentalis

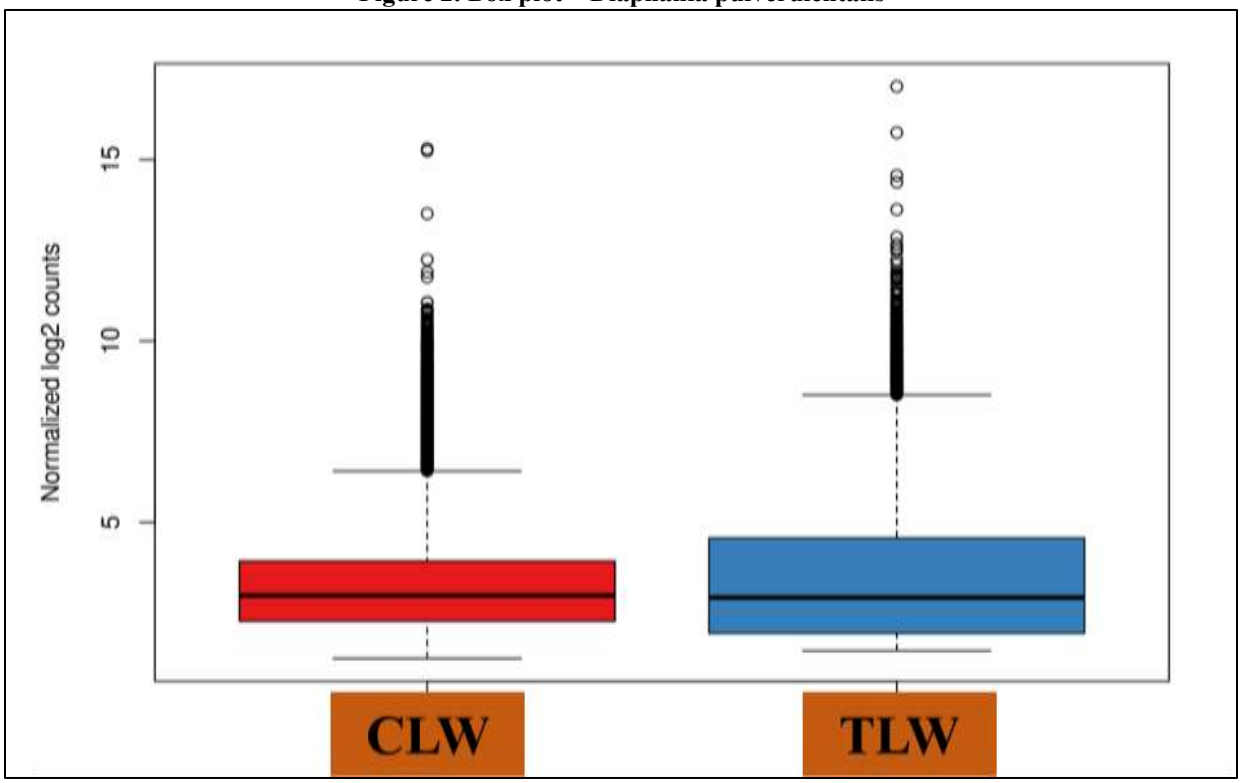

Figure 3a. Heatmap of differently expressed genes in Diaphania pulverulentalis

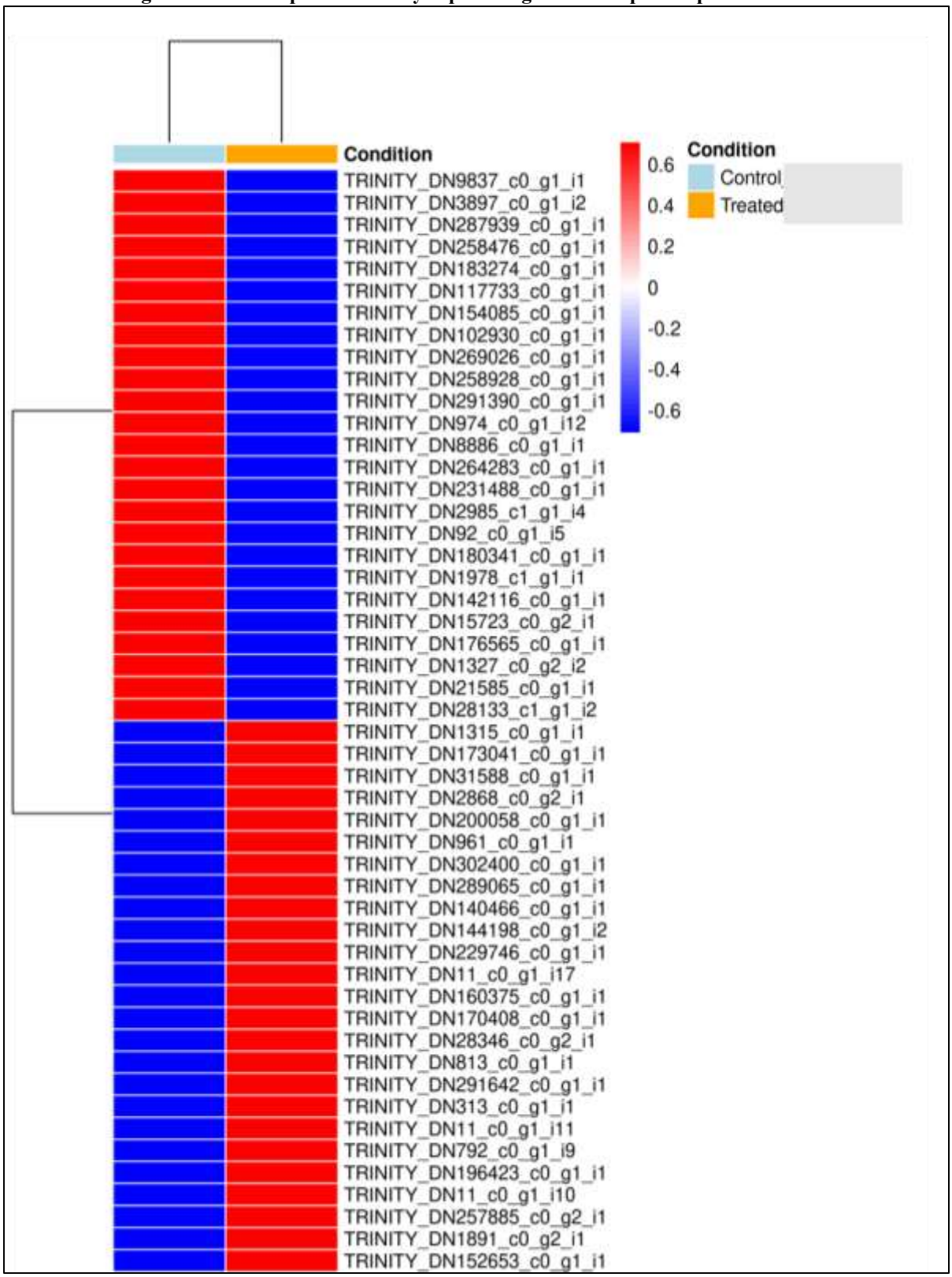

Figure 3b. Volcano plot of differentially expressed genes in Diaphania pulverulentalis

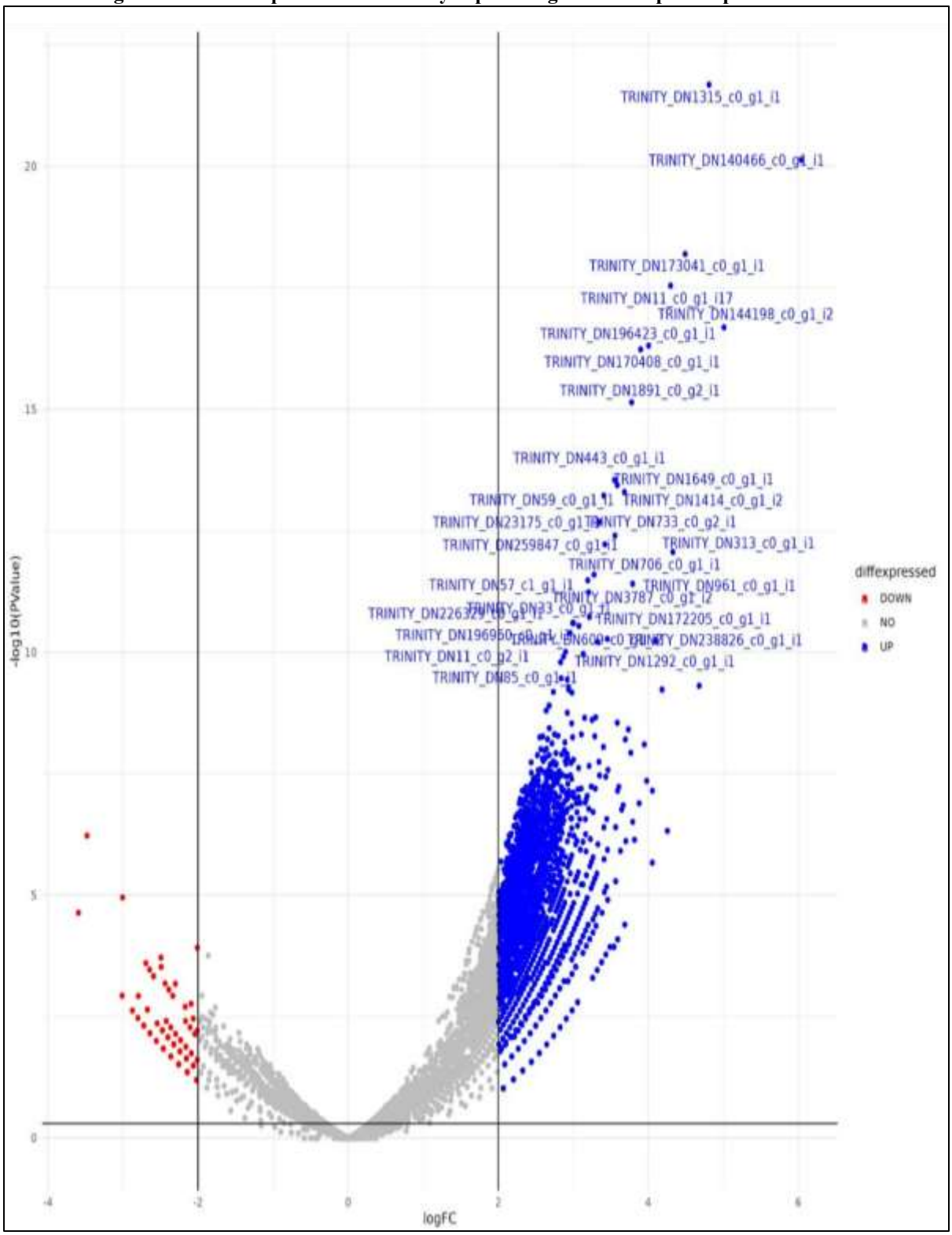

Figure 4. Sample distance map – Bombyx mori

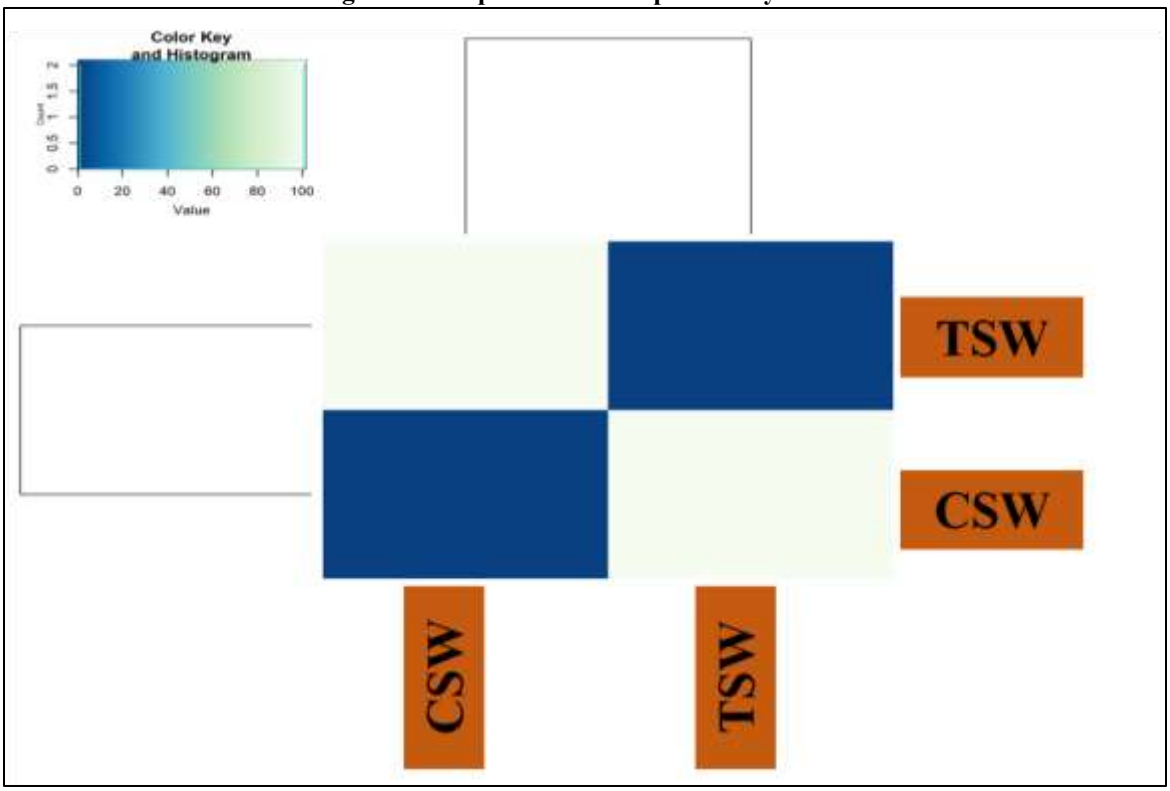

Figure 5. Box plot – Bombyx mori

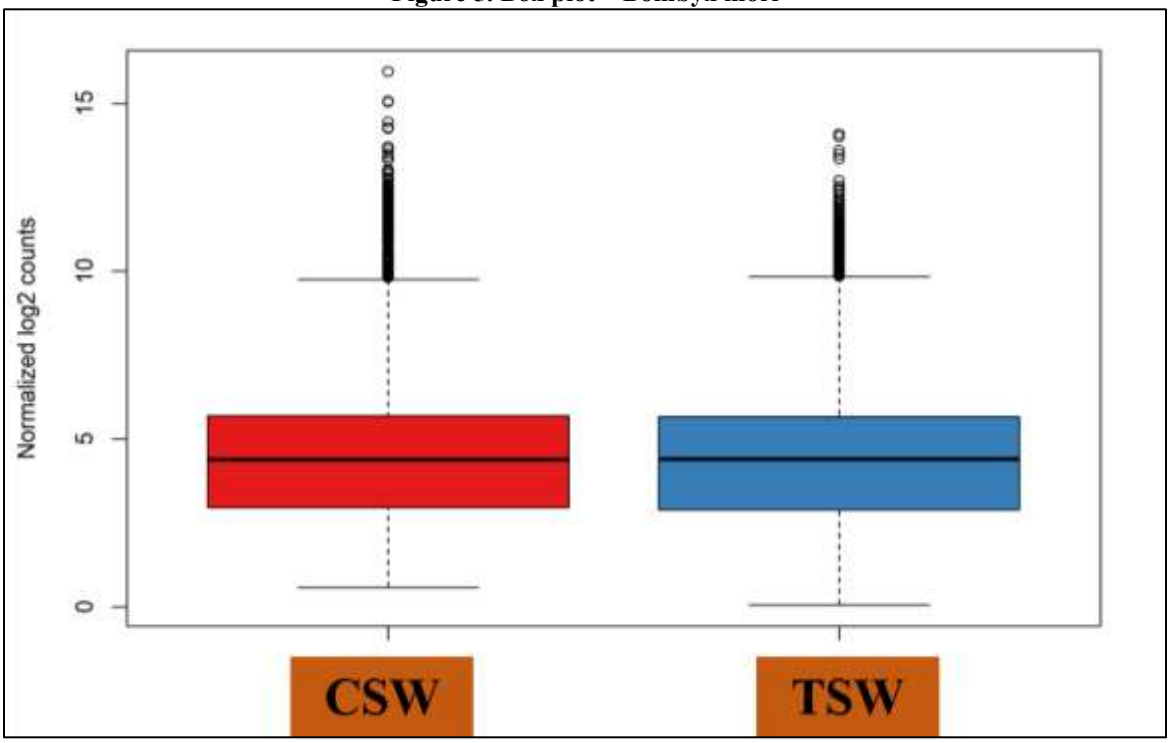

Figure 6a. Heatmap of differentially expressed genes in Bombyx mori

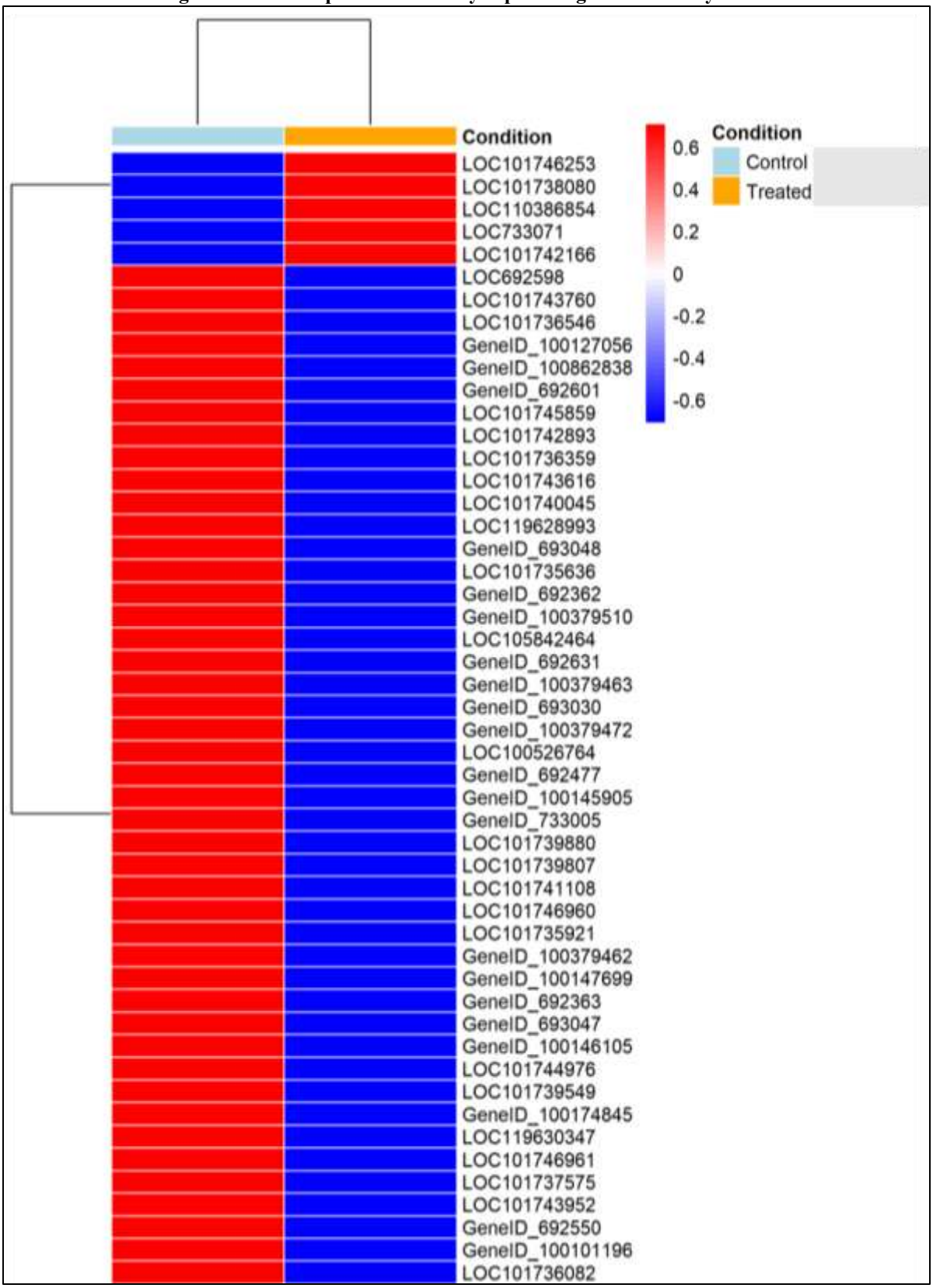

Figure 6b. Volcano plot of differentially expressed genes in Bombyx mori

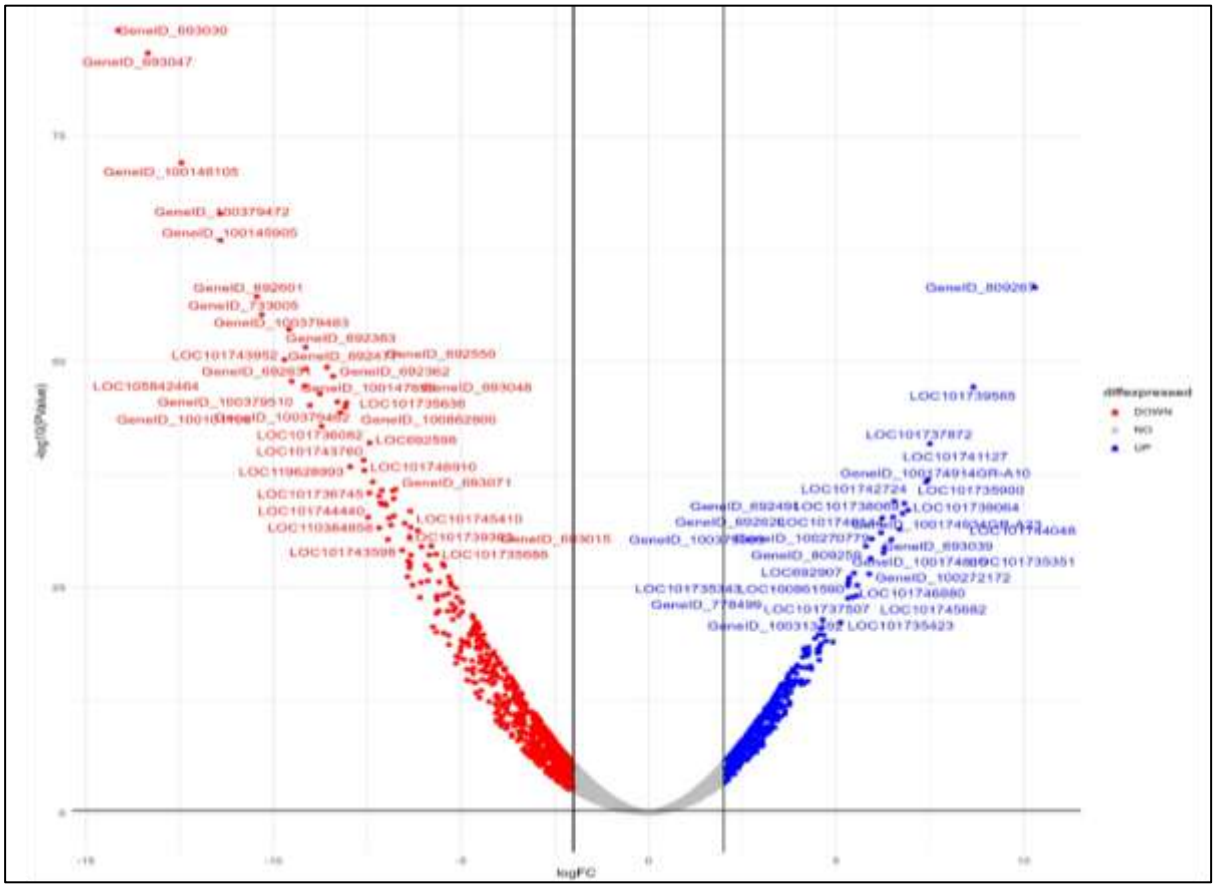

Figure 7. GO enrichment analysis for differentially expressed genes in *Bombyx mori*

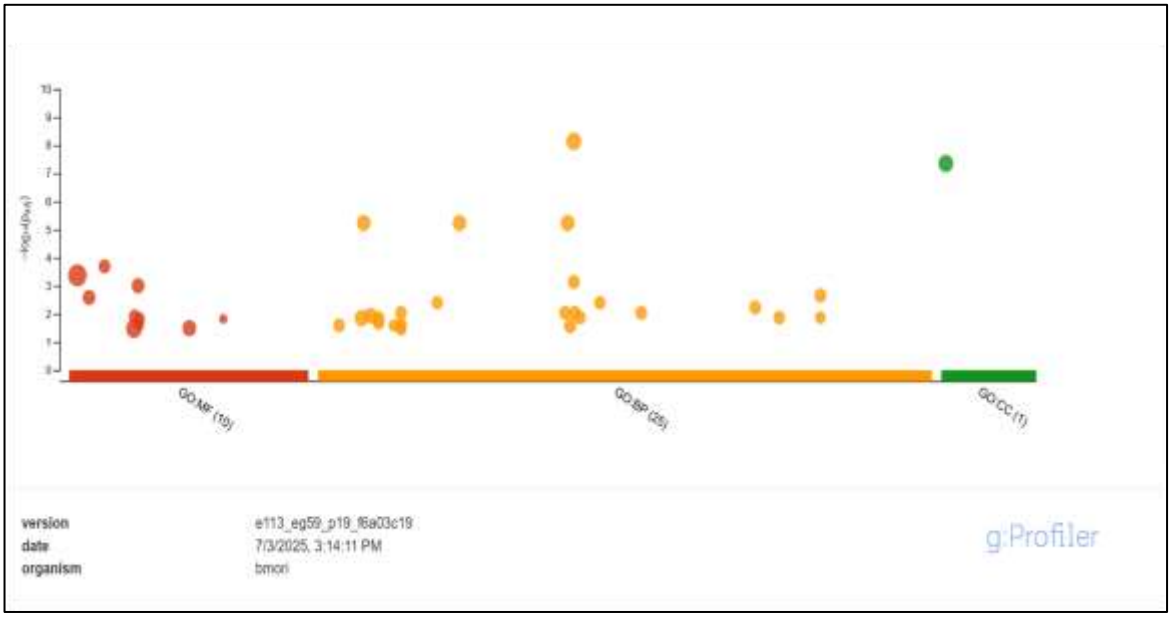

Figure 8. KEGG enrichment analysis of differentially expressed genes in Bombyx mori