

Differentially expressed microRNAs under imidacloprid exposure and identification in *Sitobion avenae*

Baizhong Zhang

Henan Institute of Science and Technology <https://orcid.org/0000-0001-6563-0554>

Jun-Jie Liu

Henan Institute of Science and Technology

Liu-yang Lu

Henan Institute of Science and Technology

Lan-Fen Xie

Henan Institute of Science and Technology

Xi-Ling Chen (✉ chenxiling456@126.com)

Shouping Zhang

Henan Institute of Science and Technology

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Abstract

BACKGROUND MicroRNAs (miRNAs) are short single-stranded non-coding RNAs that regulate the expression of target genes, especially regulation or metabolism of endogenous or xenobiotic compounds. **RESULTS** The de novo assembly of the transcriptomes was obtained through Illumina short-read sequencing technology in *Sitobion avenae*. 57 miRNAs, of which 36 were known and 21 were novel were identified. Quantitative expression levels of miRNA showed that the expression of 5 miRNAs were significant up-regulation, and the expression of 11 miRNAs were significant down-regulation in the nymph of *S. avenae* treated by imidacloprid compared to the control, respectively. The putative transcript target genes in *S. avenae* that could be regulated by these miRNAs were also carried out. The potential functions of these miRNAs in the regulation of genes involved in the metabolism, regulatory or detoxification of *S. avenae* were clarified based on Gene Ontology and KEGG pathway. The effects of these miRNAs identified api-miR-1000, api-miR-316, and api-miR-iab-4 on susceptibility of *S. avenae* to imidacloprid was determined. Modulation of the abundance of api-miR-1000, api-miR-316, and api-miR-iab-4 through the addition of inhibitors of api-miR-1000, api-miR-316, and api-miR-iab-4 to the artificial diet significantly altered the susceptibility of *S. avenae* to imidacloprid, which further proved that the regulatory role of these miRNAs in regulation or metabolism of insecticides. **CONCLUSION** It suggested that differentially expressed microRNAs under the stress of imidacloprid could play critical regulatory role in the resistance of *S. avenae* to imidacloprid.

1 Background

Micrnas (miRNAs) are a class of endogenous small non-coding RNAs of 18~23 nucleotides in length that have been shown to be responsible for the post-transcriptional regulation of mRNAs. miRNA could suppress the translation of target mRNA molecules to silence target gene expression, through binding complementarily to 3' untranslated regions (UTRs), coding sequences, or 5' UTRs.¹⁻⁵ By imperfect complementary base pairing mainly to UTRs of target mRNAs, the miRNA guide stand directs the RISC complex, as well as to exons and 5' UTRs.⁶ Though 21 to 22 bp of the guide RNA/target RNA duplex allows for loose complementarity, a near perfect match is required in the "seed" region located from positions 2 to 7 of the guide miRNA, which is considered to be related to target specificity.⁷

Studies have shown that miRNAs could regulate most of mammalian protein-coding gene,⁸ which may be key mediators in a series of developmental and physiological pathways,⁹⁻¹² i.g tissue differentiation and apoptosis, cell proliferation, and morphogenesis, and the metabolism of heterogeneous compounds.^{5, 13}

By regulating the expression of xenobiotic-metabolizing enzymes and nuclear receptors, miRNAs could mediate the detoxification metabolism of xenobiotics,⁵ i.g human UDP-glucuronosyltransferase (UGT) 1A, which participates in the metabolism of raloxifene metabolites, was proved to be post-transcriptionally regulated by mir-491-3p,¹⁴ human P450 CYP1A1 related to the metabolism of carcinogenic and metabolites can be post-transcriptionally regulated by miR-892a.¹⁵

Previous studies conducted in *Plutella xylostella* indicated that miRNAs could be involved in the resistance to chlorantraniliprole insecticides by mediating the expression levels of UGT genes.¹⁶ *UGT2B7* and *UGT2B15* genes, which was proved to be post-transcriptionally regulated by mirRNA;¹⁷ *Culex pipiens* suggested that miRNAs are involved in the resistance to pyrethroid insecticides by mediating the expression levels of P450 genes.^{18,19} However, many known about the miRNAs are involved in regulating the detoxification of xenobiotics in animals and likely have essential roles in insecticide resistance, less is understood about the regulatory roles of miRNAs in the metabolism of insecticides in insects.

The grain aphid, *Sitobion avenae*, is a destructive pest of wheat crops distributed worldwide. The control of this pest has depended largely on the use of chemically synthesized insecticides.²⁰ Imidacloprid is a chloronicotinyl insecticide that targets aphids, leafhoppers, whiteflies and other sucking pests.²¹ It has been widely used for control of wheat aphid in China. Therefore, wheat aphid has evolved serious resistance to imidacloprid.²²

In this study, we performed high-throughput sequencing of short RNA libraries constructed by control and imidacloprid treatment to detect differentially expressed microRNAs, and the potential functions of these miRNAs in the regulation of genes involved in the metabolism, regulatory or detoxification of *S. avenae*. This will help our understanding of the regulatory roles of miRNAs in the resistance of imidacloprid in insects.

2 Materials And Methods

2.1 Insect culture

The strain of *S. avenae* has been kept in the laboratory for more 10 years, and has never been exposed to insecticides. As mentioned above, the aphids were cultured on wheat seedlings, the controlled conditions are 16–25°C, relative humidity (RH) is 60-70%, and a photoperiod is 17 h L:7 h D.²³

2.2 Imidacloprid treatments

According to the methods of Chen *et al.*²⁴ with slight modifications, leaf-dipping with aphids was performed. Imidacloprid was first dissolved in acetone, and then diluted with distilled water at an LC₃₀ 0.5 mg L⁻¹ concentration. Leaf-dipping with third instar nymphs aphids were immersed in the imidacloprid solution (0.5 mg L⁻¹) for 5-10 s. And their imidacloprid-treated counterparts as the control (treated with distilled water) were collected at the same time points. Surviving aphids (N = 6 for each sample), including imidacloprid challenge and counterparts were collected at 24 h. The samples of aphids were flash frozen in the liquid nitrogen before preserved at -80°C. The experiment was repeated three times.

2.3 RNA isolation, miRNA library construction and Illumina sequencing

Total RNA was extracted from controls and imidacloprid treatments of *S. avenae* using TRIzol reagent (Invitrogen, Shanghai, China) following the manufacturer's protocol and re-suspended in nuclease-free

water 1% agarose gels were monitored to determine RNA degradation and contamination; RNA purity was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE); RNA concentration was checked using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA); RNA integrity was checked using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

The total amount of each sample was 3 µg, which was used as input material for the small RNA library. According to the manufacturer's recommendation, the sequence library is generated by using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA.), and the index code is added to the attribute sequence of each sample. 3' and 5' RNA adaptors were ligated to the RNA pool using T4 RNA ligase. Then the fragments were used for reverse transcription and subsequent PCR. The final PCR products were purified and subjected to the proprietary Solexa sequencing by synthesis method using the Illumina Genome Analyzer (San Diego, CA, USA) at the Beijing Genomics Institute (Novogene, Beijing, China).

2.4 Bioinformatics analysis, miRNA prediction, and miRNA target prediction.

2. *avenae* miRNAs, were identified by miRDeep2 software.²⁵ Raw reads from the 4 libraries, including imidacloprid treatments and controls (2 biological replicates of each sample), were submitted as input into miRDeep2, and the data of each library was analyzed separately. The default options and settings were used to performed the miRDeep2 analysis. And the sequences with polyA tails of the raw reads, the length of miRNAs ranging from 18 to 30 nt was selected for further being analyzed after the adaptor sequences was trimmed, as well as rRNA, tRNA, snRNA, and snoRNA were discarded. The sequences, mapping to the mature miRNAs in the premiRNAs and miRBase, and mapping to the *Acyrtosiphon pisum* genome would be identified to be as known mature miRNAs. The clean reads identified of *S. avenae* transcriptome has been submitted to the National Center for Biotechnology Information/Sequence Read Archive (NCBI/SRA) database (Accession number: SRP182781). Miranda performed the target genes prediction of miRNA.²⁶ The putative mRNAs that targeted by miRNAs were predicted against *S. avenae* transcriptome nucleotide sequences. 2 software programs were conducted for miRNA target prediction, miRanda (<http://www.microrna.org/>)²⁷ and RNAhybrid (<http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid/>).²⁸ The predicted miRNA target genes would be selected for further analysis. And then the target genes predicted would be aligned by the BLASTX program from NCBI (e value cut-off was 1.0E-5), as well as these gene sequences, were mapped and annotated using BLAST2GO.²⁹ All of the putative target genes identified were used to search the Gene Ontology (GO) database, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for further analyses.

2.5 Comparison of miRNA expression between controls and imidacloprid treatments of *S. avenae*

In order to obtain the miRNAs in the response of aphids against imidacloprid, differential expression of miRNAs was performed between controls and treatments of *S. avenae*. The read counts of miRNA

identified were analyzed using edgeR software (3.10.2), which can be obtained in Bioconductor version 3.1 (<http://www.bioconductor.org/packages/release/bioc/html/edgeR.html>).³⁰

The P-values was calculated by the Benjamini-Hochberg method.³¹ P-value corrected was set at 0.05 as the threshold for significantly expressed difference by default. Normalization of miRNA counts between libraries of controls and treatments was executed according to the total number of reads across libraries *in silico*. Normalized expression = Actual miRNA count/Total count of clean reads $\times 10^6$. A false discovery rate (FDR) of the miRNAs <0.05, and a fold change ≥ 2 were deemed to be significant.

2.6 Quantitative real-time PCR (qPCR) validation

To validate the miRNA data obtained by the deep sequencing, 3 miRNAs were selected to confirm their expression by qPCR. Total RNA was extracted using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. First strand cDNA was synthesized from 2 μg of total RNA using miScript II RT kit (Qiagen) following the manufacturer's instructions. SYBR Green Master Mix (miScript SYBR Green PCR Kit, Qiagen) was used for determining miRNA expression, and performed on the ABI 7500 platform (Applied Biosystems) using an SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan). And qPCR procedure was performed as previously described.³² The optimized qPCR procedure consisted of an initial step at 94°C for 2 min, 50°C for 2 min, followed by 50 cycles of 60°C for 30 s and 94°C for 15 s followed by the melting curve. After the cycling scheme, melting curves were got by increasing the temperature from 60 to 95°C (0.2°C s⁻¹) to denature the double-stranded DNA. The qPCR amplifications were performed in 96-well plates. Three biological replicates, with three technical replications for each, were evaluated for each sample. Analysis of the qPCR data was carried out using the 2^{- $\Delta\Delta\text{Ct}$} method of relative quantification.³³ U6 snRNA as an endogenous control, was used to quantify miRNA expression level. The primers used for the qPCR analysis are listed in Table 1.

2.7. MiRNA inhibitor feeding *in vitro*, and subsequent impacts on imidacloprid susceptibility

The rearing method and the artificial diet used were as described by Gong et al.³⁴ with some modifications. Transparent sterilized glass tube open at both ends (4 cm in length, 2.5 cm diameter) were used as the feeding device *in vitro*. 25% sucrose as artificial diet was sealed between 2 layers of parafilm in a 5 cm diameter feeding device, and then healthy aphids were transferred to each device for rearing covered by the mesh to prevent them from escaping. The artificial diet was used with DEPC-treated water to ensure no RNase activity. The aphids were reared under the condition described previously. Each sample contained three replications, 100 apterous adults were used in each replication.

To evaluate the inhibitory effect of miRNAs after the feeding of miRNA inhibitors, which were mixed to the artificial diet, and the final concentration of miRNA inhibitor was 2.5 mM/L. 50 healthy apterous aphids were fed with the artificial diet, and the NC-inhibitor (a negative control) were used for the control; Following the feeding of 24 h, the aphids survival were collected for RNA extraction, 3 replicates were carried out.

To evaluate the modulation effects of the miRNAs on sensitivity of *S. avenae* to imidacloprid, 50 healthy apterous aphids were placed to the artificial diet that contained imidacloprid (0.1 mg/L) mixed with miRNA inhibitor at a final concentration 2.5 mM/L, the NC-inhibitor (a negative control) were used for the control. 3 replicates were carried out, and mortality was calculated at 48 h. The miRNA inhibitors used were provided by Sangon Biotech Co., Ltd (Shanghai, China).

3 Results

3.1 Illumina sequencing data analysis

There were 14,841,584, and 13,964,533 raw reads, identified from the control and imidacloprid treatment RNA libraries, respectively (Table 2). The length of *S. avenae* sRNAs was between 18 nt and 35 nt, with 22 nt size comprising 5.28% and 5.08% of the total reads from control and imidacloprid libraries, respectively (Fig. 1A). After discarding low quality sequences and adapter, The unique reads, 6,992,451 (47.11% of total) and 6,349,363 (45.47%) remained from the control and imidacloprid treatments libraries, respectively (Table 2). The length distribution of unique filtered reads indicated a distribution in the 2 libraries (Fig. 1B), where constituted 3.75% and 3.72% of the total reads at 22 nt, respectively, where at 23 nt constituted 5.18% and 6.23% of the total reads, respectively. Where in the peak at 32 nt constituted 8.59% of the total reads of imidacloprid libraries, at 34 nt constituted 9.76% of the total reads of controls, which probably showed piRNA-like sRNAs in *S. avenae*. A little bit sRNAs had over a thousand reads, however most had fewer than 10 copies in the library (Fig. 1C). sRNAs in *S. avenae* showed a significant bias for the nucleotide U at 5' (96.99%, and 97.51%, respectively) and 3' ends (53.38%, and 55.32%, respectively) in both libraries (Fig. 2).

3.2 Sequence analysis of miRNA and predication.

According to the length distribution of the reads, miRNAs at 22 nt accounted for 43.86% of all miRNA species of sRNA libraries (Fig. 1D). The query of these reads of putative *S. avenae* miRNAs to *A. pisum* genomes and all insect miRNAs in miRBase, the results showed 36 conserved sequences were identified (Table S1). In addition, the query of *S. avenae* filtered reads against mature miRNAs in the miRBase database, the results showed that 26 miRNA sequence families were identified (Fig. 3A). It is predicted that the miR-10 family has the largest members ($n = 415$), followed by let-7 ($n = 401$), and miR-2 ($n = 180$). Insects were shared with these miRNAs, with the majority of miRNAs shared with the known *A. pisum* miRNAs, and a fewer extent trend for orthologs of other insects (Fig. 3B).

There were a total of 21 novel miRNAs potentially obtained, and named the prefix 'PC' (predicted candidate) based on the adopted nomenclature (Table S2). These specific miRNAs could be mapped to *S. avenae* transcriptome sequences. It was predicted from Mfold that stem-loop hairpin secondary structures (<18 kcal/mole³⁵) were consist of miRNAs precursor sequences, and some predicted structures in *S. avenae* were shown in Fig. S1.

Differentially expressed miRNAs between imidacloprid treatments and controls were also determined according to normalized differences of Illumina read counts, 57 *S. avenae* miRNAs, 16 of which (28.1%) were differential expression, including 11 down-regulation and 5 up-regulation (Table 3).

The PCR amplified products of api-miR-1000, api-miR-316, and api-miR-iab-4 indicated a single band as the expected size (60–100 bp). Subsequent analysis of qPCR results confirmed that the expressed level of Api-miR-1000 was 5.5- fold higher in imidacloprid treatments compared to controls. While the expression of Api-miR-316, and Api-miR-iab-4 were 0.09-, and 0.12- fold lower in imidacloprid treatments compared to controls, respectively. (Fig. S2). Overall, qPCR results of miRNAs were indicated similar ranging trends as that of the RNA-seq analyses.

3.3 Target prediction of miRNAs.

The 16 differentially expressed miRNAs in *S. avenae*, the hypothetical targets within the UTRs of transcripts from *S. avenae* for the miRNAs were predicted using Target Scan software (Table S3). Possible functions predicted of these hypothetical target genes were annotated by GO enrichment, which could be involved in molecular functions, biological processes and cellular components, like development processes (Table S4). There were 58 GO terms identified for target genes of miRNAs predicted according to GO level 2 (Fig. 4A), like the genes participated in metabolic processes.

These genes including 71 metabolic pathways predicted by KEGG enrichment analysis might be affected by miRNA regulation (targeting) (Fig. 4B). These transcript targets were predicted to orthologs of related insects, which showed that differentially expressed miRNAs between the control and the imidacloprid treatment in *S. avenae* could bind and promote the post-transcriptional regulation, including various biological pathways like xenobiotic metabolism (Table S5).

To investigate the potential roles of miRNAs in the resistance of *S. avenae* to imidacloprid, the predicted target genes for the miRNAs that could participate in xenobiotic metabolism were further focused. Interestingly, target genes for some miRNAs were found to play critical roles in insects response to xenobiotic stress, including cytochrome P450s, UDP-glucuronosyltransferase, glutathione S-transferase, etc. (Table 4). Some miRNAs predicted have a lot of putative target genes, and many putative target genes were regulated by multiple miRNAs. (Table S3).

3.4 Modulation of miRNAs consequently impacts the susceptibility of *S. avenae* to imidacloprid

The expression levels of miRNAs, api-miR-1000, api-miR-316, and api-miR-iab-4, was significantly depressed in *S. avenae* aphids fed miRNA inhibitors compared to the levels in *S. avenae* aphids fed DEPC water and NC-inhibitor as controls (Fig. 5A). Mortality decreased significantly 21.73% in *S. avenae* aphids fed api-miR-1000 inhibitor under imidacloprid exposure compared to the controls. While the mortality increased significantly by 26.75% and 21.86% in *S. avenae* aphids fed api-miR-316, and api-miR-iab-4 inhibitors under imidacloprid exposure compared to the controls, respectively (Fig. 5B).

4 Discussion

It is predicted miRNAs can regulate a lot of protein-coding genes.³⁶⁻³⁸ And it is demonstrated that miRNAs play critical regulatory roles in a lot of biological processes. In this study, miRNAs in *S. avenae* that might play regulatory roles in target genes responses to imidacloprid were identified and assessed. The identification and functional analysis of miRNAs differentially expressed in imidacloprid treated *S. avenae* were conducted, which may provide potentially insight into the regulatory/detoxifying mechanisms of imidacloprid in insects. So far, small RNA sequencing has been as a wide experimental way to identifying miRNAs from a series of organisms with the development of high throughput sequencing technology. Li et al.³⁹ identified 345 miRNAs from the winged and wingless *S. avenae*. However, we only identified 57 miRNAs in the control and imidacloprid treatments of *S. avenae*. While the results of our sequencing data could greatly enlarge the range of the available resources and provide critical basis for further studying miRNAs related to insecticide resistance in *S. avenae*.

Our analysis on expression of read counts indicated that 16 differentially expressed miRNAs were identified between the imidacloprid-treated aphids and the control aphids, which clearly indicated that imidacloprid could affect expression of miRNAs, thus suggesting a probable important role of these miRNAs in the regulation of the metabolism of insecticides in *S. avenae*. Several *S. avenae* up/down-regulated miRNAs in imidacloprid-treated aphids could be involved in the regulation of the metabolism of imidacloprid in this study. i.g api-MiR-1000, api-MiR-316, and api-MiR-iab-4 were up/down-regulated in imidacloprid-treated aphids, which suggests that they could be involved in the regulation of the metabolism of imidacloprid. *Drosophila melanogaster* miR-1000 of activity-dependent expression could indicate a mechanism of allowing neuronal activity to fine-tune the strength in the excitatory synaptic transmission, and regulate the expressed level of vesicular glutamate transporter, and load the glutamate into synaptic vesicles,⁴⁰ *D. melanogaster* dme-miR-1000 was located within the intron of musashi,⁴¹ encoding an RNA-binding protein to regulate gene translation by prior expression in the nervous system,⁴² Honeybee miR-1000 could be involved in musashi function of the nervous system,⁴³ ame-miR-1000 was as the first identified miRNA, which was expressed in a brain-selective manner of an insect. 1 cytochrome P450 and 2 glutathione S-transferase genes could be recognized by Mse-miR-316,⁴⁴ The accumulation endogenous Ubx protein could be attenuated as ectopic expression of miR-iab-4-5p, and a classical homeotic mutant phenotype could also be induced (the transformation of halteres into wings),⁴⁵ *Drosophila* dme-miR-iab-4 and dme-miR-iab-8 as bithorax-complex (BX-C) HOX clusters miRNAs could regulate neural patterning and reproduction through restricting HOX gene targets⁴⁶.

In order to further understand the effect of miRNAs on the regulation of metabolism of imidacloprid, the hypothetical target genes for miRNAs identified were predicted, and many of them annotated could participate in a lot of biological processes. At the same time, several target genes from families for miRNAs were known to be involved in the metabolism of xenobiotics.^{47,48} We found that several of the miRNAs targeting the genes related to xenobiotic metabolism were among the differentially expressed

miRNAs,^{49, 50} by combining the results of the target prediction with our differential expression analysis of miRNAs.

The mortality of aphids under imidacloprid exposure decreased significantly after their feeding api-miR-1000 inhibitor. While the mortality of aphids under imidacloprid exposure increased significantly after their feeding api-miR-316, and api-miR-iab-4 inhibitors, respectively. This showed that miRNAs differentially expressed may participate in metabolism of imidacloprid, by regulating the expression of xenobiotic metabolism genes.

In conclusion, of 57 miRNAs identified from *S. avenae*, the transcript levels of 16 miRNAs were ranged in *S. avenae* treated by imidacloprid. The prediction of targeting the genes related to xenobiotic metabolism showed that differentially expressed miRNAs may be involved in the metabolism of imidacloprid of *S. avenae*, and this represents a crucial new small RNA genomics resource for further studies.

Abbreviations

miRNAs: MicroRNAs; UGT: UDP-glucuronosyltransferase; qPCR: Quantitative real-time PCR; BLASTX: Similarity search of the NCBI protein database using a translated nucleotide query; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; nr: NCBI non-redundant protein sequences database; nt: NCBI nucleotide collection; UTRs: untranslated regions; SRA: Sequence Read Archive

Declarations

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Availability of data and materials

The transcriptome clean reads and computationally assembled sequences from this study were submitted to the NCBI/SRA database, under accession number: SRX374716.

Author contributions statement

S.Z and X.C conceived and designed the experiments, B.Z., and J.L. performed the experiments, B.Z., and S.Z. analyzed the data and wrote the manuscript text. J.L., and L.L. participated in the data analysis. All authors reviewed the manuscript.

Competing interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Author details

College of Resources and Environment, College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, P.R.China

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Tables

Table 1. Primers were used in the study

Primer name	Sequence (5'-3')	Size/bp	Application
api-mir-1000	F:AGAGCACGATATTGTCCTGTCACA	73	qPCR
	R:TATGGTTGTTCCAGACTGGTTCAC		qPCR
api-mir-316	F:TTTGGTGTCTTTTTCCGCTTTG	69	qPCR
	R:GTGCAGGGTCCGAGGT		qPCR
api-mir-iab-4	F:AGGCGAAGCGACGTATACTAAATG	68	qPCR
	R:GTGCAGGGTCCGAGGT		qPCR
U6	F:CAGCACATATACTAAAATTGGAACG	76	qPCR
	R:ACGAATTTGCGTGTCATCC		qPCR
MircoRNA inhibitor N.C	CAGUACUUUUGUGUAGUACAA	21	miRNA function
api-mir-1000 inhibitor	UACUGCUGUGACAGGACAAUUAU	22	miRNA function
api-mir-316 inhibitor	CGGCAGCAAAGCGGAAAAAGACA	23	miRNA function
api-mir-iab-4 inhibitor	UCAGGAUACAUUUAGUUAUACGU	22	miRNA function

Table 2. Distribution of miRNA reads from control and imidacloprid treatment of *Sitobion avenae*

Parameter	Controls	Imidacloprid treatments
Raw reads	14,841,584	13,964,533
Clean reads	14,224,837	13,492,898
3ADT and length filter	10,959,929	9,305,973
rRNA	253,777	247,747
tRNA	16	23
snoRNA	9,443	7,823
snRNA	25,993	24,246
Mappable reads	6,992,451	6,349,363

Note: 3ADT: reads that were removed as 3ADT was not found and length was less than 15nt and was more than 35nt. Mappable reads: reads from the raw reads that were passed by many the digital filters .

Table 3. The differentially expressed miRNAs in small RNA libraries of *Sitobion avenae*

miR_Name	Imidacloprid treatment_FPKM	Control_FPKM	P-value	Log2(Fold Change)	Difference
api-miR-998	4021.337154	14561.84926	0	-1.8564	Down
Novel-28	435.4314115	2094.17899	1.93E-286	-2.2659	Down
api-miR-306	691.567536	2386.390012	1.54E-246	-1.7869	Down
Novel-22	742.7947608	1801.967968	1.83E-120	-1.2785	Down
Novel-9	717.1811484	1704.564294	1.79E-110	-1.249	Down
api-miR-3047	332.9769618	779.2293917	3.01E-50	-1.2266	Down
Novel-25	256.1361244	657.4747992	6.09E-49	-1.36	Down
api-miR-1000	128.0680622	0	8.16E-28	8.0008	Up
api-miR-316	0	48.70183698	2.37E-13	-6.6059	Down
Novel-39	51.22722489	0	4.65E-13	6.6788	Up
Nove-35	153.6816747	48.70183698	2.31E-12	1.6579	Up
api-miR-263b	0	24.35091849	1.57E-07	-5.6059	Down
api-miR-2796	0	24.35091849	1.57E-07	-5.6059	Down
api-miR-iab-4	0	24.35091849	1.57E-07	-5.6059	Down
api-miR-3015a	25.61361244	0	2.52E-07	5.6788	Up
api-miR-3042	76.84083733	24.35091849	7.05E-07	1.6579	Up

Table 4 MiRNAs target for putative genes related to xenobiotic metabolism in *Sitobion avenae*

miR_Name	Target genes related to xenobiotic metabolism
api-miR-998	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase CYP3A19, glutathione S-transferase omega-1, glutathione S-transferase, C-terminal domain
novel-28	Microsomal epoxide hydrolase, glutathione S-transferase, C-terminal domain, P450 (GC vitamin D-binding protein), CYP4C1, CYP4V, CYP9Z4
api-miR-306	alcohol dehydrogenase 4, UDP-glucuronosyltransferase 2B10, UDP-glucuronosyltransferase 2B7, Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP4C1, Glutathione S-transferase (EEF1G elongation factor 1-gamma), CYP6A14
novel-22	UDP-glucuronosyltransferase 2C1, UDP-glucuronosyltransferase 1-7C UDP-glucuronosyltransferase 2B17, Glutathione S-transferase Mu 1, Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), CYP4g15, CYP4C1
novel-9	UDP-glucuronosyltransferase 2C1, carbonyl reductase 1, Microsomal glutathione S-transferase 1, UDP-glucuronosyltransferase 1-7
api-miR-3047	UDP-glucuronosyltransferase 2C1, UDP-glucuronosyltransferase 1-7, CYP315A1, CYP2U1, P450 (piezo-type mechanosensitive ion channel component 2)
novel-25	alcohol dehydrogenase class-3, S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase, glutathione S-transferase, C-terminal domain, CYP3A19,
api-miR-1000	UDP-glucuronosyltransferase 2B7, UDP-glucuronosyltransferase 2B10, UDP-glucuronosyl transferase 351A3, Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), Glutathione S-transferase (failed axon connections), Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP4C1, CYP6A2, CYP6A14
api-miR-316	UDP-glucuronosyltransferase 2B1, Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP4C1, CYP305a1, Cytochrome bd terminal oxidase
novel-39	Glutathione S-transferase GSTZ1
novel-35	Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP49A1, CYP6A14, CYP315A1, CYP4C1, CYP6A13, CYP6J1
api-miR-263b	UDP-glucuronosyltransferase 2C1, UDP-glucuronosyltransferase 2A1, glutathione S-transferase C-terminal domain, CYP6A13, CYP4C1, CYP4V2, CYP49A1,
api-miR-2796	UDP-glucuronosyltransferase 2B18
api-miR-iab-4	CYP314A1, CYP4C1, CYP6A13, CYP3A19
api-miR-3015a	alcohol dehydrogenase class-3, UDP-glucuronosyltransferase 2A1 dehydrogenase, glutathione S-transferase C-terminal domain, Glutathione S-transferase (phosphatidylinositol-binding clathrin assembly protein LAP), Cytochrome P450 (piezo-type mechanosensitive ion channel component 2), CYP3A19,
api-miR-	Glutathione S-transferase (EEF1G elongation factor 1-gamma)

Figures

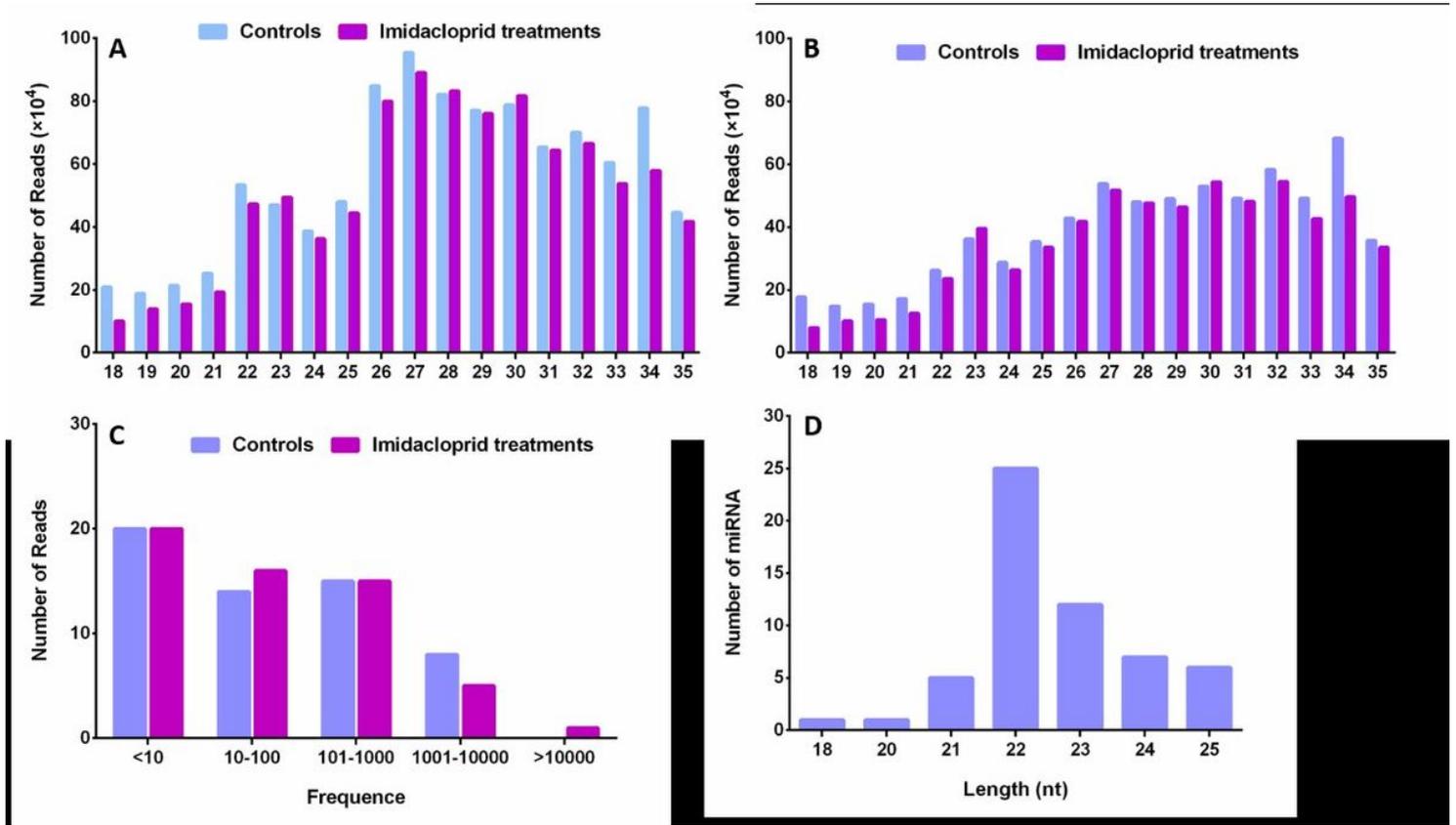


Figure 1

Characterization of miRNA sequences from *Sitobion avenae*

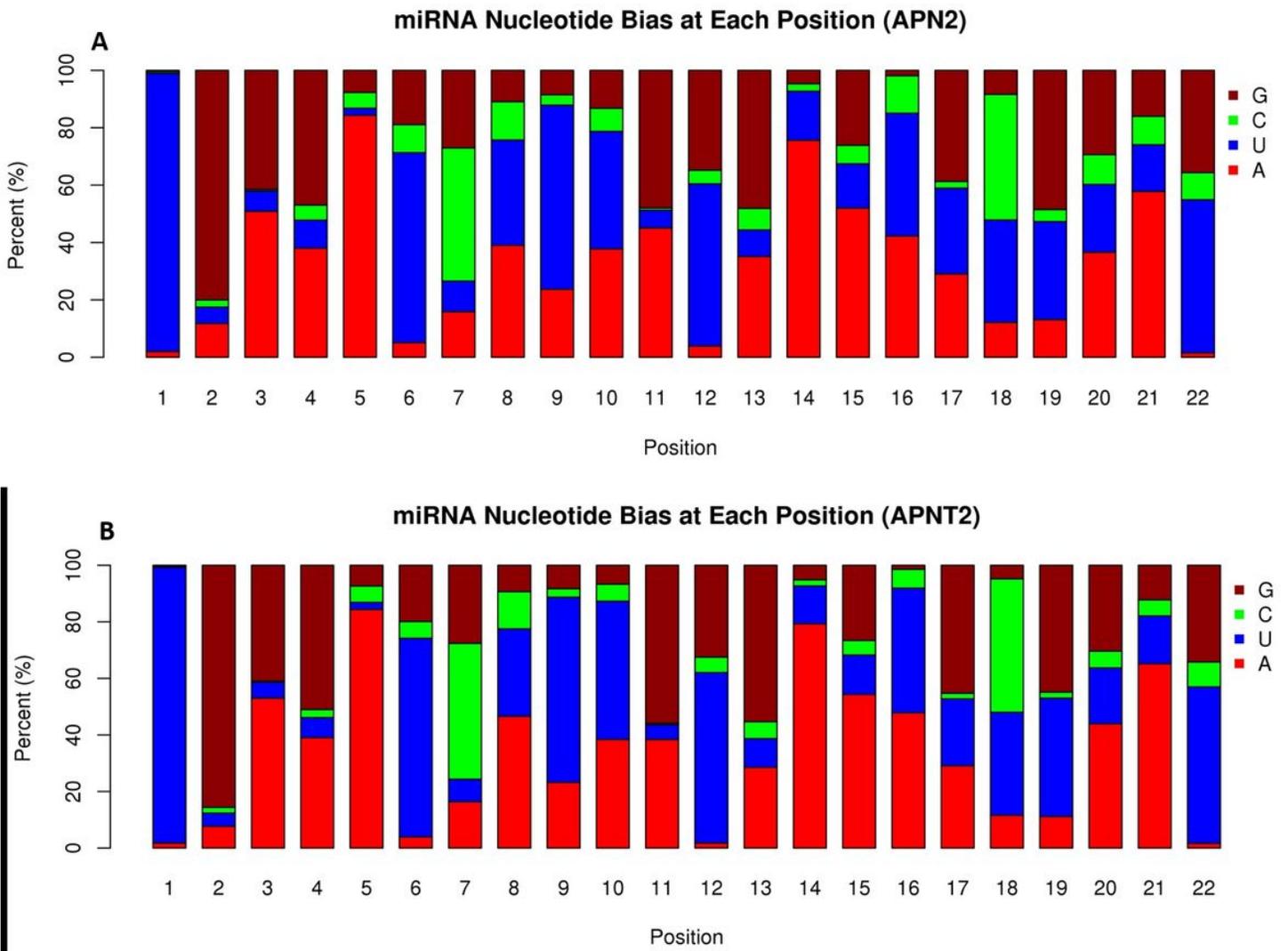


Figure 2

Nucleotide bias of predicated miRNAs from *Sitobion avenae*

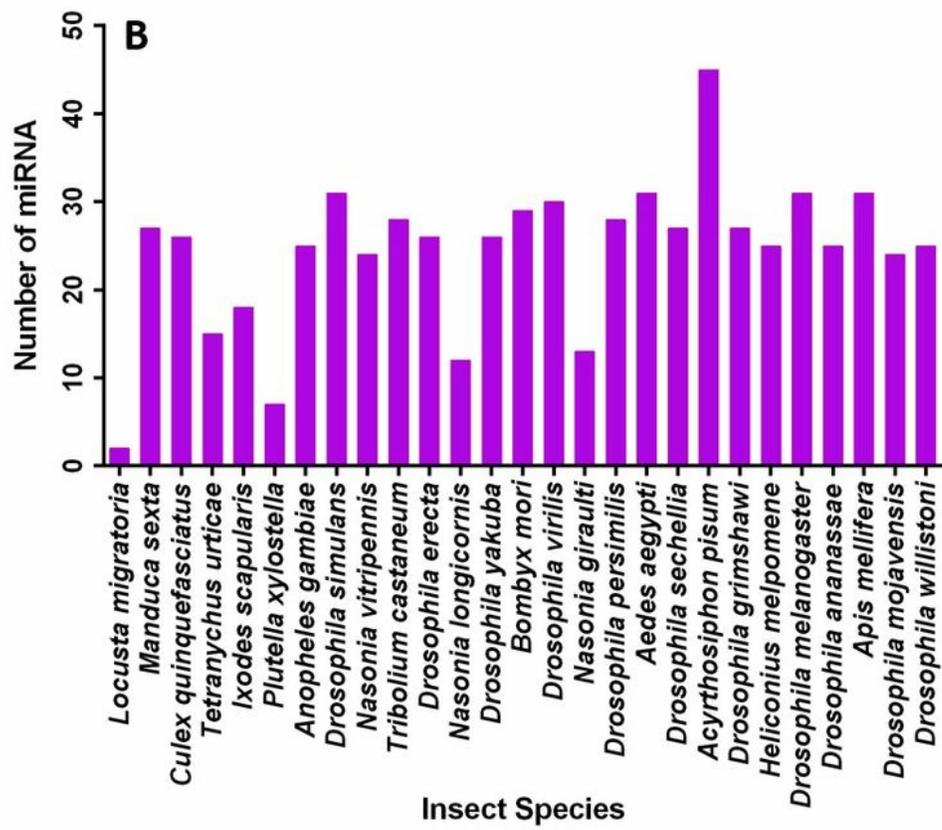
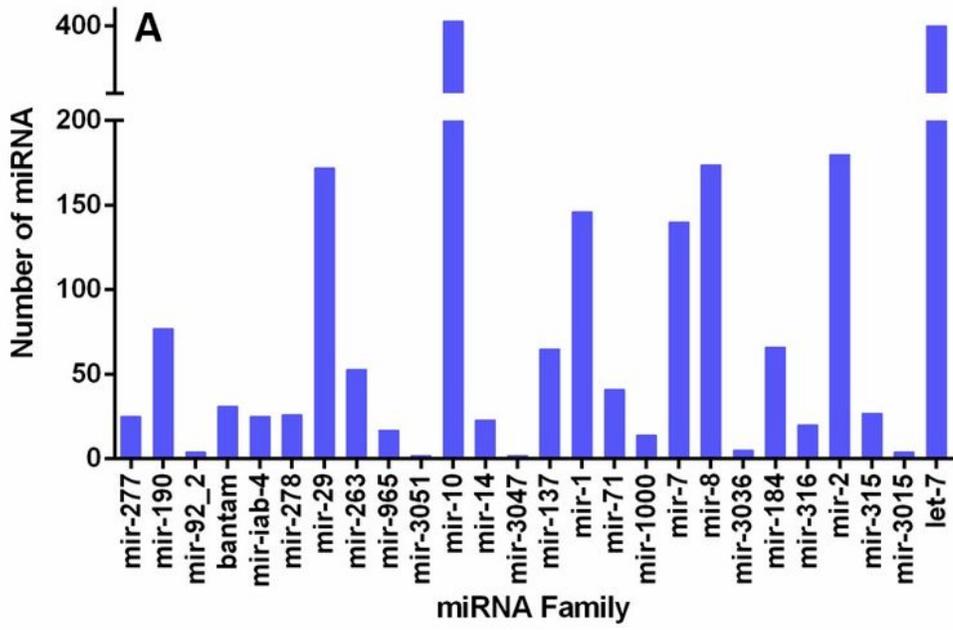


Figure 3

MiRNAs characterization of *Sitobion avenae*.

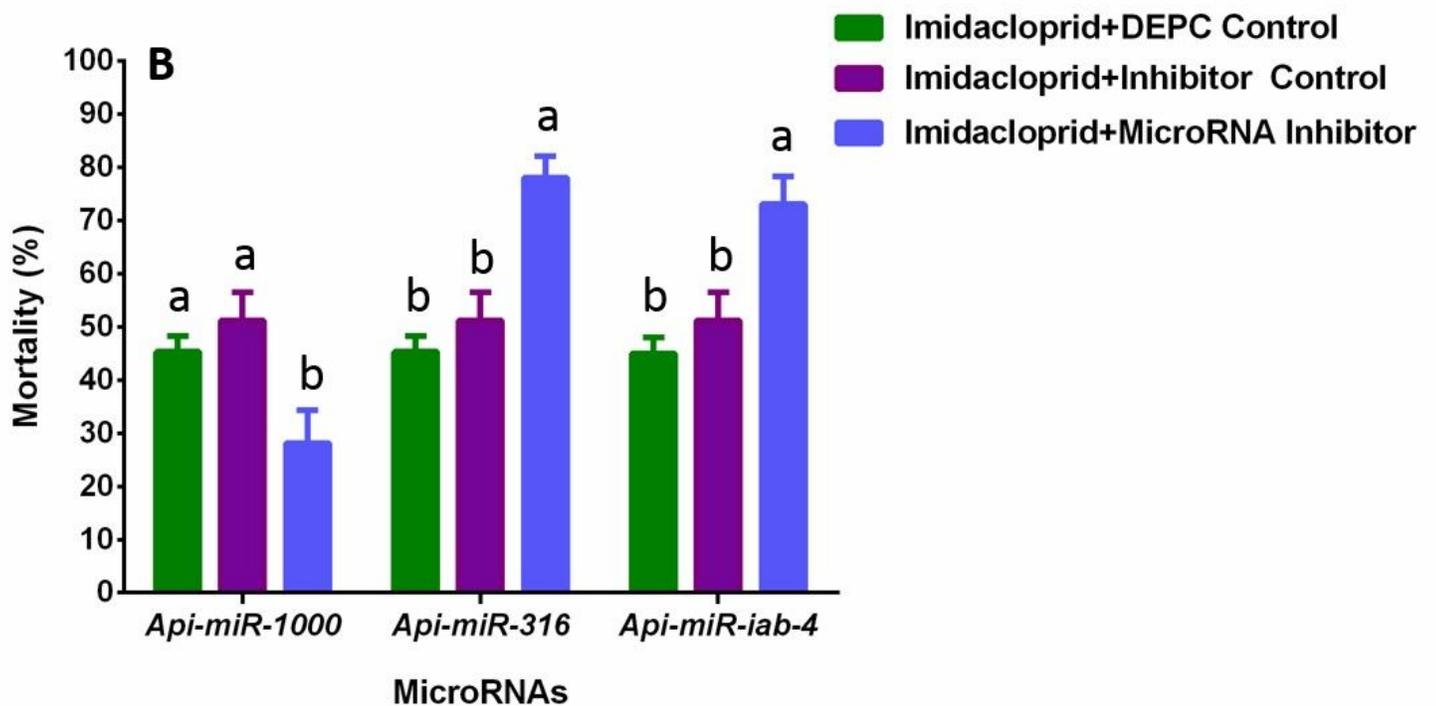
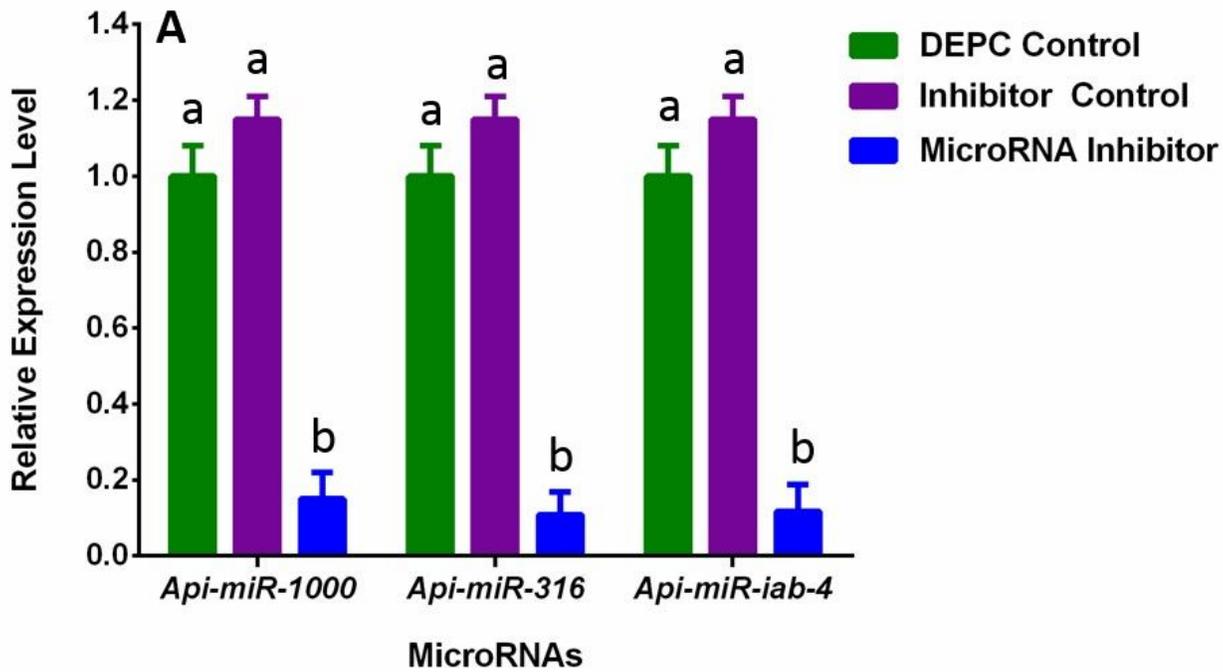


Figure 5

Effects of miRNA modulation on susceptibility of *Sitobion avenae* to imidacloprid.

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