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Molecular Study on Field Evolved Resistance of Red Palm Weevil (*Rhynchophorus ferruginous*) and its Management through RNAi

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JNA and MM performed experiments and analyzed the results. JNA, MM, ZA and SJNA wote the manuscript.

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***Rhynchophorus ferruginous*, RNA interference, cytochrome P450, Insecticide resistance management**

ABSTRACT

Molecular and proteomic study was conducted on field evolved resistance of Red Palm Weevil (*Rhynchophorus ferruginous*) against Parathyroid group of insecticide (Cypermethrin). Cytochrome P450 genes have been associated with insecticides resistance. Field collected population of *R. ferruginous* from different provinces of Pakistan was investigated for insecticides resistance development against P450 gene. Insect bioassay indicated highly significant level of LC50 values among laboratory reared and field collected population against Cypermethrin. The laboratory reared resistant population, Sindh and South Punjab collected population showed high LC50 values as compared to susceptible, Baluchistan and KPK population. The expression of P450 gene through PCR showed enhanced level of P450 gene in different Instars as well as collected population. The dsRNA specific for CYP450 gene was designed and targeted through dsRNA in thoracic region of *R. ferruginous* through micro-injection. Gene expression and proteomic study confirmed the down regulation of P450 gene and associated protein in dsRNA treated population. The application of ds RNA specific for CYP450 also reduced insecticide resistance in *R. ferruginous*. The developmental parameters were highly affected in RPW treated with dsRNA as compared to control samples treated with water or dsGFP. These results support the RNAi application as a suitable tool for the management of insecticide resistance and control of *R. ferruginous*.

INTRODUCTION

The date palm is an important fruit tree but due to severe attack of pests and diseases 30% yield losses were recorded throughout the world (El-Juhany and Loutfy, 2010). *R. ferrugineus* is regarded as tissue borer that have the ability to destroy 28 species of palm trees throughout the world. The enormous losses induced annually by this invasive pest in the Middle East are around $US 130 million (Faleiro, 2006; El-Sabea *et al*., 2009) In Pakistan, recently, red palm weevil (*R. ferruginous),* dusky cotton bug, (*Oxycarenus hyalinipennis)* and nucleopolyhedrovirus from Armyworm (*Spodoptera litura*) have been collected, molecularly identified and characterized from Pakistan (Ahmad *et al*., 2018a, b; Manzoor *et al*., 2018).

For the past six decades, synthetic pesticides are being implemented as pest control regardless of their negative impacts such as environmental pollution, health hazards, resistance development in insects (Tiwari *et al*., 2011b) and contamination of foods due to residual effect (Silva *et al*., 2012). The parasites and predators are also killed due to no target specificity of these insecticides. To handle this burning pest, molecularly constructed insecticides specific to insect species may be helpful.

RNA interference is a reliable molecular technique employed in insect pest management and operational gene expression due to the systemic nature of dsRNA (Sachs-Ericsson *et al*., 2007; Price and Gatehouse, 2008; Scharf *et al*., 2008; Huvenne and Smagghe, 2010; Whitten *et al*., 2016).Plenty of genes have been isolated and targeted for RNAi in various insects such as chitinase in *Tribolium castaneum*, *Ostrinia nubilalis*, *Nilaparvata lugens*, *Mythimna separata*, *Manduca sexta*, *Drosophila melanogaster*, *Chilo suppressalis*, *Bombyx mori*, *Anopheles gambiae* and many other economically serious pests (Shen and Jacobs, 1997; Zhu *et al*., 2008b; Khajuria *et al*., 2010; Zhang *et al*., 2011; Huang *et al*., 2012; Pan *et al*., 2012; Tetreau *et al*., 2015; Xi *et al*., 2015; Su *et al*., 2016; Cao *et al*., 2017).

Cytochrome P_{450} mono-oxygenase is one of the

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exceedingly vital groups of enzymes which are important for metabolism in arthropods. Explicit gene silencing has been directed via ingestion or incorporation of dsRNA in *Caenorhabditis elegans*, tick and flatworms, respectively (Newmark *et al*., 2003; Soares *et al*., 2005). In the same manner the gene suppression through microinjection in last instar larvae as well as adult of *T. castaneum* (Bucher *et al*., 2002) and insects such as *Ostrinia nubilalis* larvae for dsRNA delivery (Wang *et al*., 2011) has been practiced. Catalase and Olfactory Co-Receptor (Rfer Orco) down regulation in *Rhynchophorus ferrugineus* has been achieved in near past (Al-Ayedh *et al*., 2016; Soffan *et al*., 2016).

The purpose of this investigation was to evaluate parathyroid insecticide resistance level in *R. ferruginous* population in association with expression of P_{450} gene and to enhance the effectiveness of this pesticide by knocking down the expression of resistance P450 gene. This was achieved via injection of dsRNA to the thorax region of *R. ferruginous.*

MATERIALS AND METHODS

Collection and rearing of R. ferruginous

Adults, larvae and pupae of *R. ferruginous* were collected from four provinces of Pakistan, Punjab (31.1704° N, 72.7097°E) Baluchistan (28.49° N, 65.0958° E), Sindh (25.8943°N, 68.52°E) and KPK, 34.95°N, 72.331°E) from field and various palm orchards of the public and private sectors and rearing was done at Integrated Genomics, Cellular, Developmental and Biotechnology Laboratory (IGCDB), Department of Entomology, University of Agriculture, Faisalabad. Collections were made with the help of pheromone traps as well as handpicking and transferred into insect rearing boxes (15cm × 10cm × 5cm) for rearing and bioassays purposes. Laboratory susceptible (SG7-without pesticides exposure) and resistant (RG6-contiuously exposed to pesticides for 6^{th} generations (G6) population of *R. ferruginous* parathyroid insecticide were developed and maintained under laboratory controlled conditions (25±5 °C, R.H 60±5%) with 12:12 (L: D) periods. Different concentration (10-500 ppm) of tested insecticide (Parathyroid) was applied against *R. ferruginous* of laboratory and field collected population along with control and mortality data after 24h were recorded regularly.

Gene expression studies

Total RNA isolation from *R. ferruginous* samples collected from fields as well as laboratory susceptible and resistant population was done by using isolation kit (Promega WI., USA). Picodrop-200 was used to measure the quality and quantity of the RNA. The cDNA synthesis was doneusing kit (iScript synthesis kit, CA, Bio-Rad, USA). Expression of the gene P450, beta actin and gfp was determined according to the protocol of (Ahmad *et al*., 2013a; 2014). The primers for Beta actin, P_{450} and gfp (control) genes are shown in Table I. Gel electrophoresis on 1.5% gel stained with ethidium bromide was carried out in 1% TAE buffer. There were five replicates consisting of 10 insects including three technical replications.

Construction of double stranded RNA (dsRNA)

Double stranded RNA specific for P450 gene (mono-oxygenase) was synthesized according to the manufacturer protocol through RNAi-kit (Austin, TX) following the methodology of Al-Ayedh *et al*. (2016). Using reverse and forward primers with T7 promoters designed for P450 gene were amplified to get339 bp from the cDNA synthesized (Table I). There were 35 cycles consisting of 72 °C for 60 sec, 55 °C for 45 seconds and 94 °C for 30 seconds with 10 minutes elongation steps at 72 °C for the PCR amplifications.. PCR amplified product was sent for purification and sequencing to Macrogen for verification of the desired sequence. P450 ds RNAwas generated and then suspended in the DEPC water and quantified using Nanodrop (Pico-200). Agarose gel electrophoresis (1.5 %) was performed for the verification of amplified genes. . The concentration of tested genes used in RNAi experiment were made up to (1µg/uL in ddH20) and kept at -20 °C. The same protocols and methods were used for constructing of the double stranded RNA for green fluorescent protein (gfp) employed for control.

Induction of RNAi in R. ferruginous

P_{450}-dsRNA was micro-injected at a concentration of 0.5, 1.5, 2 and 3 ug/insect in the thoracic region of 5^{th}, 10^{th} instar larvae and adult of *R. ferruginous* and observed for a times period of 4h-48h (Fig. 1). For the purpose of negative and positive control, DEPC water and dsGFP were used for the microinjection. These treated insects were then dissected 24-48h after application and forgut, midgut and hindgut were extracted for further study. Three replications were made with ten larvae per replication. Treated and control larval population were placed in the cups and allowed to feed on the artificial diet.

Gene knockdown assessment through qRT-PCR

Knockdown effect for P450 gene was studied through PCR. For each test sample, about 25µl reaction mixture was prepared consisting of RNA free water (8.5 µl), cDNA template (2 µl), and primers (1µl reverse primer and 1µl forward primer) including 12.5µl PCR mix. RT-PCR (real time) was done according to Ahmad *et al*., 2013, 2014.

Table I. Genes and primers used in gene expression and RNAi study.

Purpose	Gene name	Primers sequences used
dsRNA Synthesis	P450-2 Forward	5′- TTACTGGGGCGATCTTCACG-3′
	P450-2 Reverse	5′- TGAAGCGTTCCAAGGTCTCC-3′
	β-Actin-2 Forward	5′-AAAGGTTCCGTTGCCCTGAA-3′
	β-Actin-2 Reverse	5′-ACCACAAAGCTTCCATACCCA-3′
	P450-T7 Forward	5′-TAATACGACTCACTATAGGGAGATTGACCTTGAGGACGGAATC-3′
	P450-T7 Reverse	5′-TAATACGACTCACTATAGGGAGATTGACCTTGAGGACGGAATC-3′
	dSGFP-Forward	5′-TAATACGACTCACTATAGGGAGAAAGGGCGAGGAGCTGTTCACCG-3′
	dSGFP-Reverse	5′-TAATACGACTCACTATAGGGAGACAGCAGGACCATGTGATCGCG-3′

Table II. The probit analysis showing Resistance Ratios (RR) in Lab R(G6)= laboratory resistant, FP= Field resistant population from Punjab, FK= Field resistant population from KPK, FS= Field resistant population from Sindh and FB= Field resistant population from Baluchistan of RPW through employing 0-, 10-, 50-, 100 plus 500 ppm insecticides concentrations.

Pop.	n	LC50	Lower	Upper	Slope	Intercept (Log10)	SD	SE	R^2	Chi-test(χ2)	DF	RR
Lab S(G7)	150	1.991183423	0.85078609	4.660174261	0.812	0.256	1.231	0.188	0.868	0.999	12	-
Lab R(G6)	150	355.6143702	162.328689	779.0463977	0.709	1.811	1.410	0.174	0.895	0.640	12	178.5945
FP	150	14.58985924	8.39388539	25.35941139	1.091	1.261	0.917	0.122	0.865	0.948	12	7.32723
FK	150	8.5657616	3.3274262	22.05075859	0.587	0.546	1.704	0.210	0.809	0.993	12	4.301845
FS	150	30.9031022	15.8941465	60.08512152	0.826	1.198	1.211	0.147	0.469	0.009	12	15.51997
FB	150	3.949345446	1.63997052	9.510737691	0.683	0.413	1.464	0.195	0.863	0.999	12	1.983416

PCR condition were used as 95°C for 30 seconds followed by the other cycles at 95 °C for 5 seconds and lastly at 60 °C for 30 seconds. Analysis of the melting curve was done by the final elongation step of 10 and 15 minutes at 95°C and 60°C respectively with three replications. Beta actin expression was used as standard to standardize the P_{450} expression by following the 2-ΔΔCt procedure of Livak and Schmittgen (2001).

Western blot assay

Protein was extracted from dsP450 treated and control *R. ferruginous* samples according to prescribed procedure (Ahmad *et al*., 2013b). Transverse electrophoresis was used for the blotting on PVDF (Perkin Elmer) membrane at 4°C at 100V in a glycine buffer (0.192 mM) and Tris base (0.025 mM). At room temperature, immune labeling was implemented. Tween 20 was used for the incubation of PVDF membranes for 1 hour. Membrane washing was done with Tween 20 (0.05%). Membranes were incubated with laboratory prepared polyclonal antibodies (*Anti-P450 1/4000)* for two hours and then diluted with Tween 20. PBS Tween 20 (0.05%) was used for washing (4x5 min. and 1x15min.). Chemi-luminescent reagent kit (Thermo Fisher) was used to express the labeling in the dark room. Light films (Kodak Biomax) were used to expose the membranes.

Application of pesticides

R. ferruginous were reared and maintained in cages box and fed on artificial diet for a period of two hours and then transferred to other cages. Efficacies of the dsRNA were assessed in the *R. ferruginous* applied with different concentrations (0, 0.5, 1, 1.5, 2 and 3µg/insect). The plates were soaked in the insecticides solution for 30 seconds in acetone and then air dried about 1h prior to insect placement according to the methods of Killiny *et al*. (2014). The cypermethrin at dosage rate of the 0.02 ng Al/ml (acetone) were used. The mortality data was recorded after every 24 hours. For P_{450}-dsRNA, ds-RNA-gfp was implemented as control treatment. There were five replications having five insects from RPW tested population. Each population was applied with four treatments such as RNase free water, cypermethrin treated discs, dsRNA-P_{450} treated RPW and cypermethirn.

Statistical analysis

Collected data were analyzed through ANOVA and DMR (Tukey's Multiple Range Test) used for mean's comparison at 5% significance level by SAS18. Means were expressed as standard error (SE). Insecticide resistance and activity of the P_{450} was examined through Probit analysis. Student-T significant test was used to find the comparisons among the treatments and genes expression at 5% significance level.

RESULTS

Evolution of resistance in R. ferruginous

Comparative resistance development was studied in Laboratory susceptible, laboratory resistant and four field populations (FP, FK, FS and FB) collected from four provinces {Punjab (P), Sindh (S), Baluchistan (B) and KPK (K) the Pakistan (Table II). The observed resistance ratio (RR) for laboratory resistant population of generation (G6) was 178.5945 fold as compared to laboratory susceptible generation (G7). The field populations of Sindh (FS) and Punjab (FP) depicted 15.5 and 7.32 fold more resistance whereas resistance ratio of field population of KPK (FK) and Baluchistan (FB) population was 4.3 and 1.98 fold more as compared to laboratory susceptible ones.

Fig. 1. Demonstration of the injection of dsRNA in the 5th, 10th instar larvae and adults of *R. ferruginous*.

Expression of cytochrome P_{450} gene in field collected R. ferruginous *population*

Through RT-PCR, Cytochrome P450 gene expression was studied in larval and adult population collected from different places before the application of dsRNA-P_{450}. Adult male (M), female (F) and Last stages of larval instars showed maximum expression of P_{450} gene expression as indicated by band intensity. Bands intensity in gel for various larval stages has the expression of P450 gene with increasing trend from L1 to L10. The expression of P450 was highly enhanced in larval stages L7-L10 as compared to L1-L3 as compared to constant expression of B-actin reference gene (Fig. 2).

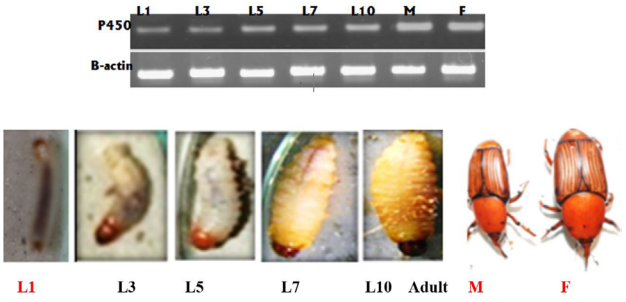

Fig. 2. P_{450} expression through PCR in several *R. ferruginous* larval instars (L1-1st instar, L3- 3rd instar, L5- 5th instar, L7- 7th instar and L10- 10th instar), male (M) and female (F).

Gene knockdown by larval injection with dsRNA

Infusion of larvae (10th instar) with dsRNA-P_{450} at different concentrations exhibited significant knockdown of P_{450} gene. After 24-48h of dsRNA-P450 injection, the knockdown effect for P450 was enhanced as dose rate increased from 0-3 µg/insect (Fig. 3). The high intensity of amplified bands was observed in the foregut of L10 with H20 and 0µg / insect (Wells 1-2) while the bands intensities was reduced gradually with increased doses showing down regulated expression of P450 gene in *R. ferruginous* (Wells 3-7). The 40-50 percent larvae was found dead after 48h and 10 percent larvae was found abnormal with passage of time as shown in Fig. 3)

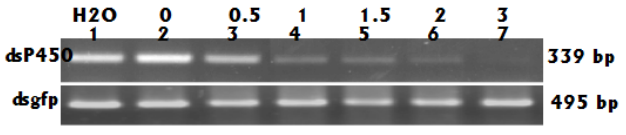

Fig. 3. P_{450} down regulation in 10th larval instars (L10) and its knockdown influence via microinjection of dsRNA Conc. (µg/insect).

Expression of P_{450} gene proteins after dsRNA- P_{450} treatment

The ds-RNA application in *R. ferruginous* (male and female) was confirmed by western blot analysis. The controlled palm weevil, *R. ferruginous* exhibited maximum amplification of P450 expression. When *R. ferruginous* was treated with different concentrations of dsRNA-P_{450}

(0.5µg, 1µg and 3µg) and constant doses of dsRNA-gfp (control), a decreasing expression of P_{450} gene was observed. The activity of P_{450} was decreased tremendously by increasing the concentration of applied dsRNA-P_{450}. The complete cytochrome P_{450} down-regulation was achieved when 3µg/ insect concentration of dsRNA-P_{450} was injected to the body of *R. ferruginous*. Although 0.5 µg/insect concentration of dsRNA-P_{450} suppresses the P_{450} gene but no significant results were noted. It was observed that target P_{450} expression levels and dsRNA treated proteins expressions are directly correlated (Fig. 4).

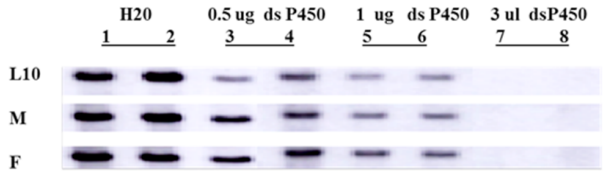

Fig. 4. Expression of P_{450} proteins by using ant-P_{450} antibodies.

Down regulation of P_{450} gene induced by dsRNA-P_{450} treatment through qRT-PCR

The expression was also studied with qRT-PCR after the application of dsRNA-P_{450} and dsRNA-gfp. The treatment with ds-RNA-P_{450} induced reduced expressions of P_{450} gene at 1-2 ug/individual. Each treatment sample of ds-RNA-P_{450} indicated positive correlation as down regulation of P_{450} expression with increased dose rate (1-2 ug/individual). The target gene P450 expression was investigated in ds-GFP treated insect samples used as control treatments. The relative gene expression of P450 in *R. ferruginous* exhibited significant down regulation (0. 2-0.5 *P<0.05) compared with dsRNA-GFP control treatments (1 *P<0.05). The dsRNA P_{450} employed with 2µg/larva displayed maximum down regulation (0.03-0.1 ***P<0.005), compared with as treated with 1µg/adult or larvae (0.2-0.3*P<0.05). But the down regulation of P450 was higher in larva (0.03-0.2) as compared to adults (0.3-0.4). The control gene Beta actin demonstrated constant expression level in all dsP450 and dsGFP treated insect (Fig. 6). Statistically no significant differential gene expression was recorded in reference gene Beta-actin. The results proved that the dsRNA-P_{450} was found target specific and induced maximum mortality.

Down regulation of Cytochrome P_{450} increased insecticide susceptibility in RPW

The effectiveness of cypermethrin against the different laboratory susceptible, resistant, and Punjab field collected larvae, male and female (SL, SM and SF; FL, FM and FF and RL, RM and RF) population of *R. ferruginous* were assessed. Its effectiveness was investigated before and after the application of dsRNA-P_{450} (Fig. 5). *R. ferruginous* Punjab and laboratory resistant populations exhibited minimum susceptibilities for cypermethrin mixed in artificial diet at LD_{50} recommended dose rates. RNase-free water treatments were used as control treatments. The pesticide treated SL, SM, SF, RF and RL populations' showed more significant (P<0.01) relationship and mortality was (Susceptible 84±3, 75±2, 73±6), (Field 63±4, 60±2, 55±5), (Resistant 38±1, 33±1, 32±2) respectively, comparing with H2O treatment pool of RPW (susceptible 11±4, 11±3, 8±2), (field 12±4, 11±2, 13±2), (resistant 6±2, 9±3, 8±1). When these population were treated with dsRNA P450 at least 12h before pesticide treatment, "Susceptible, Field and Resistant population of *R. ferruginous* showed significant interaction (P<0.005) and mortality was increased (susceptible 98±6, 95±2, 91±4), (field 90±2, 83±6, 81±7), (Rresistant 78±4, 73±4, 72±3), respectively) after the application of dsRNA-P450 (Fig. 7).

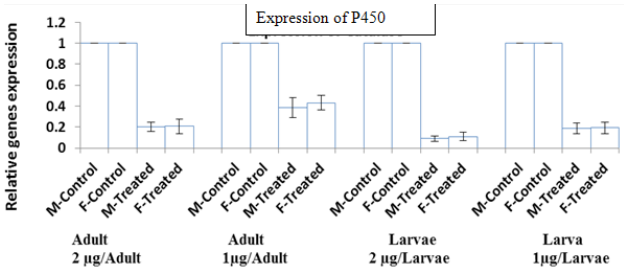

Fig. 5. Expression of P450 gene through qRT-PCR in male, female and larva of *R. ferrugineus*. (*P<0.05, **P<0.01 and ***P<0.005).

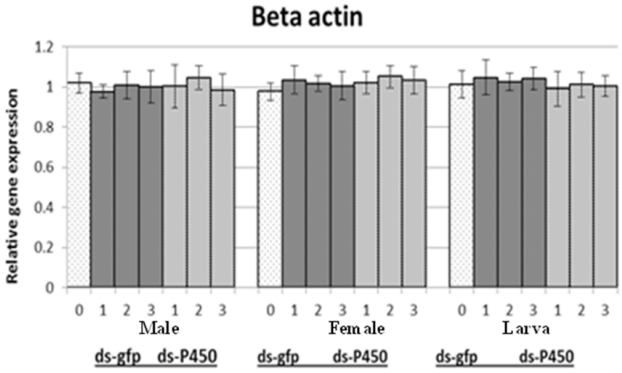

Fig. 6. Beta-actin expression in *R. ferrugineus* larvae (10th instar and Male and Female from left to right) after the application of dsRNA-gfp and dsRNA-P_{450}

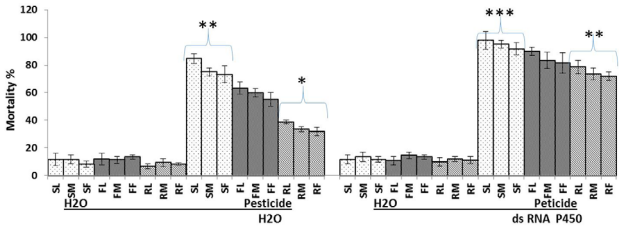

Fig. 7. Mortality percentage of *R. ferrugineus* population in response to Pesticide (Cypermethrin) and ds RNA P450 application. SL= "Susceptible larvae"- SM= "Susceptible male"- SF= "Susceptible female"- RL= "Resistant larvae", RM= "Resistant Male"- RF= "Resistant Female"-, FL= Field larva, FM=Field Male and FF=Field Female (Field population of Sindh province was used. Mortality was observed after 48h of treatment. (*P<0.05, **P<0.01 and ***P<0.00

DISCUSSION

Various genes that play an important role in the growth and development of *R. ferrugineus* are diverse in chemical composition and enzymatic properties. In *R. ferrugineus*, glycoside hydrolase 18 (GH18), playing an important role in the chitin synthesis of mature eggs of *R. ferrugineus* (Abdel-Banat *et al*., 2018).

According to Timmons and Fire (1998) feeding or micro-injection are the key methods associated with the novel RNA-interference expertise and its utilization for arthopodal control in the field conditions (Zhou *et al*., 2008; Rangasamy and Siegfried, 2012). These techniques have both benefits in addition to limitations. Passionate training is requisite in micro-injection technology comprising injection point, needle size for efficacious dsRNA penetration in test insects and appropriate calibration and optimization is needed for assortment of volume (Yu *et al*., 2013). The dsRNA-P_{450} delivered through micro-injection reduces adequacy for the induction of RNAi (Hunter, 1999). Potency of the double stranded RNA is diminished inside the gut of insect body due to harsh environmental conditions (Rajagopal *et al*., 2002; Surakasi *et al*., 2011) making it difficult for quantifying the amount of dsRNA. In the present study, *R. ferrugineus* was injected with dsRNA-P_{450} by micro-injection via ventral micro-inoculation to suppress the Cytochrome P_{450} gene in RPW.

The current work was supported by the previous work done to suppress a number of genes in insect pests including Blattodea, Hymenoptera, Neuroptera, Dictyoptera, Orthoptera, Diptera, Lepidoptera, Hemiptera and Coleoptera. These orders have been showing amenability to RNAi mediated down regulations of target genes (Tomoyasu *et al*., 2008; Bellés, 2010; Mao *et al*., 2010; Wuriyanghan *et al*., 2011; Cheng *et al*., 2012). Moreover, the Cytochrome P_{450} provides gossypol resistance to insects and is also a pesticide detoxifying gene. Turner *et al*. (2006) observed that the reduction in the mRNA in *E. postvittana* is induced by highly concentrated (4000 ppm) dsRNA but it doesn't cause mortality. Insect disease resistance against *D. citri* is supported by the metabolic mechanism which is enhanced by over-expression of gene (cytochrome P_{450}) and detoxifying enzymes (Tiwari and Siddiqi, 2012; Tiwari *et al*., 2011a, 2011b; Killiny *et al*., 2014), *Helicoverpa armigera* was subjected to dsRNA down regulation of *CYP6AE14* gene and retarded growth was observed (Mao *et al*., 2007), cytochrome P*450* monooygenase genes suppression was also noted in *Aphis gossypii* (Peng *et al*., 2016). Catalase and Olfactory Co-Receptor RferOrco down regulation in *R. ferrugineus* has been done to control this invasive pest (Al-Ayedh *et al*., 2016; Soffan *et al*., 2016).

El-Sheshay *et al*., (2013) proved that gene (P450) in the *D. citri* can be down regulated by a small amount of dsRNA (1µg/insect). However, in present work 3µg/insect concentration was sufficient to down regulate the Cytochrome P_{450} gene. The solicitation of dsRNA through micro-injection provided significant results showing shortened life span upon dsRNA in *D. citri* as compared to control. In some cases it causes 40-50% mortality of *O. nubilalis* (Wang *et al*., 2011). Double stranded-RNA target design is likewise pretty supportive in case of non-target arthropods in the fields (Whyard *et al*., 2009). In light of the fact, dsRNA specificity suggested a helpful invention to control the resistance in the *R. ferrugineus* as demonstrated in both exposed as well as resistant *R. ferrugineus* population. Contrarily, no impact of dsRNA-gfp was detected on mortality as well as Cytochrome P_{450} gene expression in control treatments. One of the most vital limitations of dsRNA is its difficult and costly for large scale. Moreover, different insect species lack the RNA interference machinery and concerned genes with high transcriptional rate so the impact of RNA interference can be evaded by them (Upadhyay *et al*., 2013). The dsRNA delivery in the *H. armigera* and *D. vergifera* introduced through plant mediated RNA interference shows positive correlations (Baum *et al*., 2007; Mao *et al*., 2007). The dsRNA could be applied in the absence of interferon regulated innate immunity for maximal effect of RNAi (Clemens and Elia, 1997). According to some researchers, the injection of bacteria with dsRNA provides a potential feasible delivery of dsRNA as well as engaging them to plants in foliar form with appropriate transfection reagent therefore, In future, dsRNA efficacy with any other microbial formulations or mixtures will be evaluated for the purpose of dsRNA application in field conditions against *R. ferruginous*.

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Statement of conflict of interest

The authors declare no conflict of interest.

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