Oxidative stress responses to sub-lethal dose of Cry toxin in the larvae of castor semilooper, *Achaea janata*

Research Project submitted to the Central University of Punjab

For the award of

Master of Science

In

Life Sciences (Specialization in Animal Sciences)

Kanika Singh

Department for Animal Sciences,

School of Basic and Applied Sciences,

Central University of Punjab, Bathinda

May, 2018

CERTIFICATE

This is to certify that the research project entitled **"Oxidative stress responses to sub-lethal dose of Cry toxin in the larvae of Castor Semilooper, *Achaea janata*" submitted by Ms. Kanika Singh (Reg. No. 16mslsas07)** for the partial fulfillment of M.Sc. Degree in Life Sciences (specialization in Animal Sciences), has been examined by the supervisor. The supervisor finds the work done by the candidate to be satisfactory and recommend that the report be accepted.

Dr. Krishna Chaitanya Rapalli
Assistant Professor, Department for Animal Sciences

DECLARATION

I declare that the project report entitled "**Oxidative stress responses to sub-lethal dose of Cry toxin in the larvae of Castor Semilooper, *Achaea janata***" has been prepared by me under the guidance of Dr Krishna Chaitanya Rapalli, Central University of Punjab, Bathinda. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institutes. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the references.

Kanika Singh
(Reg. No. 16mslsas07)
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Date:

DECLARATION

I declare that the project report entitled "**Oxidative stress responses to sub-lethal dose of Cry toxin in the larvae of Castor Semilooper, *Achaea janata***" has been prepared by **Ms Kanika Singh** bearing the (**Reg. No. 16mslsas07)**, under my guidance at the Department of Animal Sciences, School for Basic and Applied Sciences, Central University of Punjab.

Dr. Krishna Chaitanya Rapalli
Assistant Professor, Department for Animal Sciences

Abstract

Oxidative stress responses to sub-lethal dose of Cry toxin in the larvae of castor semilooper, *Achaea janata*

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Degree for which submitted: M.Sc. Life Sciences (specialization in Animal Sciences)

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Name of the School: Basic and Applied sciences.

Keywords: *Achaea janata*, *Bacillus thuringensis*, oxidative stress, antioxidants, resistance.

Development of synthetic insecticides to reduce the level of infestation led to deleterious effects on environment and human health. This lead to the development of ecofriendly pest management alternatives including *Bacillus thuringensis (Bt)*. Bt produce Crystal (Cry), Cytotoxic (Cyt) and Vegetative (Vip) proteins with insecticidal activity against different orders of lepidoptera. Of late, pest resistance against Bt is reported in countries.The reduced toxicity of Bt formulation from degradation by UV light, wash-off by rain, drying, temperature, and soil acidity as well as its chemistry. Further, insects sense pesticides through odorant receptors and move away quickly, there is always a possibility of a population of larvae to get exposed to sub-lethal doses of toxin which might exhibit variable effects and escape mortality and eventually generate resistance. Sub-lethal dose lead to the generation of oxidative stress in the insect and eventually scavenged by anti-oxidant enzymes. These stress responses would enhance our understanding of adaptations for survival and resistance development. The current study is an attempt to monitor the antioxidative responses at the transcriptional level upon sub-lethal exposure of Cry toxin in the larvae of an polyphagous pest castor semilooper, *Achaea janata.* prevalent in the Indian subcontinent.

Ms. Kanika Singh **Dr. R.K.Chaitanya**

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*I express my profound respect and regard to **parents** and **family members** for their moral support all throughout.*

Kanika Singh

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Abbreviations

Sr. no.	Full form	Abbreviation
1.	Bacillus *thuringenisis*	Bt
2.	Achaea *janata*	A. *janata*
3.	Catalase	Cat
4.	Superoxide dismutase	SOD
5.	Glutathione s transferase	GST
6.	Glutathione peroxidase	GPx
7.	Degree Celsius	°C
8.	Base pair	bp
9.	Crystal	cry
10.	Cytolytic	cyt
11	Tris acetate EDTA	TAE
12.	β –D 1 thiogalactopyranoside	IPTG
13.	Micro gram	µg
14.	Milli gram	mg
15.	Milli litre	ml
16.	Micro litre	µl
17.	Nano gram	ng
18.	Nano meter	nm
19.	Calcium chloride	$CaCl_2$
20.	Rotation per minute	rpm
21.	Non-template control	NTC

CHAPTER 1

(Review of Literature)

1.1 Castor and Castor Semilooper, *Achaea janata*

Castor (*Riccinus communis)* is an important non-edible oilseed crop cultivated in various parts of India, Mozambique, China and Brazil, responsible for 1.7 million, 68.9, 40.0 and 37.5 thousand tons, respectively (**http://www.fao.org/faostat**). Castor oil and its derivatives have many industrial uses, mainly in paints and varnishes for surface coatings, lubricants for aviation engines as a fuel which does not freeze at even 40 degrees of negative temperature, cosmetics, textile dyeing, nylon type synthetic polymers, resins, leather industry etc.. India exports 200,000 tonnes of castor meal to Japan and other countries which is used as biofertilizer. Its potential as a 'biofuel crop' is hindered by a number of biotic stress factors particularly, pests which degrade the crop quality and productivity (i.e., 973 kg of seeds per ha) (**Ogunniyi, 2006).**

The main defoliator of castor crop is castor semilooper, *Achaea janata*. Its occasional hosts include economically important plants like mustard, sugar cane, cabbage, rose, tomato, banana and tea (**Sujatha *et al.,* 2009**). *A. janata* L. (Noctuidae: Lepidoptera) is prevalent on castor during July–October in India. The older larvae are voracious feeders which often totally defoliate the plants during the outbreaks and compel the farmers to abandon the fields. Its life cycle consist of 4 stages i.e. egg, larvae, pupa, and adult. The larva consists of 5 instars and each instar is of 3 days. Larva is eclosed into pupa and finally adult emerges from the pupa (**Budatha *et al.,* 2007**). This pest has developed resistance to the chemical insecticides. Hence, various genetically-modified insect resistant Castor varieties are under development.

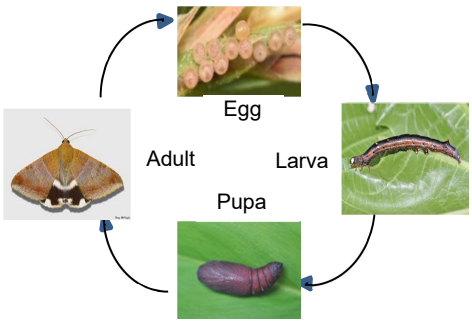

Fig.1. Life cycle of A. *janata*

1.2. Bt and its mode of action

Bacillus thuringiensis ssp*. kurstaki* is by far the most successful microbial preparation used for insect pest biocontrol. Crops such as cotton, maize, sorghum that are genetically modified with Bt have effectively countered the pests. The insecticidal activity (toxin) of *Bt* is contained within a very large structure called a parasporal crystal, which is synthesized during bacterial sporulation. When a parasporal crystal is ingested by a target insect, the Cry protoxin is activated within its gut by the combination of alkaline pH (7.5 to 8.0) and specific digestive proteases, which convert the protoxin into an active toxin with a molecular mass of approximately 60-70 kDa. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar before inserting into the membrane. Toxin insertion creates an ion channel, which leads to an excessive loss of cellular ATP. About 15 minutes after this ion channel forms, cellular metabolism ceases; the insect stops feeding within a few hours, becomes dehydrated, and eventually dies (in about 2 to 5 days). For Cry toxins, at least four different binding proteins/receptors have been described in different lepidopteron insects; a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (**Bravo *et al.* 2007)**

Using various criteria such as sequencing, serotyping, phage susceptibility and plasmid profiles 100 Bt subspecies have been identified and classified approximately. The Bt strains produce three types of insecticidal toxins, crystal (Cry) toxins, cytolytic (Cyt) toxins and vegetatively expressed insecticidal proteins (vip). Till 2012, a total of 229 cry toxins (Cry1Aa to Cry72Aa), 11 cyt toxins (cyt1Aa to cyt3Aa) and 102 vip toxins (vip1Aa1 to vip4Aa1) have been discovered. A total number of 342 Bt toxin genes are available for research to develop insect resistant GM crops. These toxins are biodegradable, highly insect-specific and doesn't affect human, plant and vertebrate populations **(Bravo *et al.,* 2015).**

1.3 Resistance to Bt

Recently, Bt resistance is being widely reported. Foliar spray of Bt formulation often results in the loss of toxicity from degradation by UV light, wash-off by rain, drying, temperature, and soil acidity as well as its chemistry. Further, insects sense pesticides through odorant receptors and move away quickly. Under these conditions, there is always a possibility of a population of larvae to get exposed to sub-lethal doses of toxin which might exhibit variable effects and escape mortality and eventually could develop resistance **(Melo *et al.,* 2016).**

One of the best-known and widely accepted resistance mechanisms to Bt is the reduction in binding of Bt Cry toxins to their specific midgut receptors i.e. Aminopeptidases N (APN), Cadherins, Alkaline phosphatases (ALP) and ABCC transporters due to mutations in these proteins which results in cross-resistance to Cry toxins sharing recognition of the altered receptor site **(Endo *et al*., 2017).** Recently emerging non-receptor related resistance mechanisms involving stem cell mediated regeneration in midgut epithelium, pathogen-response (REPAT) and arylphorin genes and detoxifying proteins/enzymes pose an additional threat to Bt crops, since they could affect steps common to all Cry toxins and would result in cross-resistance to a wide range of Bt insecticidal proteins **(Pachecho *et al.,* 2017).**

1.4. Insect stress responses

Throughout the life cycle, insects thrive in pathogen-rich environments, manage harsh weathers and are exposed to a number of allochemicals which elicit oxidative stress responses and counter responses by the complex systems of detoxification/antioxidant enzymes. Insects exposed to different stress conditions are known to generate differential responses. Midgut cells exposed to Cry-toxin induced oxidative stress are protected from the damage by numerous detoxifying enzymes including superoxide dismutases (SODs), which transform superoxide anions into hydrogen peroxide. Peroxidases (eg. Catalase and Glutathione peroxidase) detoxify

hydrogen peroxide into water molecule. Redox enzymes (eg. Glutathione-S-transferase) maintain cellular redox homeostasis **(Pavani *et al.,* 2015**). Elucidation of these stress responses would enhance our understanding of the key phenomena like adaptations for survival and resistance development. The current study is carried out to monitor the antioxidant gene changes at the transcriptional level upon sub-lethal exposure of Cry toxin in the larvae of castor semilooper, *A. janata*.

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CHAPTER 2

(Methodology)

2.1. Rearing and maintenance of *A. janata* larvae

A. janata were obtained from the fields with no prior exposure to any *Bt* pesticide and were reared in the laboratory conditions. The larvae were reared on sterile castor leaves and raised for three generations at 27 ± 2°C under a 14:10 h (light:dark) photoperiod and 60-70% relative humid conditions.

2.2. DOR- Bt1 treatment

DOR Bt-1 formulations consists of cry1 (cry1Aa, cry1Ab, and cry1Ac) and cry2 (cry2Aa and cry2Ab) genes with broad-spectrum potential against lepidopteran, and dipteran insects (Reddy et al., 2012). The reported IC_{50} value of the formulation is 247.52 µg/ml for a 3rd instar larvae **(Vimala Devi and Sudhakar, 2006)**. One tenth of IC_{50} value was used as a sub-lethal dose (i.e., 24.75 µg/ml of water) in the present study. Toxin-coated castor leaves were fed to the triplicate insect larvae groups (n=50 each) were maintained. Each group was continuously exposed to toxin-coated castor leaf discs (145 cm^2; 0.170 µg/cm^2) till 72 h. Control larvae were maintained on water coated leaf discs.

2.3. Midgut tissue extraction

Both the control and treated larvae were narcotized on ice and following which an incision was made with the help of sterile blade in the abdominal region of the larvae and extended through its length. The midgut was isolated, rinsed in sterile PBS and transferred immediately into Trizol reagent containing tubes.

2.4. RNA isolation from the midgut

100 µg of midgut tissue was homogenized in 1 ml of TRI Reagent (Sigma chemicals) follwed by addition of 200 µl chloroform and incubation for 15 min at room temperature. The mixture was centrifuged at 12,000 g for 15 min at 4 ºC. The upper

aqueous phase was collected to which 500 µl of isopropanol was added, mixed and incubated at room temperature for 10 min. The RNA pellet was collected post centrifugation at 12000 g for 20 min at 4 ºC. The pellet was washed thrice with 75 % ethanol, air dried and dissolved in 20 µl of diethyl pyrocarbonate (DEPC) treated water and immediately used.

2.5. Total RNA integrity

RNA integrity was checked using Nanodrop (ND-1000) spectrophotometer. A solution with an absorbance at 260 nm of 1 contains ~ 40 µg of single stranded RNA/ml. Using this, the concentrations of various RNA samples was calculated. The absorbance of the sample was also monitored at 280 nm (A280) to check for any protein interference. The purity of the sample was determined by calculating the ratio of A260/A280.

For agarose gel electrophoresis, the RNA sample (1 µg) was mixed with 12.5 µl of formamide, 2.5 µl of10 x formaldehyde gel buffer [0.2 M MOPS, 80 mM sodium acetate and 10 mM EDTA (pH 8.0)] and 4 µl of formaldehyde in a total volume of 25 µl. The mix was denatured at 65° C for 5 min followed by snap cooling on ice for 2 min. To this mix, 2.5 µl of gel loading dye (50 % glycerol, 1 mM EDTA, 0.25 % bromophenol blue and 0.25 % xylene cyanol) was added and gel loaded. The RNA samples were electrophoresed on 1 % agarose-formaldehyde denaturing gel. The electrophoresis was carried using 1 x formaldehyde gel buffer at voltage 5V/cm^2 until the dye reached the end of the gel. The EtBr stained gels were visualized under UV-transilluminator and analyzed using UVP-gel documentation system.

2.6. First strand cDNA synthesis

The first strand cDNA was synthesized using 1 µg of total RNA. Total RNA, 1 µl of oligo $(dT)_{20}$ (50 µM), 1 µl of dNTP (10 mM) mix and DEPC water were added to make a final volume of 10 µl in a PCR tube and incubated at 65 ºC for 5 min. To this tube, a

mix of 2 µl 10 x RT buffer, 4 µl of $MgCl_2$ (25 mM), 2 µl of DTT (0.1 mM), 1 µl of RNase OUT™ (40U/µl) and 1 µl of Superscript™ III RT (200U/µl) was added to make a 20 µl reaction mixture. The mixture was incubated at 50 ºC for 50 min and terminated at 85 ºC for 5 min. To this, 1 µl of RNase H (2U/µl) was added and incubated at 37 ºC for 20 min. The cDNA synthesized was stored at -20 ºC till further use.

2.7. Cloning of partial fragments of SOD, Catalase, GPx & GST

Based on the accession numbers available on the Pubmed database () specific primers were designed (using Primer BLAST) for partial amplification of SOD, Catalase, GPx, GST. The lists of forward and reverse primers used for the study are given below (Tab.1.). The cycling conditions for PCR amplification are initial denaturation at **95 °C** followed by denaturation at **95 °C** annealing at **60 °C** and extension at **72 °C for 35 cycles** rS7 gene was used as internal control. No template control was set up to monitor contamination and primer-dimer formation to eliminate any false positive results. The PCR products were cloned into p-GEM-T vector.

Gene	Forward primer	Reverse primer
SOD	CGCACTTCAACCCCACGAAGT	GCGTGTACTACGAGAGTGCGG
CAT	GATGGATACAGGCATATGAACGGTTATG	GTCACCCTTGGCAATGGCATTATAC
GPx	GCCGCGTATAACGGTCATGTG	GGTCTAGATCCAGGCAATCCAGAAG
GST	CGTGGCTTGCTGGTGATGAAG	AGGCACCTAAAAGCTCAACTCCTG

Tab.1. List of forward and reverse primers used for cloning of partial CDS of *SOD, CAT*, *GPx, GST*.

2.8. Agarose gel electrophoresis of DNA

The amplified DNA was electrophoresed on 0.8 % agarose gel polymerized using 1 x TAE [40 mM Tris-acetate and 1 mM EDTA (pH 8.0)]. The electrophoresis was carried

using the 1 x TAE buffer at voltage 5V/cm2 until the dye reached ¾th of the length of the gel.

2.9. DNA extraction from the agarose gel

The gel piece corresponding to the amplified DNA was excised and transferred to a microcentrifuge tube. To this, 3 volumes of Buffer QG was added (Buffer QG contains guanidium thiocyanate) that solubilizes the agarose gel slice and provides appropriate conditions for binding of DNA to the silica membrane of the spin column. Following binding, the column was washed with 0.75 ml of PE buffer [10 mM NaCl, 50 mM MOPS (pH 7.0) and ethanol phase] that removes any unwanted impurities such as salts, enzymes, unincorporated nucleotides, ethidium bromide etc. The DNA was eluted with 50 µl of buffer EB [10 mM Tris-HCl (pH 8.0) with 1 mM EDTA].

2.10. Ligation of amplified DNA and the cloning vector

A reaction mixture of 6 µl of gel purified DNA (100 ng/µl), 3 µl of vector pGEM-T (55 ng/µl), 4 µl of 5 x ligation buffer, 1 µl of T4 DNA ligase (5U/µl) and 6 µl of nuclease-free water was set up. The ligation reaction was terminated after overnight incubation at 16 °C in the ligation bath. After ligation, the ligation mixture was transformed into DH5α competent cells. The transformation reaction was carried out at 42 °C for 90 sec. The mixture was plated onto LB agar plates containing ampicillin (100 µg/µl). Positive colonies were picked up based on blue-white screening.

2.11. Bacterial transformation

A single bacterial colony (DH5α) was picked from a LB agar plate and incubated for 16-20 h at 37 ºC. The colony was inoculated into 100 ml of LB broth. The culture was incubated for 3 h at 37 ºC with vigorous agitation and continuous monitoring of the growth. For efficient transformation, it is essential that the number of viable cells does not exceed 10^8cells/ml, which for most strains of E. coli is equivalent to an OD_{600} of ~

0.4. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 ºC. The pellet obtained was resuspended by in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM MgCl2 and 20 mM CaCl2). The cells were recovered again by centrifugation at 4000 rpm for 10 min at 4 ºC. and the pellet was resuspended freshly in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. At this point the cells were directly used for transformation. The vector with or without DNA insert were incubated with competent cells for an hour on ice and later cultured in 1 ml of LB broth for an hour at 37 ºC, centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 50 µl of fresh LB broth and plated on LB Agar for blue-white screening.

2.12. Blue-white screening

The blue-white screening allows for detection of recombinant bacteria. Cells transformed with vectors containing recombinant DNA will produce white colonies and those transformed with non-recombinant plasmids (i.e. only the vector) produce blue colonies. The method is based on the principle of α-complementation of the β-galactosidase gene. The host *E. coli* strain carries the *lacZ* deletion mutant (*lacZΔM15*) which codes for the ω-peptide, while the vector harbor the *lacZα* sequence which encodes the α-peptide of β-galactosidase,. Neither of the peptides is functional by itself. However, when the two peptides are expressed together i.e. when a vector containing the *lacZα* sequence is transformed into *lacZΔM15* cells, they form a functional β-galactosidase enzyme. The blue/white screening method is based on disruption of this α-complementation process. The vector carries within the *lacZα* sequence an internal multiple cloning site (MCS). This MCS within the *lacZα* sequence is where the foreign DNA is inserted thereby disrupting the gene that produces α-peptide. Consequently, in cells containing the vector with DNA insert, no functional β-galactosidase is formed. The presence of an active β-galactosidase is detected by addition of X-gal, a colorless analog of lactose that is cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form an insoluble blue product, 5,5'-dibromo-4,4'-dichloro-indigo. Blue colonies contain a vector with an

uninterrupted *lacZα* (without insert), while white colonies indicate the presence of an insert in *lacZα*.

2.13. Plasmid isolation

TheDH5α strain containing the recombinant plasmid was grown in LB-Amp broth (100 µg ampicillin per ml LB broth) for 14-16 h and the plasmid DNA was isolated. The cells were collected after centrifugation at 3,000 g for 10 min and suspended in 250 µl of P1 buffer [100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM EDTA, 0.2 % (w/v) BSA and 20 mg/ml RNase A]. To this suspension, 250 µl of P2 buffer [30 % polyethylene glycol (PEG 6000) and 3 mM NaCl] was added, and incubated at room temperature for 5 min followed by addition of 100 µl of buffer N3 (100 mM NaCl, 100 mM Tris-HCl pH 7.5 and 25 mM EDTA). The mix was centrifuged at 3,000 g for 10 min to separate supernatant from the pellet containing plasmid DNA and was loaded on QIAprep column. The column was washed with 750 µl of PE buffer [10 mM NaCl, 50 mM MOPS (pH 7.0) and ethanol phase]. The plasmid DNA was eluted with 50 µl of buffer EB [10 mM Tris-HCl (pH 8.0) with 1 mM EDTA]. The recombinant plasmids containing partial DNA sequences of SOD, GPx, GST and catalase were sequences and analyzed.

2.14. Quantitative PCR

Control and Cry-toxin treated midguts collected at different time points were dissected in ice-cold TRI® reagent and the total RNA isolation was carried out immediately. All total RNA samples were treated with DNase I prior to first strand cDNA synthesis. Reverse transcription was carried out with 0.5 µg total RNA using random hexamer primers and Superscript® III reverse transcriptase according to manufacturer's protocol. Based on the partial DNA sequences of antioxidant genes, the primer sets for qRTPCR were carefully designed. Standard curve for each gene was plotted with serial dilutions of respective primers and cDNA. Gene expression was assessed by SYBR green qRT-PCR (Applied Biosystems) in ABI7500 fast real-time PCR system

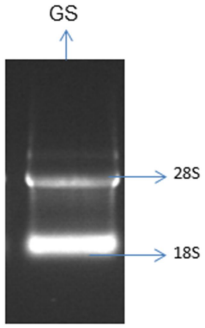
(Applied Biosystems). A 40-cycle two-step PCR was carried out in triplicates with 10.0 uL reaction volume containing the following components: 1.0 µL of cDNA template, 1.0 µL of forward and reverse primers each, and 5.0 µL of 2x master mix. Dissociation or melting curve analysis was performed for all the genes to check for specific amplification. The amplification efficiency was 95%-99% with slope of the curve ranging between -3.0 to -3.3. During each cycle of the PCR, fluorescence accumulation resulting from DNA amplification was analyzed and converted into cycle threshold (C_t) by the sequence detection system software (Applied Biosystems). Relative quantification results were normalized with conserved ribosomal protein S7 as endogenous control. Ct values were obtained from the exponential phase of PCR amplification. All the results are represented as change in the transcript levels relative to the reference values obtained for the control and were normalized to that of endogenous control gene (S7) C_t values using the $2^{-\Delta\Delta Ct}$ method **(Schmittgen and Livak, 2008).**

CHAPTER 3

(Results)

3.1. Quality control analysis of midgut total RNA

The total RNA from the midgut was isolated immediately after dissection as the gut tissue is rich in nucleases and proteases which may result in RNA degradation and eventually affect the quality and the quantity. The integrity of extracted ribonucleic acid (RNA) is assessed by gel electrophoresis and subsequent analysis of the ribosomal RNA (rRNA) band. Although ribosomal RNA integrity is not an accurate measure of mRNA quality, it is useful as a readily available indicator of the general state of the purified RNA. The electrophoretic rRNA profile of insects differs significantly from the standard benchmark since the 28S rRNA of most insects contains α and β fragments, which remain hydrogen-bonded together. Upon denaturation, the masking hydrogen bonds are disrupted, releasing two similar sized fragments that both migrate closely with 18S rRNA. Usually, the ratio of 28S to 18S in intact RNA is around 2:1 simply because 28S is around double the size of 18S and incorporates more dye. The resulting rRNA profile of 28S to 18S in the ratio 1:2 thus reflects the endogenous composition of insect rRNA and not degradation **(Winneback *et al*., 2010).** The total RNA concentration obtained from 100 mg of midgut tissue was 1.3 µg/µl and the 260/280 and 260/230 ratios are >2 indicating the purity of the RNA isolated (Fig.2.).



A.

Sample RNA concentration	1343.9 ng/µl
260/280	2.01
260/230	2.34

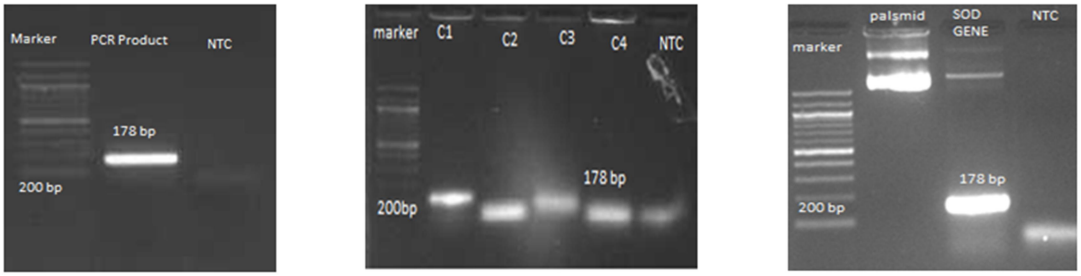
B.

Fig.2. Quality, purity and concentration of midgut total RNA. A) The image represents the qualitative estimation of isolated RNA from the gut of A. *janata*. The lighter band represents the 28s rRNA and darker band represents the 18s rRNA B) Quantitative estimation of RNA using Nanodrop spectrophotometer. (GS= gut sample).

3.2. Cloning of partial fragment of *SOD*

Based on the nucleotide sequence of *A. janata SOD* available on PubMed (KP939036), a partial fragment of 178 bp was amplified using specific primers and confirmed using Colony PCR and Plasmid PCR. The variations observed in the band size during colony PCR could be due to the amplification of various *SOD* isoforms. The nucleotide sequence was obtained by DNA Sequencer. BlastP analysis of amino acid sequence of the putative *SOD* gene showed the presence of a conserved Cu-Zn Superoxide dismutase superfamily (Fig.3.)

A.



B.

SOD PARTIAL CDS:

CGCACTTCAACCCCACGAAGTTGGACCACGGCGCGCCCGACGCCAGCATCCGTCACGTCGGAGATCC
GGCAACATTGAGTCGTATGGAGGCGTCACTAAGGTGTGCATCCAAGACTCCCAGATTTCCCTGGCCCCT
GGCACCAACAACATCATCGG**CCGCACTCTCGTAGTACACGCG**

AMINO ACID SEQUENCE

MEASLRCASKTPRFPWPLAPTTSSAALS

C.

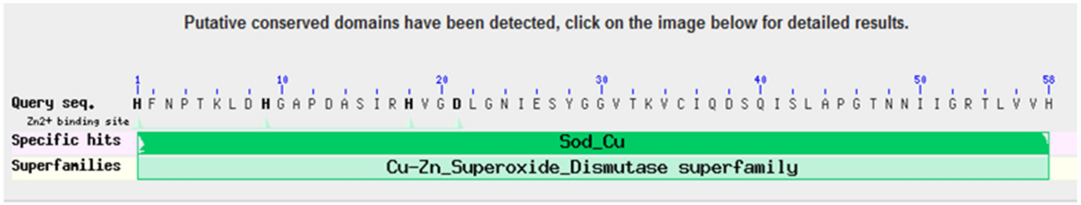
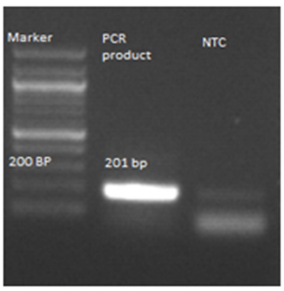


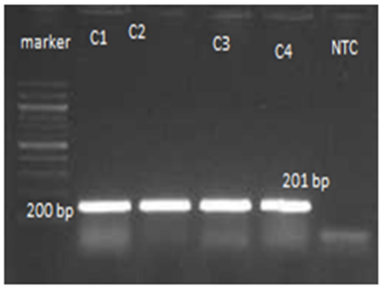
Fig.3. Cloning, confirmation and analysis of *SOD*. A) Agarose gel depicting PCR amplification of *SOD* using specific primers; Colony PCR for the same gene; Plasmid isolation and amplification was performed from the cultured colony of DH5α strain B) Partial CDS of *SOD* and its corresponding amino acid sequence (C) BLAST P analysis of aminoacid sequence of SOD .

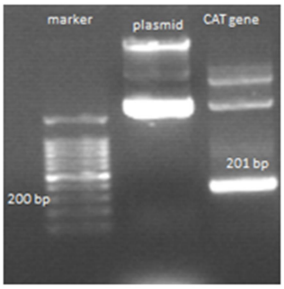
3.3. Cloning of partial fragment of *Catalase*

Based on the nucleotide sequence of *A. janata Cat* gene available on PubMed (KM063183), a partial fragment of 201 bp was amplified using specific primers and confirmed using Colony PCR and Plasmid PCR. The nucleotide sequence was obtained by DNA Sequencer. Blast P analysis of amino acid sequence of the putative *Cat* gene showed the presence of a conserved catalase-like superfamily domain (Fig.4.).

A.







B.

CAT PARTIAL CDS

GATGGATACAGGCATATGAACGGTTATGGATCACACACATTCAAGCTTGTGAATGCCCAAGGTGTCG
CTCACTGGGTGAAGTTCCACTTCAAGACCAACCAGGGTATTGAGAACTTACCAGTTGACAAAGCTAAT
GAGTTGGCTTCCTCTGATCCTGATTATGCTATTAGGGATTT**GTATAATGCCATTGCCAAGGGTGAC**

AMINO ACID SEQUENCE

MNGYGSHTFKLVNAQGVAHWVKFHFKTNQGIENLPVDKANELASSDPDYAIRDLNAIAKGD

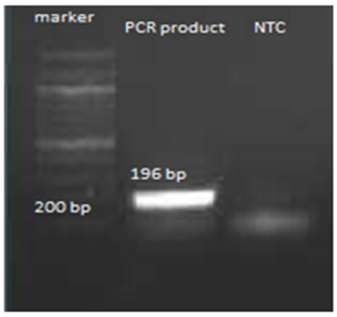
C.

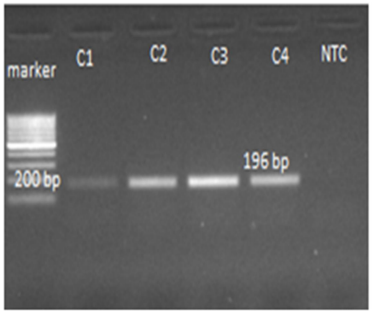
Fig.4. Cloning, confirmation and analysis of *Cat* A) Agarose gel depicting PCR amplification of *Cat* using specific primers; Colony PCR for the same gene; Plasmid isolation and amplification was performed from the cultured colony of DH5α strain B) Partial CDS of *Cat* and its corresponding amino acid sequence (C) BLAST P analysis of aminoacid sequence of CAT .

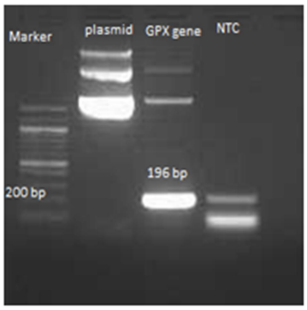
3.4. Cloning of partial fragment of *GPx*

Based on the nucleotide sequence of *A. janata GPx* available on PubMed (KP939037), a partial fragment of 196 bp was amplified using specific primers and confirmed using Colony PCR and Plasmid PCR. The nucleotide sequence was obtained by DNA Sequencer. BlastP analysis of amino acid sequence of the putative *GPx* gene showed the presence of a conserved Thioredoxin like superfamily domain (Fig.5.)

A.







B.

GPx PARTIAL CDS

GCCGCGTATAACGGTCATGTGTGTATCATCGTGAATGTTGCTTCAATGAGCGGCCTTGAGTGCCTCC
TTGCTTGTATTGAGGAACTTATACAAAGAGTAGCTGAAAAGGGATTATCTATTCTCTCATTCCCATGCA
ACTTCATTTTTCGATCTTTACCAAAGCATACGATTT**CTTCTGGATTGCCTGGATCTAGAC**

AMINO ACID SEQUENCE

MSGLECLLACIEELIQRVAEKGLSILSFPCNFIFRSLPKHTISSGLPGSR

C.

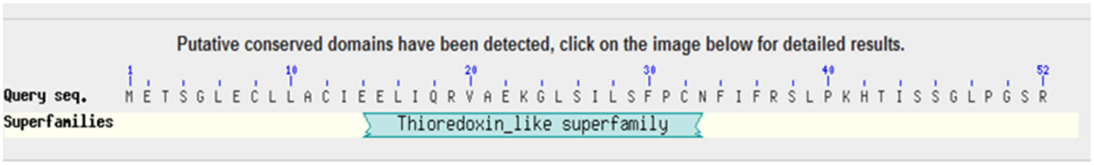


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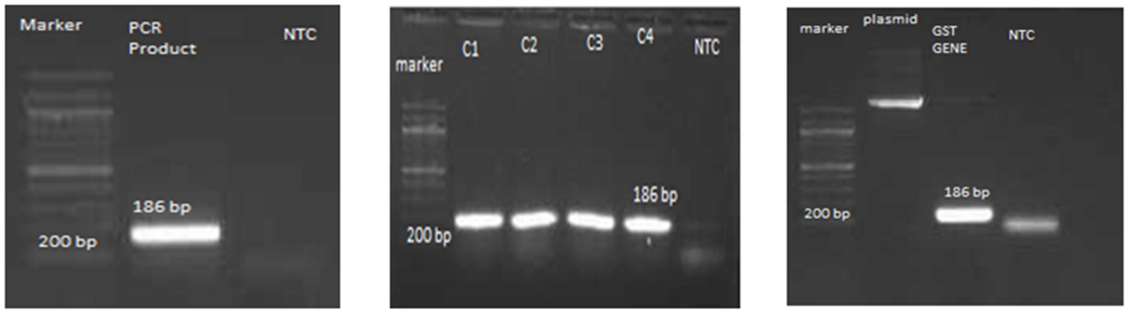
Fig. 5 Cloning, confirmation and analysis of *SOD*. A) Agarose gel depicting PCR amplification of *GPx* using specific primers; Colony PCR for the same gene; Plasmid isolation and amplification was performed from the cultured colony of DH5α strain B) Partial CDS of *GPx* and its corresponding amino acid sequence (C) BLAST P analysis of aminoacid sequence of GPx.

3.5. Cloning of partial fragment of *GST*

Based on the nucleotide sequence of *A. janata GST* available on PubMed (KM063184), a partial fragment of 186 bp was amplified using specific primers and confirmed using Colony PCR and Plasmid PCR. The nucleotide sequence was obtained by DNA Sequencer. BlastP analysis of amino acid sequence of the putative *GST* showed the presence of a conserved GST like superfamily domain (Fig. 6.)

A.



B.

GST PARTIAL CDS

CGTGGCTTGCTGGTGATGAAGTAACATTGGCAGATATTTGTTGCGTCAGCAGCATCAGTTCAATGAA
TGTGATTCTGCCCATTGATGAAAATAAGTACCCCAACATAACGGCCTGGCTTGATCGTTGTACTCAGT
TGGAGTATTATAAAACTAAGAACCAAC**CAGGAGTTGAGCTTTTAGGTGCCT**

AMINO ACID SEQUENCE

MNVILPIDENKYPNITAWLDRCTQLEYYKTKNQPGVELLGA

C.

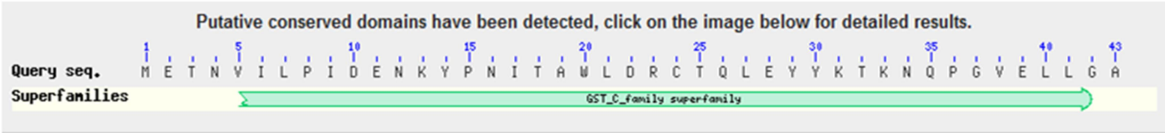


Fig. 6. Cloning, confirmation and analysis of *SOD*. A) Agarose gel depicting PCR amplification of *GST* using specific primers; Colony PCR for the same gene; Plasmid isolation and amplification was performed from the cultured colony of DH5α strain B) Partial CDS of *GST* and its corresponding amino acid sequence (C) BLAST P analysis of aminoacid sequence of GST .

3.6. Relative expression of antioxidant enzyme genes, *SOD, Cat, GPx, GST* during sub-lethal Cry toxin exposure

Compared to control, the antioxidant genes showed differential expression pattern upon sub-lethal Cry toxin exposure. The transcript levels of *SOD* remains unaltered although marginally diminished expression was observed at 48 and 60 h post treatment. Catalase mRNA levels showed non-significant decline from 12 – 60 h but thereafter reclined to normal levels (comparable to control) by 72 h post treatment. *Gpx* levels were very highly elevated (> 20-30 fold) at 48 and 60 h following exposure. *GST* levels exhibited a pattern similar to that of the *Cat* gene where the expression diminished till 60 h followed by retrieval to normal levels (similar to that of control). Overall, significant elevated levels of *GPx* in the midgut during sublethal exposure of Cry toxin to the insect larvae was the most prominent observation (Fig.7.). The C_t values obtained in triplicates for each sample are documented (Fig.8.)

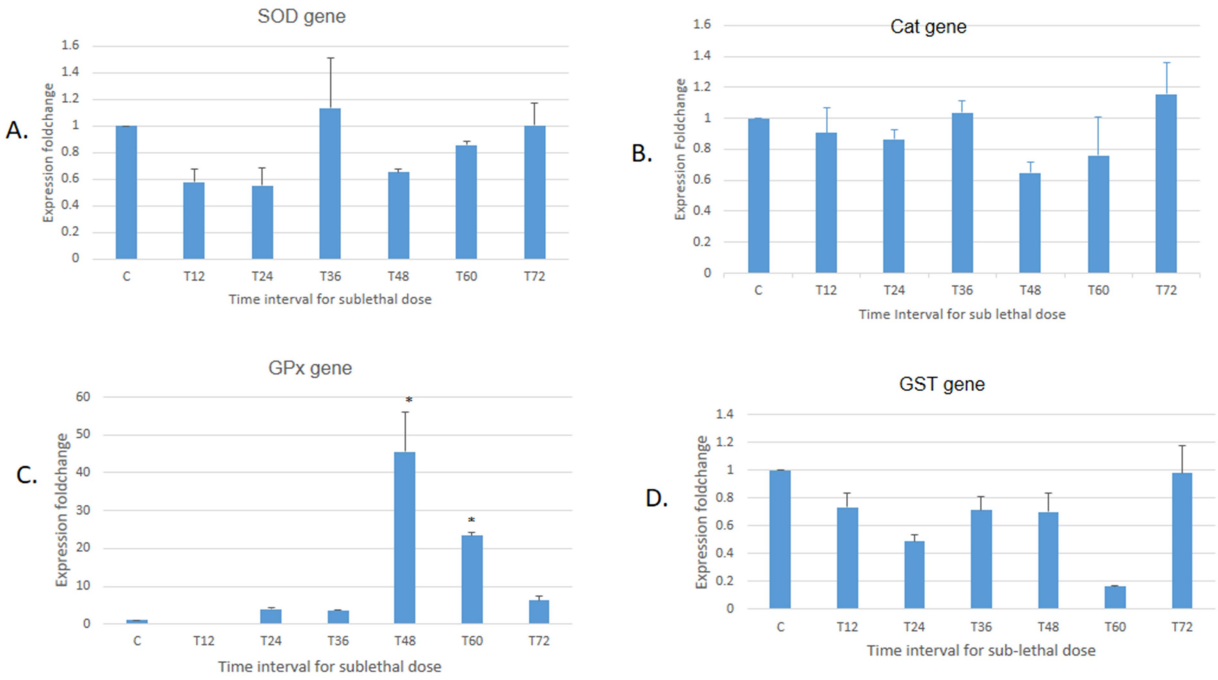


Fig.7. Relative expression of antioxidative genes upon sublethal Cry toxin exposure. X-axis represents time intervals C: Control, T12, 24, 36, 48, 60, 72 represent the samples collected post treatment in hours. Y-axis represents relative

expression of A) *SOD* B) *Cat* C) *GPx and* D) *GST.* All the experiment are done in triplicates and * signifies P value > 0.5

	RS 7	GST	GPx	SOD	Cat
C12	19.93725586	23.78487015	19.82914162	22.31977081	19.89628983
	19.51820374	23.98354149	19.79645729	22.17634506	19.66055489
	19.52838898	23.82338715	19.91617012	21.93806267	19.65234566
T12	19.51820374	23.88346672	27.1229229	22.88896751	19.95077133
	19.51820374	23.98865509	26.96811295	22.43122292	19.53385544
	19.55378723	23.6302433	27.10639763	22.82872772	19.46608353
T24	19.22770119	23.96995926	17.0686124	22.36096363	19.2923336
	19.09329033	24.07940483	17.14722443	22.78692055	19.34479141
	19.13810539	24.05467796	16.90994263	22.13372612	19.32912254
T36	19.55378723	23.8888855	17.52131241	21.73333168	19.54107857
	19.51820374	24.08227158	17.60822525	21.31010628	19.47537804
	19.81393814	23.95705414	17.88139534	22.62561607	19.60158157
T48	19.40589142	23.48401451	13.46715355	22.36687469	19.9822731
	19.13093567	23.73007202	13.57966518	22.10388374	19.90075493
	19.16145515	23.61472511	13.87067032	22.22696304	19.59873581
T60	18.97541237	25.39480019	14.331604	21.57657814	19.99578667
	18.91590881	25.41131783	14.20097446	21.58119011	19.14263916
	18.89722252	25.29070091	14.30111313	21.46164894	18.90450478
T72	19.35211182	23.05964279	16.69535637	21.58507347	19.08882523
	19.47752762	23.19978905	16.46659088	21.73694038	18.99740028
	19.0904808	23.35484695	16.48207664	21.76132965	19.12673187

Fig.8. C_t values in triplicates of the rs7 (internal control gene) and *SOD, Cat, GPx, GST*

CHAPTER 4

(Discussion)

The presence of SOD implies that superoxide free radical is produced in cell during normal metabolism. SOD scavenges superoxide free radicals to less toxic hydrogen peroxide. However, various forms of SODs including Fe/Mn SOD, Cu/Zn SOD 2, Cu/Zn SOD 3A, Cu/Zn SOD 3B are reported in insects and their expression levels vary with varied stress stimuli **(Dunning *et al*., 2013).** The partial fragment of SOD cloned in this study reveals similarity to multiple isoforms. Hence, the observation that the mRNA levels of SOD remain unaltered post toxin exposure highlights the necessity to monitor the expression of each of the isoforms of SOD after toxin exposure. Further, the transcript levels of catalase were relatively constant in toxin treated larvae. However, the GPx expression levels were significantly upregulated manifold. Intracellular H_2O_2 levels are tightly regulated by the activity of H_2O_2 metabolizing enzymes, Cat and GPx **(Baud *et al*., 2004).** Low levels of H_2O_2 triggers adaptive responses that eventually increase cell resistance to oxidants by enhancing the expression of antioxidant enzymes. On the contrary, high H_2O_2 levels are toxic which trigger generation of hydroxyl radicals in the presence of redox-active transition metal (**Martin and English *et al*., 2013).** Further, catalase is of particular importance when the clearance of H2O2 in high concentrations is warranted. The mammalian GPx enzymes are selenium-dependent and broadly classified into five categories: the cytosolic GPX, plasma GPX, gastrointestinal GPX, phospholipid hydroperoxide GPX and sperm nuclei GPX. However, PHGPxs reported in insects encode selenium-independent form. Recently, a selenium-dependent GPx was identified and characterized in *Rhodnius prolixus* (RpSeGPx). Moreover, silencing of this RpSeGPx did not alter the gene expression of catalase but it reduced the levels of the dual oxidase and NADPH oxidase 5 transcripts **(Mittapalli *et al*., 2006)**. Hence, it can be presumed that sub-lethal toxin exposure leads to low level hydrogen peroxide generation and involvement of selenium dependent GPx and this hypothesis needs to be further tested. The future direction of this work could be a) to monitor whether the activities of the antioxidant enzymes are in corroboration with the gene expression reported in the present study b) To perform antioxidant genes' RNAi-mediated knockdown studies to elucidate the specific role of each gene c) To clone and monitor the expression and activities of various SOD isoforms during sublethal

toxin exposure d) To focus on in- depth analysis of the contrasting/co-operative roles of Catalase and GPx in stress responses during sub-lethal toxin exposure e) Also, to clone and characterize the selenium-dependent and selenium independent GPx in the model of study **(Dias *et al*., 2016).**

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