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Expression of Heat Shock Protein (Hsp) Genes in the Stored Grain Pest, *Sitophilus oryzae* (L) (Coleoptera:Curculionidae)

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Abstract

Heat shock proteins (Hsp) are a family of proteins that help organisms to modulate stress response and protect them from environmentally induced cellular damage. They act as molecular chaperones, promoting correct refolding and preventing aggregation of denatured proteins.The expression of different Hsp genes are induced and modulated in insects in response to environmental inputs like abiotic stresses such as heat shock, ultraviolet radiation,chemical pesticides, as well as biotic stresses such as viruses, bacteria, fungi and other insects. In the present study, HSP_{60} gene of *Sitophilus oryzae* treated with acetone extract of the plant leaves of Glycosmis pentaphylla was down regulated compared to control.It indicates the development of stress condition in the treated insects due to plant extract treatment. So it could be integrated into pest management system.

Keywords: *Sitophilus oryzae*, *Glycosmis pentaphylla*, Hsp60 gene, gene expression, RT-qPCR

1. Introduction

Insects have been causing tremendous losses not only to the crops growing in fields but also to post-harvest commodities during storage. There is a continuous need to protect the stored products against deterioration, especially loss of quality and weight during storage. Chemical pesticides are most important among them. These result in serious health problems in non target organisms including man. Injudicious use of chemicals as a pest management method have lead to the development of resistance in insects towards them and have a high degree of residual effect due to their nondegradable nature (Dwivedi and Sonia Venugopalan, 1998) Plants may provide potential alternatives to currently used insect-control agents because they constitute a rich source of bioactive chemicals (Wink, 1993. Many plants are proved to be effective in controlling the stored grain pest *Sitophilus oryzae.*

Many environmental factors such as temperature, humidity chemicals and heavy metals (Gibbs, 2003; Macnair, 1997) affect the distribution of species in nature. Temperature variation act as a important selection force leading to genetic divergence among local populations (Angilletta, 2009) A particular set of proteins, called heat stress proteins (Hsps),that are expressed under stress conditions like exposure to heat, cold, metal ions, pesticides etc (Peter et al,1997).Hsps are seen in all living organisms, from bacteria to humans (Nevins, 1982). On the basis of molecular weight and homology of amino acid sequences, Hsp can be divided into several families including Hsp90, 70, 60, 40 and small Hsp (Feder and Hofmann, 1999) Hsp60 is mostly located in mitochondria of eukaryotic cells (Gatenby et al, 1991).

The plant products produce a stress condition in the insects and there is the expression of certain heat shock proteins (HSPs).Hsps are highly-conserved molecules (Richter et al, 2010). Expression of Hsps is altered when the cells are exposed to stress condition (Kiang and Tsokos, 1998).The heat shock response was first discovered in *Drosophila melanogaster* Meigen (Drosophilidae) (Ritossa, 1962). Hsp expression can vary with the physiological state of the organism and developmental stage (Feder and Hofmann, 1999).HSPs usually act as molecular chaperones that promote protein folding and assembly, and prevent the aggregation of denatured proteins or newly-synthesized polypeptides (Feder and Hofmann, 1999).

The present study was aimed to elucidate the expression of Hsp 60 genes in the rice weevil, *Sitophilus oryzae* treated with acetone extract of the plant leaves of *Glycosmis pentaphylla*

2. Materials and methods

Test insects

The pest, *Sitophilus oryzae*, was collected from stored rice from a local shop. Fresh rice was washed and dried in sunlight. This rice was taken in containers and the insects were transferred to it. Thus stock cultures were prepared. Holes were drilled on the container lid for permitting the passage of air. The culture was maintained at room temperature. For getting newly emerged adults, 100 insects, including both male and female, from the stock were transferred to fresh rice. They were allowed to lay eggs on fresh rice. Then after 2 weeks, they were removed, and the rice containers were kept undisturbed. On the sixth week, new insects began to emerge in the containers. These insects were used for further studies. The stock culture was cleaned by sieving once in five days. This helps to remove the food waste and faecal matter of the insects to avoid fungal attack.

Plant used for the study

Acetone extract of the plant leaves *Glycosmis pentaphylla* were prepared using the soxhlet apparatus.50 gm of powdered leaves of the plant was weighed and tied in a thin cloth and placed in extraction tube.500 ml acetone was taken in the glass flask and was boiled at 55°C continuously. Boiling was continued for six to eight hours till the extract become pale green.On completing the boiling, the extract was allowed to cool and stored in air tight containers for further use under refrigerated condition.The acetone extract obtained was treated as 100% concentration.

Treatment of adult insects

The effect of acetone extract was analysed by using residual film method. No.1 What man filter paper were cut in round shape and placed in the plastic containers. Sub lethal doses of the extract (3%) was applied to these filter papers using a micropipette and allowed to dry so that the solvent may evaporate completely.Then 100 gm of feed was weighed out and fifty newly emerged adult insects were placed in the containers so that each would get about 2gm of feed.

For each treatment control was also set up without applying plant extract.Solvent (acetone) alone was used in the control. Five replicates were kept for each treatment and its control. 100 mg of treated and control insects were homogenised in Trizol and taken for RNA isolation.

Isolation of Total RNA (Trizol method)

Total RNA was isolated using the total RNA isolation kit according to the manufacture instruction (Invitrogen – Product code 10296010). Addition of Trizol solution causes the disruption of cells and the release of RNA. Chloroform extraction following centrifugation, exclusively in the aqueous phase whereas proteins are in the interphase and organic phase. On mixing with isopropanol, RNA gets precipitated as a white pellet on the side and the bottom of the tube.

1ml of trizol reagent was added to the 100mg tissue sample and homogenized until it formed a fine paste. The contents were then transferred to a fresh sterile eppendorf tube.200 µl of chloroform was added and shaking was done vigorously for 15 seconds and incubated for 2-3minutes at room temperature, followed by centrifugation at 14000 rpm for 15 minutes at 4°C. The aqueous layer was collected and 500 µl of100% isopropanol was added. It was incubated for 10 minutes at room temperature and then centrifuged at 14000 rpm for 15 minutes at 4°C. Supernatant was discarded and pellet thus obtained was washed with 200 µl of 75% of ethanol (Merck). It was then centrifuged at 14000 rpm for 5 minutes at 4°C in a cooling centrifuge (Remi CM12). The RNA pellet was dried and suspended in TE buffer.

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Gene expression analysis by RT-qPCR

Total RNA was extracted using TRI Reagent (Sigma) from tissues. The purity and the concentration of total RNA was determined. Template complementary DNA was synthesized using the cDNA preparation kit (Thermoscientific, Product code- AB1453A, Verso cDNA Synthesis kit). Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (Applied Biosystem, Life technologies). All reactions were performed in triplicates and data were analysed according to $\Delta\Delta C_t$ method. The primer sequences used were summarized in table1

Table 1. Primer sequences used for study

OLIGO NAME	FORWARD		REVERSE	
	SEQUENCE (5'->3')	Tm	SEQUENCE (5'->3')	Tm
LB-ACTIN	GGGACGATATGGAGAAGATCTG	60.3	CAGGCTCTGTGAGGATCTTC	59.4
LoHsp 60	CCCGGAGGTGGTACTGCTT	61.0	GCCATCTACGCCTGCGTTT	58.8

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method for separating and visualizing DNA fragments. The fragments are separated by charge and size and move through agarose gel matrix, when subjected to an electric field. The electric field is generated by applying potential across an electrolyte solution (buffer).When boiled in an aqueous buffer, agardissolve and upon cooling solidifies to a gel. Agarose gel electrophoresis was performed to check the purity of isolated mRNA. 1% agarose gel was prepared in 1x TE buffer and melted in hot water bath at 90°C.Then the melted agarose was cooled down to 45°C. 6µl of 10mg/ml of ethidium bromide was added and poured in to gel casting apparatus with the gel comb. After setting, the comb was removed from the gel. The electrophoresis buffer was poured in the gel tank and the platform with the gel was placed in it so as to immerse the gel. The amplified RNA sample was switched on and it was observed that RNA bands started migrating towards the anode. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen) and the mean density was determined using Image J analysis software.

3. Observation and Results

The result indicates that the HSP_{60} gene is down regulated in treated insects compared to control.It indicates the development of stress condition in the treated insects due to plant extract treatment. (Figure 1)

Table.2. Expression fold change

Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
24.56	4.27	2.70	1.57	0.3360311
-				

Table.3 Relative expression of HSP 60 in *Sitophilus oryzae*

Hsp 60 Gene Expression	
Control	Treated
1	0.3360311

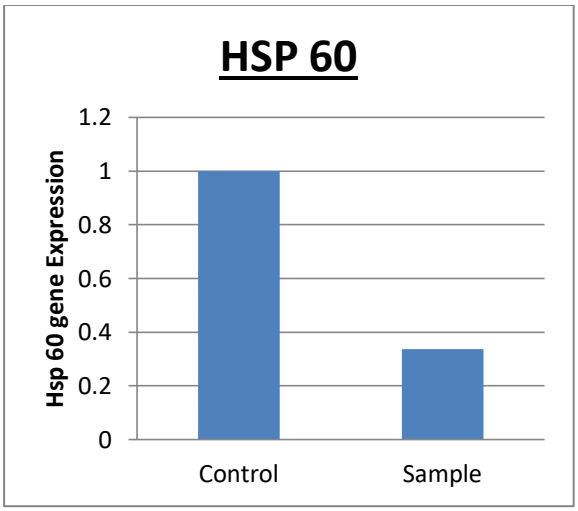

Fig 1: Graphical Representation of Expression Analysis of gene HSP 60 using qRT-PCR

4.Discussion

It is observed that the expression of HSP_{60} gene is found to be depleted in treated insects compared to control. Many workers obtained similar findings .Inhibited response of *Hsp70* also reported in the leafworm moths, *Spodoptera litura* exposed to zinc(Shu et al, 2010).As well as being induced by a variety of stresses, Hsp expression can vary with the physiological state of the organism and developmental stage (Feder and Hofmann, 1999). Altered gene expression also reported in the stored-product insects, the psocid, *Liposcelis entomophila*, (Guedes et al, 2008)

In the gypsy moth, *Lymantria dispar* (L.) (Erebidae), altered expression of Hsp70 was reported (Yocum et al, 1991)Heat shock proteins also help to protect cells from apoptosis, stabilize the cytoskeleton and contribute to proteostasis as housekeeping proteins (Morrow and Tanguay, 2012)Structure and function of hsp60 gene is highly conserved (Schlesinger, 1990).In the flesh fly,*S. crassipalpis*, Hsps were altered during diapause. Hsp60 were down-regulated during the diapause of *L. oryzophilus* (Rinehart and Denlinger, 2000).Hsp60 response of *B. plicatilis* was altered when it exposed to stress(Wheelock et al, 1999)Expression of Hsp60 was changed in *P. patulus* exposed to heat (Rios-Aranal et al, 2005)

Hsp60, *Hsp70*, and *Hsp90* play critical roles in insect development (Sharma et al,2007) Many heat shock proteins *are* regularly express during development stages. For example, in *Neoseiulus cucumeris* , *Hsp90* increases significantly in eggs and adult stages, and *Hsp70* expression is the highest in eggs (Chen et al, 2015)In *Spodoptera exigua* (Lepidoptera: Noctuidae) and *Chilo suppressalis* (Lepidoptera: Crambidae), *Hsp70* is highly expressed in first instar larvae and second instar larvae (Xu et al, 2011;Lu et al, 2014)

Under stress condition,transcriptional and translational level expression of hsp gene has been extensively studied in *Drosophila melanogaster* (Hoffman et al, 2003; Sorensen et al, 2007) observed a similar response in the induction of heat shock in *L. sativae hsp60* using real-time quantitative PCR methods, and the expression of *L. sativae hsp60* was inhibited. The induction of *Hsp* genes varies with the intensity of stress and the physiological state of the insect (King and MacRae, 2015)

Hsp60 act as a molecular chaperone in many species and it is essential for the proper functioning of cells under normal and stress conditions (Goloubinoff et al, 1989;Hartl, 1996).Hsp60 plays a central role in the folding of newly imported and stress-denatured proteins (Martin et al,1992). Yeast containing mutated mt-Hsp60 do not grow at elevated temperatures (Cheng et al, 1989)and show irreversible aggregation of a large number of newly imported proteins(Dubaquie et al, 1998)

A tissue-specific variation in the expression of Hsp60 was also reported in grasshopper (*Spathosternum prasiniferum*), cockroach (*Periplanata americana*) and gram pest (*Heliothis armigera*) (Singh and Lakhotia, 2000)The level of Hsp60 in *L. cuprina* was significantly altered in some tissues (Sunita et al, 2006)This may cause some negative effects on growth, development, survival and fecundity (Krebs and Feder, 1998) suggesting that the expression of Hsp may relate to physiological processes(Huang and Kang, 2007) Hsp plays a major protective role against cellular damage.

5. Conclusions

In the present study it can be concluded that the down regulation of Heat shock protein (hsp60) genes in the treated insects may be due to the stress condition created by insecticidal compounds present in the acetone extract of plant leaves *Glycosmis pentaphylla* .They have significant insecticidal effect, so it could be a potential grain protectant against *Sitophilus oryzae*.

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