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Expression of the heat shock protein genes in the adults of *Callosobruchus chinensis* due to *Centella asiatica*

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ABSTRACT: Medicinally important plant *Centella asiatica* was ckecked against the stored product pest *Callosobruchuschinensis*. HSP gene is more expressed in the treated adult insects, indicating the development of stress condition in the treated insects due to plant extract treatment. The presence of insecticidal compounds like rotenoids and steroids present in the plant *Centella asiatica* may stimulated the immune system of the insect and caused over expression of HSPs.

KEY WORDS: heat shock protein genes, *Centella asiatica*, *Callosobruchuschinensis*

INTRODUCTION

Different kinds of pesticides are used for checking the stored product pest *Callosobruchus chinensis.* Chemical pesticides are the most important among them.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 and plant compounds are proved to have pesticidal activity against various pests.Many medicinal plants are proved to be effective in controlling the stored pest *Callosobruchus chinensis.* These plants produce a stress condition in the insects and there is the expression of certain heat shock proteins. Heat Shock Proteins (HSPs) are a family of proteins that are produced by cells in response to exposure to stressful conditions. Extracellular and membrane bound heat-shock proteins, especially Hsp70 are involved in binding antigens and presenting them to the immune system (Nishikawa *et al.,* 2008). HSP70 is a key protein that is closely related to the molecular mechanism of insect resistance to the environment. Thus, understanding the differential expression of HSP70 may provide insight into how insects react to the stress environment and provide specific information about the mechanisms of resistance to various stress (Ling Wang *et al.,* 2015).More reports about insecticide inducible HSPs in insects have been published (Sharma *et al*.. 2008, Gupta *et al*., 2007). Many populations of *Frankliniella occidentalis* have developed resistance to various classes of insecticides, including avermectin (Immaraju *et al.,* 1992) which is now widely used for thrips control in China (Gao *et al.,* 2012).

A well-known mechanism used by organisms to cope with environmental stresses is the expression of heat shock proteins HSPs. HSPs usually act as molecular chaperones that promote protein folding

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and assembly, and prevent the aggregation of denatured proteins or newly-synthesized polypeptides (Gehring and Wehner, 1995; Sorensen *et al*., 2003; Feder and Hofmann, 1999). HSPs may be induced when a cell or organism undergoes any number of diverse environmental stresses, such as exposure to heat, cold, metal ions, pesticides, desiccation, or hypoxia (Kregel, 2002; Morrow *et al*., 2004; Joanisse *et al*., 1998).

Present study is an attempt to analyze the expression of HSP 70 genes in *Centella asiatica* acetone extract treated adult insects of *Callosobruchus chinensis.* The plant is widely used for medicinal purposes. Centella is mildly antibacterial, antiviral, anti-inflammatory and anti ulcerogenic. *Centella asiatica* extracts have been used traditionally for wound healing, asiaticoside, a constituent in *Centella asiatica*, has been reported to possess wound healing activity by increasing collagen formation and angiogenesis.

MATERIALS AND METHODS

Experiments were conducted in the Entomology Research Laboratory, Department of Zoology, University College Thiruvananthapuram. Pulse beetle, *Callosobruchus chinensis* adults were reared at normal room temperature and a relative humidity of 40% on clean and un-infested green gram (*Vigna radiata* L).The seeds were made pesticide free by washing with clean water. Newly emerged adults were used for the study.

Acetone extract of *Centella asiatica* was prepared with 20 g of powdered leaves of the plant was weighed and tied in a thin cloth and placed in the soxhlet apparatus. 200 ml acetone was taken in the glass flask and boiled at 55^0 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 effect of acetone extract was analyzed by using residual film method. No.1 Whatman filter paper were cut in round shape and placed in the plastic containers. Sub lethal dose of the extract (2% or 20 g) was selected by analyzing the lethal dose by probit analysis and the dose below lethal dose was taken as sub lethal dose (lethal dose for acetone extract of the plant is 25 g - Table 2,3 and Fig. 2) was applied to these filter papers using a micropipette and allowed to dry so that the solvent may evaporate completely. Then 50 g of feed was weighed out and twenty five one day old adult insects were placed in the containers so that each would get about 2g 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 homogenized in Trizol and taken for RNA isolation. LD_{50} was calculated using probit analysis (Muhammad Akram Randhawa, 1944).

Total RNA was isolated using the total RNA isolation kit per the manufacture instruction (Invitrogen, USA). 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. 200 µl of chloroform was added and shaken vigorously for 15 seconds and incubated for 2-3minutes at room temperature. Then the sample was centrifuged at 14000 rpm for 15 minutes at $4^{æ%}$C. The aqueous layer was collected and 100% 500 µl of isopropanol was added. It was incubated for 10 minutes at room temperature. Supernatant was discarded and pellet was collected, washed with 1ml of 75% of ethanol (Merck). It was then centrifuged at 10000 rpm for 5 minutes at $4^{æ%}$C in a cooling centrifuge (Remi). The RNA pellet was dried and dissolved in TE buffer. The purity of extracted RNA was determined using flurimeter Qubit 3.1 (Life Technologies, USA)

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction laboratory commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however an RNA strand is first reverse transcribed into its DNA complement (Complementary DNA or cDNA) using the enzyme reverse transcriptase and the resulting cDNA is amplified using PCR or real time PCR. RT-PCR technique was performed using primer designed specifically for amplified gene. Verso One step RT PCR kit of Thermoscientific, USA was used for the cDNA synthesis and amplification. About 5 ìl of RNA, 1ìl of enzyme mix, 2.5ìl of RT Enhancer, 2ìl of forward primer and reverse primer were added to an RNAse free tube. To this mixture 25 ìl of primer RT PCR premix was added. Then the total reaction volume was made up to 50 ìl with the addition of sterile distilled water. The solution was mixed by pipetting gently up and down. The thermal cycler (Eppendorf Master Cycler) was programmed to undergo cDNA synthesis and amplification. The cycling conditions followed were as per -

Gene	Primer sequence
Callosobruchus HSp70 F	GGCATCGGAATGAACAGACA
Callosobruchus HSp70 R	GGATTTCCAGACACAAGACTCC

	Temperature	Time	No. cycles
cDNA synthesis	50^0C	15 min	1
Verso inactivation	95^0C	15min	1
Denaturation	95^0C	20sec	
Annealing	50-60^0C	30sec	35-45
Extension	72^0C	1min	
Final extension	72^0C	5min	1

Gene fragments were separated by charge and size and move through agarose gel matrix, when subjected to an electric field through agarose gel electrophoresis method. The electric field is generated by applying potential across an electrolyte solution (buffer). When boiled in an aqueous buffer, agar dissolve and upon cooling solidifies to a gel. Agarose gelelectrophoresis 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 90^0C. Then the melted agarose was cooled down to 45^0C. 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 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.

RESULTS AND DISCUSSION

HSP gene is more expressed in treated insects compared to control. It indicates the development of stress condition in the treated insects due to the presence of insecticidal compounds in the plant extract (Fig. 1). The band is distinct in the treated insects compared to control. In the relative expression of HSP 70 in *C. chinensis* the mean intensity was higher (5610.26) in the treated individuals and it was low (3278.66) in the control.

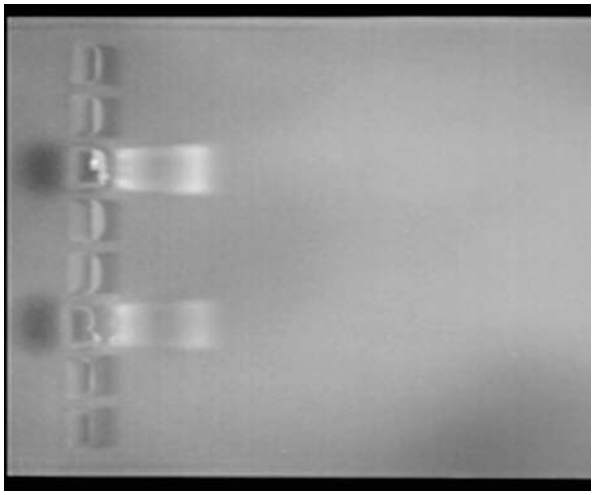

Fig.1. Expression of HSP gene in treatment and control

LD_{50} was calculated using probit analysis (Table 2, 3 and Fig. 2). Log LD 50 is 1.37 and LD 50 is 25 g.

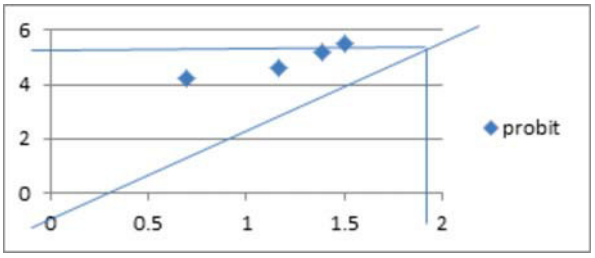

Fig. 2 LD_{50} using probit analysis

Table 2. Mortality of adults with *C. asiatica*

Dose(%)	Mortality (%)
0.5	22±0.02
1.5	38±0.01
2.5	58±0.03
3.5	70±0.02

Table 3. Probit analysis of adult insects on treating with *C. asiatica*

Group	Dose g/kg	Log dose	% dead	% corre-cted	Probit
1	5g	0.69	22	22	4.2
2	15g	1.17	38	38	4.6
3	25g	1.37	58	58	5.2
4	35g	1.50	70	70	5.5

In the present study there is a significant expression of HSP gene in the plant extract treated insects. It suggests that plants represent a promising source of insecticidal compounds. Over expression of HSP indicates the resistance developed in insects to overcome the stress aroused due to plant extract treatment. Previous studies have suggested that insect HSPs play protective roles in response to abiotic and biotic stresses (Zhao and Jones, 2012). The presence of insecticidal compounds like rotenoids and steroids present in the plant *C. asiatica* may stimulated the immune system of the insect and caused over expression of HSPs. The expression of HSPs has been reported to be induced and modulated in response to pesticides (Sonoda and Tsumuki, 2007). Wang Hai-hong *et al.* (2013) investigated the expression of HSPs in thrips after exposed to a pesticide avermectin. They found that avermectin is seemed to regulate the expression of *F. occidentalis* HSPs in two different ways. Five Fo-HSPs were induced at low concentrations of avermectin. The upregulation was approximately 12-fold for Fo-HSP28.9 for *F. occidentalis* exposed to bean discs treated at 0.002 ppm compared with the untreated control. Jatuporn Tungjitwitayakul *et al.* (2015) studied the expression of heat shock protein genes in different developmental stages after temperature stress in the maize weevil. Yasir H Siddique *et al.* (2013) reported the the effect of chemical, cyclophosphamide in the expression of HSP 70 in *Drosophila melanogaster*. Tang *et al.* (2012) reported that Stress-induced HSP70 from *Musca domestica* plays a functionally significant role in the immune system.

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