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FUNCTIONAL ANALYSIS OF *BRACON HEBETOR* VENOM ON TARGET AND NON-TARGET INSECT CELL LINES

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Abstract

Insect cell cultures are widely used in studies of insect cell physiology, toxicology, developmental biology and microbial pathology. The lethal effects of crude venom extracted from the ectoparasitic wasp *Bracon hebetor* were examined with three cultured insect cell lines; *Spodoptera frugiperda* (Sf9), *Tribolium castaneum* (TcA) and *Aedes aegypti* (Aag-2). Venom caused cells to round-up, swell and eventually die. Despite similar sensitivities and overlapping LC_{50} values [(0.00125-0.00695) venom reservoir equivalents (VRE)/µl], significant differences were noted at the onset of cytotoxicity among the three insect cell lines. Cells from the *Tribolium castaneum* (TcA) and *Aedes aegypti* (Aag-2) showed little sensitivity to the venom: 0.0046 VRE were needed to induce 50% mortality in TcA [50% lethal concentration (LC_{50}) = 0.0046 VRE/µl], and 0.0069 VRE were needed to induce 50% mortality in Aag-2 [50% lethal concentration (LC_{50}) = 0.0069 VRE/µl). Over 80% of the Sf9 cells were nonviable within 1 h after the addition of an LC_{99} dose of venom, whereas the other cells required a 5-10-fold longer incubation period to produce mortality above 50%.

KEY WORDS: *Bracon hebetor,* cell line, venom, VRE, parasitoids

Introduction

The venoms of parasitic Hymenoptera play a vital role in regulating the development of the insect host. The complicated interactions between hymenopteran parasitoids and their insect hosts result in alterations to the development of the host for the advantage of the parasitoid (Coudron, 1991). Venoms of ectoparasitoid species affect different functions, such as paralysis stimulation and/or regulation of immune responses of host, development and metabolism (Doury *et al*., 1997; Abt & Rivers, 2007).

Most studies of the venom of species that produce different degrees of paralysis have demonstrated effects on the nervous systems of their hosts (Piek, 1986). *Bracon hebetor* (Say, 1836) (Hymenoptera: Braconidae) is a vital biocontrol agent, particularly for the lepidopteran insect pests of different crops. Promising success has been achieved by the use of this biological control agent against insect pests. Therefore, to establish the realistic probability of using biocontrol agents, mainly *B. hebetor,* in insect pest management (IPM), there is a need to set the basic principles relevant to the operational aspects for the management of many lepidopteran pests in general and *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) in particular. *Bracon hebetor* is one of the most important larval parasitoids that attacks more than 130 hosts (Yu *et al*., 2012).
Bracon hebetor is a gregarious, arrhenotokous, ectoparasitic idiobiont wasp that attacks the larvae of lepidopterans (Cline *et al*., 1984; Gul & Gulel, 1995; Heimpel *et al*., 1997; Darwish *et al*., 2003) and the alfalfa weevil *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) (Khalil *et al*., 2016), as well as some Chrysomelidae (Coleoptera) and Cynipidae (Hymenoptera) (Yu *et al*., 2012). The most detailed work regarding venom has been done with the genus *Bracon (= Microbracon, Habrobracon)* which induces permanent paralysis (Edwards & Sernka, 1969; Piek & Engels, 1969; Tamashiro, 1971; Beard, 1978). The venoms produced by these species act at the neuromuscular synapse level and cause somatic muscular paralysis (Beard, 1952; Piek, 1966; Piek & Thomas, 1969; Piek *et al.,* 1974).

In vitro cytotoxicity assays measure whether a test compound is toxic to cells in culture, usually by determining the number of viable cells remaining after a defined incubation period. The desired approach is to use a convenient and cost-effective method that predicts *in vivo* toxicity. Dye exclusion methods are traditionally used to assess cell viability, with trypan blue being one of the most common. Trypan blue is a vital stain that leaves nonviable cells with a distinctive blue color when observed under a microscope, while viable cells appear unstained (Stoddart, 2011). We investigated the cytotoxicity of crude venom extract from *B. hebetor* on three different insect cell lines using this dye exclusion method.

Materials and Methods

Parasitoid rearing

Bracon hebetor was reared on its preferred host, the greater wax moth *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae), under controlled conditions. Wax moth was collected from infested bee hives located at the campuses of the University of Agriculture, Faisalabad, Pakistan. Honeycomb was used as a natural diet to rear *G. mellonella*. Host and parasitoid cultures were maintained in separate growth chambers at 28±2°C, 60±5% RH and 16:8 (L:D) photoperiod and 27±2°C, 65±5% RH and 16:8 (L:D) photoperiod, respectively.

Venom extraction

Bracon hebetor females of mixed ages were collected individually and put in glass vials for later anesthetizing on ice. The whole reproductive tract of adult females was removed by grasping the ovipositor tip with fine forceps while holding the abdomen of the female with other forceps in a drop of phosphate-buffered saline solution, pH 7.4 (PBS; sodium chloride, sodium phosphate, potassium chloride, potassium phosphate) under microscope. The venom glands and reservoirs obtained from each female were subsequently separated from the ovaries and other unnecessary tissues using fine micro dissecting needles and were transferred to a 20-µl drop of ice-cold PBS. The venom reservoirs were gently opened with fine needles to allow the diffusion of venom in a drop of PBS solution. The crude extract was centrifuged at 5000 g for 5 min at a temperature of

4°C to remove any fragments of tissue. Then the supernatant was recovered and transferred to a sterile Eppendorf tube. The supernatant was finally diluted with PBS solution in order to obtain the needed concentration of venom reservoir equivalents (number of reservoirs processed as described above, VRE)/µl.

Insect cell cultures

The Sf9 cell line originated from the IPLBSF-21 cell line, derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), the Aag-2 cell line came from the larvae of *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae) and the cell line BCIRL-TcA-CLG1 or TcA came from late pupae of the red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) and were used for *in vitro* toxicity assays maintained at 27°C in the Insect Virology lab, University of Kentucky, USA. The media used was Ex-Cell 401 serum free media (SFM) (JRH Biosciences, USA), or Sf-900 II SFM (GIBCO BRL, ThermoFisher Scientific, USA).

Venom assays

Cells from each line were counted with a hemocytometer and seeded ($1x10^4$ cells/well) into 96-well plates (Falcon) with 100 µl of appropriate cell medium as mentioned above. Venom in PBS was added to each well with 10 wells for each concentration (0.05, 0.03, 0.01, 0.005, 0.003, 0.001-VRE). Cells were incubated at 27°C for 24 h. Cell monolayers were washed with PBS (pH 7.4) by removing spent culture media, adding 100 µl PBS and then gently rocking the plate for 10-20 s before discarding the buffer. After the wash, 100 µl of fresh insect medium were added to each well. Cell viability was assessed by vital staining with trypan blue dye (Sigma, USA) on a hemocytometer as previously described (Rivers *et al.,* 1993). Ten µl of 0.1% (w/v) stock solution were added to each well and incubated for 3-5 min at room temperature as previously described (Rivers *et al*., 1993). The LC_{50} (concentration required to induce 50% mortality) and LC_{99} (concentration required to induce 99% mortality) were determined using the method described by Finny (1971). LT_{50} (time required to kill 50% of cells) was determined in the same manner as for LC_{50}. Cell viability was determined after the introduction of an LC_{99} dose of venom at the following time intervals: 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300 min and also after 24 h.

Results

The lethal effects of crude venom extracted from the parasitoid *Bracon hebetor* were examined using three insect cell culture lines. The venom showed markedly differential toxicities towards the viability of different cell lines. For example, LC_{50} for *S. frugiperd*a (Sf9) cells was 0.00125 VRE/µl, which was quite low as compared to *T. castaneum* (TcA) cells, which had 0.00466 VRE/µl toxicity and *A. aegypti* (Aag-2) cells with LC_{50} of 0.00695 VRE/µl. LC_{99} for Sf9 cells was recorded as 0.527 and for TcA and Aag-2 cells 0.189 and 0.378 VRE/µl, respectively (Table I, Fig. 1). The LC_{50} and LC_{99} values for lepidopteran cells were quite low as *Spodoptera* species are one of the major hosts for *B. hebetor*, while the other cell lines showed less sensitivity.

Timing of cell responses against toxicity of venom was also calculated. For this purpose, LC_{99} concentrations were selected to calculate the LT_{50} and LT_{99} for all the cell lines given above. The LT_{50} for Sf9 was the lowest as compared to the other cell lines. The LT_{50} for the Sf9 cell line was 6.94 min, which means that half of the cells were dead due to the effect of crude venom after treatment with $LC_{99,}$ which was calculated before. The LT_{50} for TcA cells was 109.83 min and for Aag-2 cells it was 154.8 min. To kill 99% of the cell population,

LT_{99} for Sf9 cells was 41.78 min and for TcA cells it was more than 6 h (almost 403 min) and for Aag-2 cells was 466.4 min. These results showed that because of host specificity, the wasp venom was highly toxic and specific towards the lepidopteran cell line as compared to the other cell lines.

Table I. Susceptibility of different insect cell culture lines to the crude venom of *Bracon hebetor* (Say). Values in parenthesis are showing the 95% confidence intervals determined by probi.t analysis.

Cell Line	Origin of cell line	LC_{50} (VRE/µl)	LC_{99} (VRE/µl)	LT_{50} (Min)
Sf9	Pupal ovarian	0.00125 (0.0010,0.0015)	0.05277 (0.0398,0.0744)	6.943 (4.938,8.703)
TcA	Pupae	0.00466 (0.0042,0.0052)	0.18930 (0.1410,0.2680)	109.83 (103.0,116.4)
Aag-2	Larvae	0.00695 (0.0062,0.0078)	0.37840 (0.2690,0.5678)	154.80 (148.2,161.5)

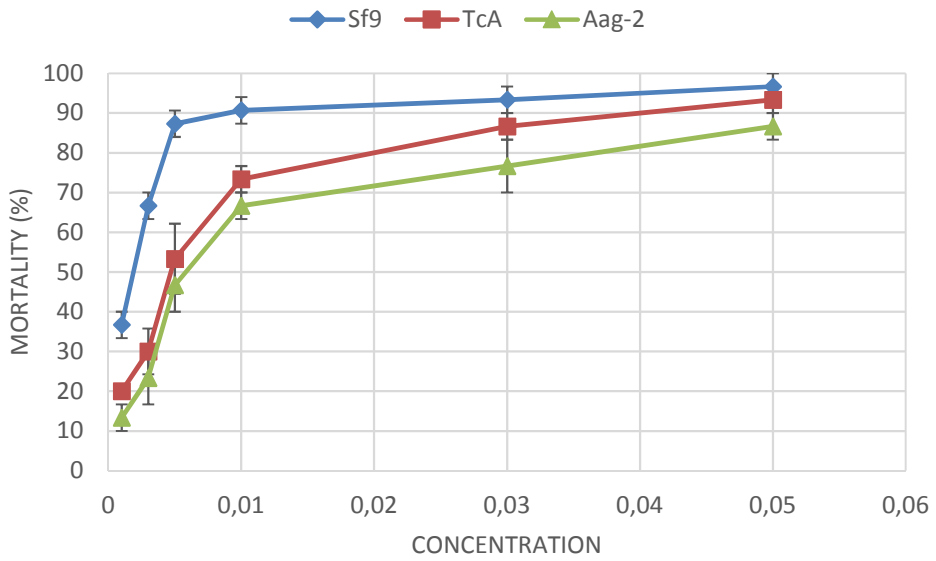

Figure 1. Cytotoxicity response of different concentrations of crude venom from *Bracon hebetor* towards cells of *Spodoptera frugiperda* (Sf9), *Tribolium castaneum* (TcA) and *Aedes aegypti* (Aag-2).

Discussion

Selectivity of a chemical is achieved if both pest and non-pest species do not have the same target (Feyereisen, 1995). Although the use of insecticides is an effective solution for the control of insect pests, it has many side effects. Many scientists are trying to introduce compounds that are effective, cheaper and more environmentally friendly in agroecosystems for the management of insect pests (Pretty & Bharucha, 2015). Biopesticides are considered a safer alternative to chemical pesticides as they do not cause any serious harm or create any environmental pollution (Leng *et al*., 2011). Many parasitoid venom toxins can effectively be employed in biopesticide engineering as they contain toxins that are active against insects (Gurevitz, 2010).

Few of the hymenopteran venoms tested are selective for target animals. In several genera of social Hymenoptera, venoms display almost no specificity and induce indiscriminate cell lysis, often resulting in paralysis (Piek & Spanjier, 1986; Quistad *et al.,* 1992). In contrast, venoms from parasitic Hymenoptera display some selectivity *in vivo* (Drenth, 1974; Coudron & Puttler, 1988).
In the present study, bioassay tests on different insect cell lines were performed in order to evaluate the effectiveness of the crude venom extracted from *B. hebetor*. The results of our study clearly demonstrated that the venom from *B. hebetor* is more effective against the cell line derived from *Spodoptera frugiperda* as even with a 0.00125VRE/µl concentration, the mortality was up to 50% and cytotoxicity was observed within 10 min after exposure to LC_{99} concentrations of venom (Fig. 2). This toxicity for Sf9 cells (0.00125VRE/µl) was high as compared to TcA cells, with 0.00466 VRE/µl and Aag-2 cells with an LC_{50} of 0.00695 VRE/µl. According to Beard's calculation, a concentration of 0.005 ppm in the hemolymph is sufficient to provoke permanent paralysis. Lower concentrations of venom resulted in delayed paralysis, a reduction of the percentage of paralyzed larvae and an increased rate of recovery (Beard, 1952), cit. Piek, 1966).
Many researchers have already confirmed the effectiveness of the venoms extracted from different parasitoids against different insect cell lines (Rivers *et al*., 1993, 1999; Zhang *et al*., 2005). For example, in one study, the toxicity of venom from *Nasonia vitripennis* (Hymenoptera: Pteromalidae) was assessed for cultured insect cells (Rivers *et al.,* 1993). When different concentrations of venom were tested *in vitro,* the venom caused the death of cultured Lepidoptera (TN-368) and Diptera (NIH SaPe4) cells. The LC_{50}s were 0.0014 and 0.0010 VRE/µl for TN-368 and SaPe4 cells, respectively. Cytotoxicity was observed within 10 min after exposure to LC_{99} levels of venom, with 100% cell mortality at 100 min for the NIH SaPe4 cells and at 24 h for TN-368 cells. As *N. vitripennis* is an endoparasitoid of dipterans, its venom showed more toxicity to NIHSaPe4 cells. In the same way, the venom from *B. hebetor* showed more toxicity to Sf9 cells as *Spodoptera* species are its favored target hosts.

In another study, the lethal effects of crude venom obtained from the ectoparasitic wasp *Nasonia vitripennis* (Walker, 1836) (Hymenoptera: Pteromalidae) were examined with cultured cells from six insects and two vertebrate species (Rivers *et al.,* 1999). Venom caused cells from *Sarcophaga peregrina* (Robineau-Desvoidy, 1830) (Sarcophagidae: Diptera) (NIH SaPe4), *Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae) (CRL 1963), *Trichoplusia ni* (Hubner, 1803) ((Lepidoptera: Noctuidae) (TN-368 and BTI-TN-5B1-4), *Spodoptera frugiperda* ((J.E. Smith, 1797) (Lepidoptera: Noctuidae) (SF-21AE) and *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Noctuidae) (IPL-Ldfbcl) to round-up, swell and eventually die. Despite similar sensitivities and overlapping LC_{50} values [0.0004-0.0015 (VRE)/µl], significant differences were noted at the onset of cytotoxicity among the six insect cell lines: over 80% of the NIH SaPe4 and SF21AE cells were nonviable within 1 h after the addition of an LC_{99} dose of venom, whereas the other cells required a 5-10-fold longer incubation period to produce mortality approaching 100%. Our results also showed that over 80% of the Sf9 cells were nonviable within 1 h after addition of an LC_{99} dose of venom (Fig. 2), as these cells are a

specific target of *Bracon hebetor* venom, whereas the other cells required a 5-10-fold longer incubation period to produce mortality above 50%.

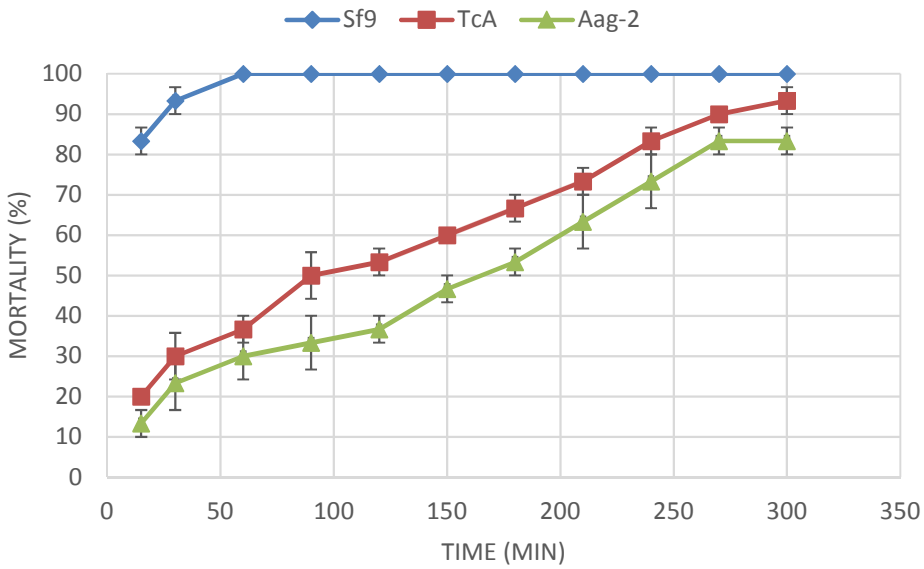

Figure 2. Cytotoxicity response of crude venom from *Bracon hebetor* towards cells of *Spodoptera frugiperda* (Sf9), *Tribolium castaneum* (TcA) and *Aedes aegypti* (Aag-2) after different time intervals.

Zhang *et al*. (2005) also checked the responses of Tn-5B1-4 and Ha cells to venoms from *Pteromalus puparum* (Linnaeus, 1758) (Hymenoptera: Pteromalidae) at 10 different concentrations *in vivo*. Venom showed highly lethal effects on the Tn-5B1-4 cells: the LC_{50} after venom treatment for 0.5 and 4 h was 0.0084 and 0.0074 VRE/µl, respectively, and the LC_{90} was 0.0520 and 0.0468 VRE/µl. For Ha cells, the LC_{50} after venom treatment for 0.5 and 4 h was 0.0024 and 0.0022 VREµ/l, respectively, and the LC_{90} was 0.0040 and 0.0040 VRE/µl, respectively. Our results are also more consistent with the phenomenon that *B. hebetor* prefers to parasitize lepidopteran hosts as compared to other hosts.

It is possible that the venom component responsible for *in vivo* and *in vitro* activities may be different, but results from the cell culture work suggest that this method offers a promising assay for quickly screening venom samples. The high susceptibility of Sf9 cells of Lepidoptera and other orders to the venom, as well as its novel (paralytic) action, suggest that it may have considerable potential for development as a biopesticide.

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ФУНКЦИОНАЛНА АНАЛИЗА ОТРОВА ВРСТЕ *BRACON HEBETOR* НА ОДАБРАНЕ ЋЕЛИЈСКЕ ЛИНИЈЕ ИНСЕКАТА

АТИФ МАНЗУР, ЗАИН УЛ АБДИН, ХУР ШАИНА, БРУС ВЕБ И АМЕР ЏАМИЛ

Извод

Ћелијске културе инсеката се често користе у многим истраживањима о ћелијској физиологији инсеката, токсикологији, биологији развића и патолошкој микробиологији. У раду су испитивани летални ефекти сировог отрова узетог из ектопаразитске осе *Bracon hebetor* на три линије ћелијских култура инсеката; *Spodoptera frugiperda* (Sf9), *Tribolium castaneum* (TcA) и *Aedes aegypti* (Aag-2). Под утицајем отрова, ћелије су заузимале округли облик, повећавале запремину и на крају умирале. Упркос сличном осетљивошћу и преклапању LC_{50} вредности [(0.00125-0.00695) еквивалети резервоара отрова (VRE)/µl], значајне разлике су забележене у почетку цитотоксикологије у оквиру три ћелијске линије инсеката. Ћелије врсте *Tribolium castaneum* (TcA) и *Aedes aegypti* (Aag-2) су показале ниску осетљивост на отров: 0.0046 VRE је било потребно да изазове 50% смртности код TcA [50% леталне концентрације (LC_{50}) = 0.0046 VRE/µl], и 0.0069 VRE је било потребно да изазове 50% смртности код Aag-2 [50% леталне концентрације (LC_{50}) = 0.0069 VRE/µl). Преко 80% Sf9 линије је било невијабилно након једног сата третирањем LC_{99} дозом отрова, док је за остале ћелије било потребно пет до десет пута дужи период инкубације да би се достигла стопа смртности од 50%.

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