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DIFFERENTIAL APYRENE SPERM ACTIVATION ELICITED BY VARIOUS REGIONS OF MALE REPRODUCTIVE SYSTEM OF *SPODOPTERA LITURA* (F)

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ABSTRACT

In this study, the sperm activation potential of various regions of male reproductive system of *Spodoptera litura* (F.) has been evaluated by in-vitro bioassay. This has been done using their respective secretions as sperm activator for the sperm derived from the duplex of 2-3 days old virgin male moths. The results revealed that the prostatic part elicited a significantly pronounced sperm activity profile (in terms of proportion of active sperm and their activity intensity), as compared to the subregions of the prostatic part. The sperm activity profile by the prostatic part secretions was further observed to be reinforced by the addition of secretions of accessary glands. The sperm activity peak plateau reached around 30-90 min during the sperm activation bioassay. Further studies are in progress towards identification and characterization of potential biomolecule(s) responsible for sperm activity in the secretions of male prostatic part of the ejaculatory duct. This study has pragmatic application in exploring the anti-reproductive potential of various biorationals and optimization of radiogenetic IPM methods that are based on sperm dynamics and mating competitiveness.

Key words: *Spodoptera litura*, sperm activity, ductus ejaculatorius duplex, male reproductive system, noctuid moth, prostatic part, in vitro bioassay, biorational molecules, IPM

Spodoptera litura (F.) is a serious polyphagous pest that causes significant damage to many crops like soya bean, cotton, tobacco and vegetables and has attained global importance. Development of resistance to various major classes of insecticides i.e., organophosphates, organochlorines and synthetic pyrethroid has been reported against *S. litura* in many parts of the world (Ramakrishnan et al.*,*1984; Saleem et al., 2008; Tong et al., 2013; Gandhi et al., 2016). Moreover, the injudicious and indiscriminate use of pesticides has worsened the situation causing environmental pollution, elimination of natural controlling agents and outbreak of secondary pests (Vreysen et al., 2006; Seth et al., 2016b). Hence, a considerable emphasis is being laid to tackle such serious pests by evolving some biorational tactics that would be ecofriendly. Activity of sperm is a crucial parameter for success of reproduction in any species and sperm behaviour might be an important target for achieving success in lepidopteran pest control strategies. Sperm dimorphism in lepidopterans provides a wide window to develop methods that might influence the sperm dynamics. In lepidopterans, the males produce two distinct types of sperm- nucleated eupyrene and non-nucleated apyrene sperms. *Spodoptera litura* is a noctuid moth and is reported to exhibit sperm polymorphism (Seth et al., 2016a). The apyrene sperm of *S. litura* generally comprise about 75% of the sperm in the ejaculate (Seth et al., 2016b). Research has been conducted to find out proteins responsible for sperm activation in *Bombyx mori*. Sperm plays an important role in reproduction and can be targeted in either male (before copulation) or female (after copulation in spermatophore). The sperm passage from male testes to female eggs till fertilization undergoes a variety of modifications and is affected by many factors like pH, enzymes, gland secretions etc. (Werner and Simmons, 2008). The present study ascertains the differential potential of secretions of different parts of the male reproductive tract in eliciting activation of apyrene sperm derived from ductus ejaculatorius duplex (herein after referred to as 'duplex') of *S. litura* so that an appropriate region in male reproductive tract could be identified to extend the research on identification of biomolecule(s) responsible for sperm activation.

MATERIALS AND METHODS

The culture of *S. litura* (F.) was maintained in the laboratory (27± 1°C, 75± 5% RH, and a 12:12 hr L:D photoperiod with lights on at 06.00 hr and lights off at 18.00 hr) on a chickpea based semisynthetic diet (as described by Seth and Sharma, 2001). Proper care was

taken to avoid microbial infection in the culture. The freshly emerged adults in 4-5 pairs were transferred to the perspex cages (20x20x20cm) with 10% honey solution to feed on and castor leaf to act as ovipositional trap. The eggs were collected daily and incubated for hatching in to the first instar larvae (L1) that moulted five times during larval phase, followed by larval-pupal moult and adult eclosion. For invitro sperm activation assay, 2-3days old virgin adult male of *S. litura* was dissected, and reproductive tract was taken out on a glass slide in Belar's saline (0.6g NaCl, 0.2g KCl, 0.2g $CaCl_2$, 0.2g Na_2CO_3 and water to make volume upto 1l). The duplex, prostatic part and accessory glands were dissected out separately. The prostatic part was further divided into upper and lower prostatic parts for sperm bioassay. The duplex was minced in 50µl of 0.3 M of HEPES- KOH buffer at pH 7.0 containing 20mg/ ml bovine serum albumin to extract sperm. The sperm solution was kept in parafilm container placed on moist tissue paper at room temperature.

The prostatic part whole, its different regions (upper and lower) and accessory glands were separately minced in 40µl of 0.03 M ammonium bicarbonate buffer with a pH of 7.0. The extracts were then transferred in to 1.5ml micro centrifuge tube and centrifuged at 6000 rpm for 10 min at 4°C. The supernatants of the prostatic region, its subregions and accessory glands were collected to act as an individual activator in invitro sperm activation assay. Then, 5µl of a specific supernatant (activator) was mixed with 5µl of sperm solution from the duplex. The solution was put on a glass slide, covered with coverslip and observed under microscope at 400x magnification. Two parameters related to sperm motility were observed and recorded upto 135 min (with a frequency of 15-30 min). These parameters included- (i) % sperm active (proportion of sperm that elicited activity out of the total number of sperm observed) and (ii) sperm activation intensity (number of to and fro motions or undulations of activated sperm/ sec). Each data point comprised of '2' phases: (i) computing the number undulations/ sec and (ii) transforming the number of undulations into a scale from 0-4 ('0'= no undulation, '1'=1-5 undulations/ sec, '2'= 6-10 undulations/sec, '3'= 11-15 undulations/ sec, '4'= > 15 undulations/sec).Ten replicates were performed for assessing each parameter and each replicate comprised an average of 10-15 readings from different microscopic views pertaining to one insect. The data were replicated ten times, and subjected to one way ANOVA; % data was transformed using arcsine √x value before ANOVA, but data shown in tables and graphs are back transformed. The significance level was set at $p \leq 0.05$, and LSD test was performed to determine significant differences among the treatments (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

Invitro bioassay was conducted to study the sperm activation potential of various regions of male reproductive system of *S. litura* using the secretions of different parts of reproductive system as sperm activator for eliciting the activation in the sperm derived from the duplex of 2-3days old virgin male moth. The secretions from the prostatic part elicited a significantly pronounced sperm activity profile assessed as proportion of active sperm, as compared to the secretions from different sub-regions of the prostatic part. The sperm activity was substantially enhanced at 15 min during the assay and the peak plateau of the sperm activity reached around 30-90 min. with >70% of the sperm eliciting activity, during the sperm activation bioassay. During sperm activation bioassay, significantly enhanced sperm activity was exhibited by whole prostatic part as compared to the lower and upper prostatic parts or accessory gland, although lower prostatic part elicited a better response. Further, the sperm activity by the prostatic part was found to be reinforced due to addition of the accessory gland secretions, that caused instant sperm activity due to addition of activator, with activity profile reaching the plateau faster than the activity profile induced by prostatic part alone. Similar pattern of sperm undulation intensity during sperm activation bioassay was noticed vis-à-vis the profile of % active sperm (Fig. 1; Table 1). The lower prostatic part seemed to elicit the sperm undulation intensity better than the upper part, and the sperm activation intensity elicited by prostatic part was further pronounced by addition of secretions from the accessory glands. The peak plateau of sperm activity intensity due to prostatic secretions ranged from 3-4 scale, amounting to 11-15 undulations/ sec or more.

Various regions of the male reproductive tract eliciting a varying degree of sperm activity, can be arranged in descending order as prostatic part (as a whole) > lower prostatic part > accessory gland > upper prostatic part (Table 1; Fig. 1).The involvement of various regions of male reproductive system was ascertained towards apyrene sperm activation in *S. litura* by the sperm activation bioassay (as per method described by Seth et al., 2016a). The presence of sperm activator biomolecules in the prostatic part of *S. litura* has been reported earlier by Seth et al. (2016a), whereas in the present study the male accessory glands secretions

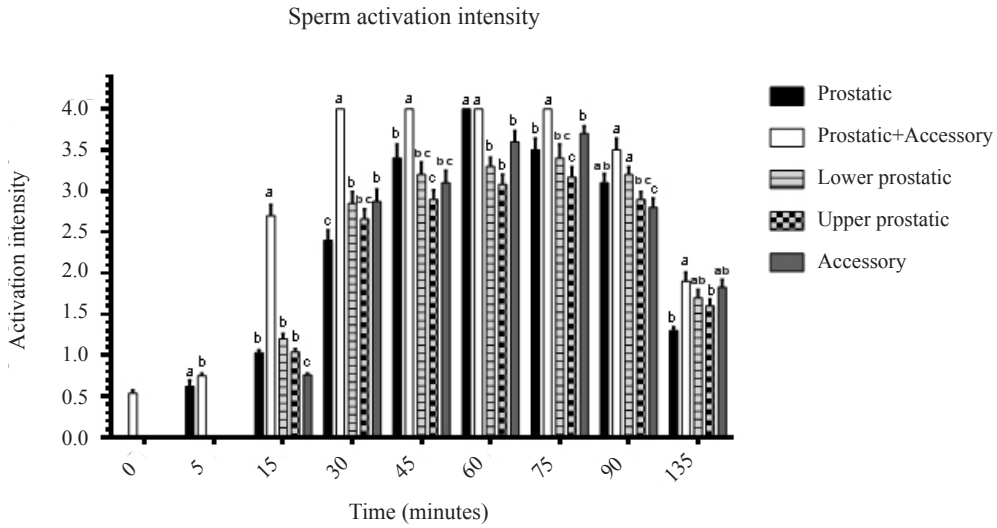

Fig. 1. Sperm activity was determined as sperm activation intensity (in-vitro assay) in sperm from duplex mixed with activator from different regions of male reproductive system; Means followed by the same alphabets among various regions with respect to each specific time during the assay are not significantly different at P<0.05 level (ANOVA followed by LSD post-test). Activity intensity was assessed by transforming the number of undulations into a scale from 0-4 ('0'= no undulation, '1'=1-5 undulations/ sec, '2'= 6-10 undulations/sec, '3'= 11-15 undulations/sec, '4'= > 15 undulations/sec).

Table 1. Sperm activity (in vitro assay) elicited by different regions of male reproductive system

	0	5	15	30	45	60	75	90	135
Prostatic	0	19.98a± 2.6	66.59a± 2.89	89.41a± 3.23	86.21ab± 4.26	84.46ab± 3.04	83.11ab± 3.5	81.55ab± 4.17	30.77ab ± 2.92
prostatic+ accessory	16.53± 2.68	27.83b± 2.46	73.83a± 4.32	92.16a± 2.81	91.62a± 2.56	87.16a± 3.34	86.20a± 3.55	84.06a± 2.08	36.16a± 3.18
Lower prostatic	0	0	52.16b± 2.02	76.83b± 2.07	85.54ab± 2.76	82.12ab± 3.43	81.23ab± 2.7	80.80ab± 2.69	28.83ab± 2.39
Upper prostatic	0	0	46.16b± 2.89	72.38b± 2.72	79.90b± 2.92	77.08b± 3.56	75.97b ± 2.9	74.40b± 2.78	26.82b± 2.42
Accessory	0	0	49.11b± 3.22	73.04b± 4.83	82.77b± 3.50	83.90ab± 3.54	84.57ab± 3.41	74.76b± 3.1	32.12ab± 2.55
F value		18.180	66.315	22.293	6.354	2.52	2.71	2.93	11.583

Sperm activity was determined as % sperm eliciting activity (in-vitro assay) in sperm from duplex mixed with activator from different regions of male reproductive system; Means± SE followed by the same alphabets within a column are not significantly different at *P*<0.05 level (ANOVA followed by LSD post-test). % data was transformed for ANOVA and data in the table is untransformed.

were also found to reinforce the sperm activity. Stephens et al. (2018) reported the presence of trypsin like protease in the male accessory gland of *Culex pipiens* and *Culex quinquefasciatus*. Their further studies suggested the likely involvement of accessory gland serine proteases in activating *Culex* sperm motility during or after transfer to the female reproductive tract (Stephens et al., 2018).

Sperms are non- motile in the testis and become motile only during copulation or after deposition in the female as spermatophore (Davis, 1965; Hughes and Davey, 1969). The process of spermatogenesis in males differs between eupyrene and apyrene sperms as reported by Friedländer, 1997. The apyrene sperm must have a specific function in the reproductive process (Silberglied et al.,1984).The apyrene sperm may have multiple functions, such as regulating female receptivity (Cook and Wedell, 1999; Swallow and Wilkinson, 2002), assisting eupyrene sperm for fertilization (Till-Bottraud et al., 2005), assisting the migration of eupyrene sperm to the spermatheca (Holt and North, 1970), as carriers of nutrition for eupyrene sperm (Boggs, 1995), etc. During mating in *S. litura*, the eupyrene sperm (as bundles) and apyrene sperm (as individual, dissociated from bundles) are transferred within a spermatophore to the bursa copulatrix of female. Eventually, movement of dissociated apyrene

as well as eupyrene sperm occurs into the spermatheca (Seth et al., 2002).

The present study revealed the presence of apyrene sperm activator molecules in accessory gland and different regions prostatic part with a pronounced effect due to the secretion from the prostatic part. The combination of secretions from prostatic part and accessory gland showed an additive effect. It was noticed that the initiation of sperm activity was instantaneous due to combined secretions of prostatic part and accessary glands, while there was a little latency of 2-5 min in the initiation of sperm activity when individually secretions of prostatic part or accessory gland were used as sperm activator in separate bioassays. This infers that presumably increase in concentration of sperm activator molecules might induce faster sperm activation. Both % active sperm and sperm intensity data analysis showed similar profile of sperm activity elicited by different specific regions of the male tract. With the progression of time after initiation of sperm activation bioassay, the number of activated apyrene sperm increased in the present study, indicating that activator molecule might be a self-catalytic molecule, as suggested by Nagaoka et al.(2012). A sharp increase in activation intensity in apyrene sperm was noticed from 15 min onwards showing a peak plateau at 30-90 min. (Fig. 1). Sperm activator molecules were found to be predominantly present in the prostatic part.

Sperm activity is a promising domain for devising novel biorational IPM strategies to target male reproductive competence. Sperm viability matters in insect sperm competition under different stress conditions, and sperm competition can be a potent selective force acting on an array of male reproductive traits. The frequency of successful insemination can be considered an important indicator of competitiveness, especially for lepidopteran insects (LaChance et al., 1978). Sperm viability is a major male fitness component, as increased sperm viability would be associated with enhanced sperm competitiveness (Tourmente et al. 2019). Pest control can be exercised in many ways by using radiation (Seth et al., 2016), using protein inhibitors (Zhao et al., 2019; Singh et al., 2020), gene mutation (Zhang et al., 2019; Xu et al., 2020) and RNAi technology (Jayachandran et al., 2013; Singh et al., 2020). Radiation induced F1 sterility technique has been proposed for *S. litura* (Seth and Sharma, 2001; Seth et al., 2016a, b). Formulating pest control methods to target sperm activation molecules would be ecofriendly in nature as it might not affect non targeted organisms and be compatible with other pest management tactics to be used in IPM. The role of accessory gland secretions in activation of moth sperm has been evaluated in the present study. Further studies are in progress for identification and characterization of potential biomolecule(s) in the male prostatic part of the ejaculatory duct. This study has practical implications in terms of (i) assessing and establishing the antireproductive or sterilant role of various pesticides, xenobiotics, other stresses (viz., radiation, temperature, environmental pollution) (ii) devising novel biorational pest control tactics, using sperm activity as target through biochemical inhibitors(to check sperm activation) and RNAi technology, that might be coupled effectively with radiation mediated genetic control methods against lepidopteran pests.

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AUTHOR CONTRIBUTION STATEMENT

RKS conceived and designed research. PY conducted experiments. SA and CKS assisted in the conducting experiments and validation of data. RKS and PY analyzed the data and involved in writing, editing and reviewing the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST

No conflict of interest.

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