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EFFECT OF IONIZING RADIATION ON PHEROMONE BIOSYNTHESIS ACTIVATING NEUROPEPTIDE *(PBAN)* GENE EXPRESSION AND ITS PHOTOSENSITIVE RHYTHM IN FEMALE *SPODOPTERA LITURA* (F.)

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ABSTRACT

In this study, the effect of ionizing radiation was studied on the pheromone biosynthesis activating neuropeptide *(PBAN)* gene expression in female moths of a noctuid pest *Spodoptera litura* (F.), that would trigger pheromone production and its release needed for calling behaviour. The *PBAN* gene expression in the radiosterilized female moths (at 130 Gy) showed a decline with respect to control (unirradiated moths), and *PBAN* expression was drastically reduced at higher dose, 200 Gy. The photosensitivity and diel rhythm of *PBAN* gene expression, indicating higher expression during peak hours of scotophase and lowest expression during photophase was maintained at 130Gy, which deemed this gamma dose a suitable sterilizing dose for female moths which seemed to retain reproductive competence. This study might support the radiosterilized female moths for their combined release with substerilized males, using 130Gy to effectively operate the F1 sterility technique proposed to control *S. litura*

Key words: *Spodoptera litura*, F1 sterility, radiogenetic pest control, PBAN, gene expression, photosensitivity, diel rhythm, parabiological control, pheromone reduction, calling behaviour

Spodoptera litura (F.) (Lepidoptera: Noctuidae), the common cutworm, is a serious polyphagous pest economically devastating a variety of crops all across India (Moussa et al., 1960). Continuous application of traditional pest control method of using chemical pesticides has led to the development of resistance in this insect while also harming the environment in the process (Armes et al., 1997). The sterile insect technique (SIT), a biorational pest control method, though successful in dipteran pest control, has been less efficient in eradicating lepidopteran pests due to their high radioresistance as lepidopterans require large doses of radiation for 100% sterilization, leading to somatic damage and reduced competitiveness in the irradiated insect (Anisimov et al., 1989). Hence, a modified form of SIT (F1 sterility technique) has been proposed for the control of lepidopteran pests including *S.litura* (Bughio, 1988; Carpenter et al., 1983; 1986, Carpenter, 1987; North, 1975; North and Holt, 1969; Seth and Sharma, 2001; Seth et al., 2016a,b.), wherein the lower (partial sterilizing) dose of gamma radiation is used that would lead to sub-sterile male and eventually sterile F1 progeny in the field. Conventional SIT and F1 sterility technique make use of irradiated male moths and their release in the pest infested field (North, 1975). The use of treated females in the SIT/IS programmes has also been proposed for simultaneous release in various studies and simulation models (Hight et al., 2005; White et al., 1976; Vreysen, 2006). The inclusion of irradiated females along with males might be beneficial in ultimately eradicating the laborious process of sex-based pupal or adult segregation (Braganca et al., 1998). Simultaneous male and female release might also be effective in overwhelming the pest population already present in the field as both treated sexes would compete with wild males and females at the same time (Marec and Vreysen, 2019).

Due to radiogenetic damage caused by gamma radiation in the insects, their reproductive fitness might be affected, that would ultimately affect their performance in the field by hindering their competitiveness against the wild population. Therefore, analysis of effect of radiation on reproductive fitness of the irradiated females is necessary to understand their mating competitiveness with the wild population and contribution in pest control. The premating efficacy of moths is an important indicator of their reproductive fitness concerned with mating ability and mating success. The sexual communication between sexes in lepidopteran species is mediated mainly by sex pheromones, which are volatile compounds used by

the female to attract potential mates from a distance (CSIRO, 1982). Sex pheromones play an important role in the elicitation of mating behaviour in moths and are, therefore, crucial for successful mating. Therefore, understanding the mechanisms that underlie sex pheromone production and the factors influencing it carries a great relevance.

Mating in moths usually occurs during a discrete period of the photophase/scotophase cycle, and in most cases is nocturnal (Jurenka, 2017). Sex pheromone biosynthesis in moths is affected by a variety of exogenous and endogenous factors such as temperature, photoperiod, host plants, age and mating, radiation as well as by endocrine and neuroendocrine factors (Eliyahu et al., 2003; Babilis, 1992; Raina et al., 1991; Xu et al., 1995, Flint, 1983). Raina and Klun (1984) reported that pheromone production in female *Helicoverpa* (then *Heliothis*) *zea* moths was controlled by a cerebral neuropeptide, which was termed pheromone biosynthesis activating neuropeptide (PBAN). PBAN was found to be a 33-amino acid, C-terminally amidated neuropeptide and the peptide was termed Hez-PBAN (nomenclature according to Raina and Ga¨de). It is generally presumed that pheromone production in many lepidopteran species is controlled by PBAN, as is the case in *S. litura* (Abernathy et al., 1995; Choi et al., 2009 and 2012; Chang, 2014; Fabrias et al., 1994; Matsumoto et al., 1995; Lu et al., 2015). In this insect, PBAN released by the brain+ suboesophageal ganglia (brain+ SOG complex) is transported through the haemolymph and targets the PBAN-receptor present on the pheromone glands to initiate secretion of pheromones in the same (Masler et al., 1994; Rafaeli, 2009; Jurenka, 2017). PBAN is produced by the *PBAN* gene which is photosensitive and shows a specific circadian rhythm in production and release depending on light (Zavodska et al., 2009; Iglesias et al., 1999, 2002).

In this study, the light dependent circadian rhythmicity of *PBAN* gene expression was analysed in the sterilized female moth, *S. litura* in view of the exclusively nocturnal mating behaviour exhibited by these moths. The female moths in this study were irradiated with 130 Gy identified as an appropriate complete sterilizing dose, which was also a partial sterilizing gamma dose proposed for male moths for use in F1 sterility technique) (Seth and Sharma, 2001; Seth et al., 2016 a,b; Seth et al., unpublished). Further, a higher dose of 200 Gy was also evaluated in female moths, to assess the gradational response of radiation. These irradiated female moths were studied for relative *PBAN* gene expression at scotophase and photophase to compare the effect of radiation on rhythmic expression of the gene.

MATERIALS AND METHODS

The culture of *S. litura* was maintained at 27±1ºC, 70± 5% RH and 12 hr light : 12 hr dark as photoperiodic regimen in an insectary on semisynthetic diet (Seth and Sharma 2001). The adult moths were placed in pairs of four in each cage made of Perspex and nylon (size, 20 x 20 x 20 cm) for the purpose of mating. In small plastic containers cotton swabs saturated with 10% (w/v) honey was placed as adult feed. The swabs were replaced every day. The leaves of castor were put in each cage to serve as ovipositional trap. The eggs were collected, surface sterilized and kept for incubation in high RH (80%). The larvae were reared on semi-synthetic diet till pupal stage, after which the eclosed adults were placed in the cages for pairing to continue the lifecycle.

For the present study, 0-1 day old adult females were exposed to gamma radiation doses (130 and 200 Gy) at a dose rate in the range of 0.625-0.429 KGy/hr in a Co^{60} research irradiator, Gamma Chamber 5000 (GC 5000) located at Institute of Nuclear Medicine and Allied Sciences (INMAS) of Defence Research and Development Organization (DRDO), Delhi-110054. For control, the non-irradiated female moths were exposed to the same conditions (light, temperature etc.) as the irradiated females except the radiation exposure. The relative gene expression study for pheromone biosynthesis activating neuropeptide (PBAN) was conducted by excising the head and upper thorax (brain+ SOG complex) of the adult female moths (treated and untreated). The sampling was conducted under different radiation doses (0, 130, 200 Gy) and two light (photophase and scotophase) regimens. The sampling was performed 4-6 hr after onset of photophase and scotophase as per the respective regimen.

Total RNA was isolated from the above tissues using Trizol reagent followed by phenol-choloroform extraction method and treated with DNase I. The RNA concentration in each sample was measured through NanoDrop 2000 C (Thermo Fischer Scientific). 1 µg of pure RNA treated with DNase was used for single stranded cDNA synthesis by Revert Aid First Strand cDNA synthesis kit following manufacturer's protocol. Gene-specific direct primers were designed for *PBAN* gene. FASTA sequence for mRNA obtained from NCBI and online program of primer quest tool was used to

design the primers and used for qPCR (quantitative real-time PCR). The primer used had a forward sequence 5'- CTCGGCAGGACGATGAATTT -3' and a reverse sequence 5'- CTGTTGGTACTCCTGACCATTC -3'. The quality and specificity of the primer was tested with the respective tissue by performing RT-PCR and the primer pair with a single melt curve peak was selected. qPCR was performed using SYBR green on Applied Biosystems ViiA7 real-time PCR system using standard run procedure. *EF1* was taken as reference gene for normalization with insect specific primers (Forward sequence 5' GACAAACGTACCCATCGAGAAG-3', Reverse sequence 5'-GATACCAGCCTCGAACTCAC-3'). The reference and gene of interest for all treatment groups were run in duplicates with each treatment group containing 6 replicates (n=6) in a single plate to avoid variations. Each 384-well PCR plate included the non-template and sample controls without reverse transcriptase, and an endogenous control of *EF1* gene (housekeeping gene). The relative expression of the targeted gene was determined by ΔΔCt method (Livak, 2001). This method used the difference in Ct value between reference and target genes to calculate an estimated fold change in the target gene. The negative of the resultant ΔΔCt value powered to 2 ($2^{-\Delta\Delta ct}$) was plotted as relative mRNA expression of the target gene. All statistical analyses were performed using Graph Pad Prism software (version 9.0), with 6 replicates, as specified in the text. One-way ANOVA, followed by Tukey's multiple comparisons was performed on each data set to analyze the differential effect of radiation on gene expression. The student's t-test was performed to compare the effect of light phases on gene expression under different radiation regimens with respect to control.

RESULTS AND DISCUSSION

F1 sterility technique for the control of a serious lepidopteran pest, *S. litura,* would usually involve the irradiation of male moths with proposed partially sterilizing gamma dose (like 130 Gy), and the radio-sterilized male moths would then compete with the wild males to mate with wild females leading to fully sterile or almost fully sterile F1 progeny and eventually pest suppression. With an aim to release both sexes simultaneously, 130 Gy (a proposed partial sterilizing gamma dose for male moths) was identified as complete (100%) sterilizing dose for female moths (Seth, unpublished) in view of differential radio-sensitivity of both sexes (female moths being more radiosensitive than male moths) (Traut,1977). The combined release program has been proposed to exclude the tedious procedure of sex-based segregation before irradiation treatment in the traditional SIT and F1 sterility technique (Kittayapong et al., 2018), but this release program should also be able to provide reproductively competent irradiated moths to the pest infested field to transmit the sterility effectively into the wild population. Therefore, it was necessary to understand the effect of female irradiation on its reproductive fitness. One of the crucial factors determining female reproductive fitness would be to produce pheromones and exercise the calling behaviour. PBAN (pheromone biosynthesis activating neuropeptide) is an important neuropeptide which triggers the biosynthesis of pheromone in the female moths.

Therefore, for the inclusion of irradiated female moths in the F1 sterility programs, it was imminent to study the effect of radiation on *PBAN* gene expression which might consequently affect the pheromone production at the time of mating. In this study, the *PBAN* gene expressed in the brain+SOG complex of female *S. litura* was quantified in scotophase and photophase with the moths exposed to different radiation doses (130 Gy and 200 Gy). The relative gene expression showed a declining trend as the radiation dose increased and the decrease was statistically significant in irradiated insects with respect to control [photophase (One way ANOVA, $F_{(2,15)} = 13.10$, $p < 0.05$), scotophase (One way ANOVA, $F_{(2,15)} = 4.69$, $p < 0.05$)] (Fig. 1). It has been seen in various nocturnal moths

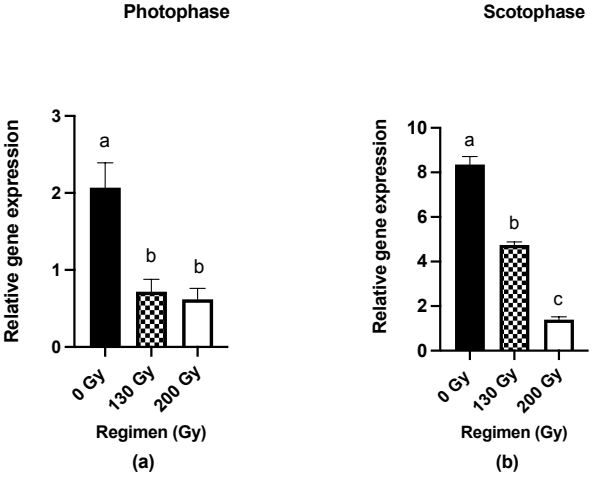

Fig. 1. *PBAN* gene expression of irradiated female *S. litura* moths in different light phases. (a) *PBAN* gene expression in photophase, (b) *PBAN* gene expression in scotophase. Means+SE followed by same letters not statistically different at $p<0.05$ (One-way ANOVA followed by Tukey's multiple comparisons)

that pheromone production which is triggered by *PBAN* gene expression is usually high at night (Levi-Zada, 2021). In this study we found similar results that the *PBAN* gene expression was higher at night (scotophase) than in the day (photophase) by female *S. litura,* that corroborated to the earlier studies. This diel rhythmicity is one of the factors responsible for maintaining the circadian rhythm of mating at night in the nocturnal insects (Zavodska et al., 2009; Bloch et al., 2013). This study showed the effect of radiation on the *PBAN* gene expression rhythmicity in the irradiated female *S. litura.* The irradiated insects showed a significantly reduced expression in photophase and scotophase in comparison to control. The diel variations of the *PBAN* gene expression was distinctly maintained in the irradiated females at 130 Gy (photophase vs scotophase, t-test, $p<0.05$) in comparison to control (0 Gy), whereas at higher dose of 200 Gy, the *PBAN* expression was markedly reduced in photophase and scotophase but diel variation was apparently noticed (Fig. 2).

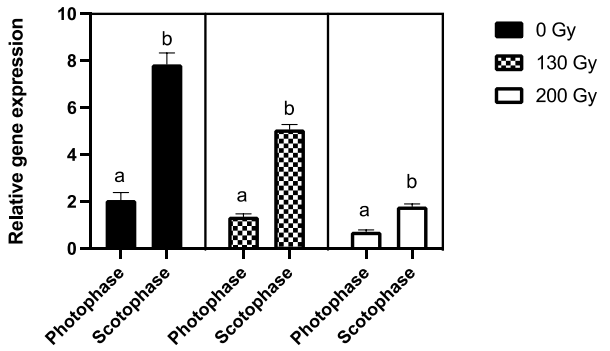

Fig. 2. Profile of *PBAN* gene expression with respect to photophase and scotophase in female moths irradiated at different doses. Means+ SE followed by different letters within each irradiation regimen statistically different at $p<0.05$. (Student's t-test for each radiation regimen comparing scotophase and photophase).

The *PBAN* gene expression observed in this study was influenced by ionizing radiation dose, and the decline in *PBAN* expression was apparent at 130 Gy but it was drastic at higher dose of 200 Gy, which validated the gradational response of gamma radiation and led to the selection of a preferable lower dose that was enough to completely sterilize the female moths. The gene expression analysis carried out in this study confirmed the diel rhythm of *PBAN* expression, and its predominant expression in scotophase corroborated with nocturnal behaviour. The current study provides an insight into the photosensitive expression of *PBAN* gene under different light regimen. In *S. litura,* the onset of mating was during the scotophase and the mating activity was not observed in the photophase. This rhythmicity of mating was also apparently reflected in the premating behavior that included the process of pheromone production, its release and male moth activation, followed by mating during the scotophase. These findings support the use of 130 Gy for complete sterilization of the female moths wherein *PBAN* gene expression profile observed was indicative of the circadian rhythmicity of the same, although the relative gene expression was less than control. This study on *PBAN* expression and its diel variations vis-a-vis control supported the reproductive competence of radio-sterilized female moths (130Gy) and their employment in the simultaneous release of both irradiated sexes in F1 sterility technique.

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AUTHOR CONTRIBUTION STATEMENT

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

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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