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Improved bioassay method for evaluation of oviposition deterrents against Old World bollworm, *Helicoverpa armigera* (Hübner)

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Old world bollworm *Helicoverpaarmigera* (Hübner) is one of the serious pests of agricultural crops with more than 184 recorded hosts including cotton. In cotton, *H. armigera*usually causes yield losses up to 40% with 20-80% damage intensity. In the Indian context it has already developed resistance to most conventional classes of insecticide and its survival on *Bt* cotton also has been reported in some isolated places. Under such situation, application of semiochemicals can serve as an alternative management option. Among the semiochemicals, oviposition deterrent ones are known to be the most effective as they minimize the infestation at first line of attack by deterring the female moths and protecting the host from oviposition. However, before applying at field level, it is important to develop and standardize a bioassay method for evaluation of oviposition deterrent compounds under laboratory condition. Here, we report a suitable improved bioassay method for evaluation of effect of oviposition deterrents against *H. armigera*. The five days duration of bioassay method was finalized according to the peak activity of adult moth in terms of mating and fecundity. This investigation presents a method, for finding promising oviposition deterrent compound which will be helpful for researchers to identify the most potent molecule/compounds against *H. armigera*.

Keywords: Cotton, Fecundity, Mating, Pest management; Semiochemicals

Cotton is one of the major cash crop grown across the globe for fibre and seed oil thus, designated as 'White gold'. The Old World bollworm, *Helicoverpaarmigera* (Hübner) (Lepidoptera: Noctuidae) is the major insect pest of cotton[1] with a wide spread distribution in Africa, Asia, Australia, Southern Europe[2] and in South America with the great possibility to reach towards North America[3]. Prior to introduction of *Bt* cotton in India, bollworm complex was responsible for the yield loss up to 40%[4]. Importance of these menaces can be justified by the fact that in India more than half of the insecticides are used solely for control of bollworms[5]. As a result, *H. armigera*has developed resistance to almost all groups of conventional insecticides *viz.*, carbamates, cyclodienes, organophosphates and pyrethroids[6-12]. Worldwide introduction of *Bt*cotton had made a big difference in overall management of bollworms. But the effectiveness of this technology slowly declined over the period and simultaneously increased the risk of development of resistance. In India also, *H. armigera* developed resistance[13] and successful reproduction on *Bt*cotton[14] has been recorded a decade ago. Variation in the expression levels of toxins in different cultivars might have helped *H. armigera*in developing resistance to *Bt*cotton[15].

Owing to difficulties in management of *H. armigera* on cotton, some alternative, environment friendly, pest management strategies need to be worked upon for proper management of this pest. Use of semiochemicals; to disrupt the feeding, mating and oviposition behavior of insects are the acceptable alternative for the management of insects as it alters the behaviour of insect[16]. Among the semiochemicals, generally oviposition deterrents (ODs) have been explored from many insects[17] including coleopteran[18], dipteran[19] and lepidopteran[20,21]insects pests. To assess the efficacy of these compounds as an oviposition deterrent, preliminary evaluation under laboratory condition is prerequisite before its field application at large scale. To address this issue, we have developed an improved bioassay method to determine effectiveness of test compounds under laboratory condition. Laboratory evaluation of oviposition deterrent compounds comprises few critical steps *viz.*, environmental conditions for experiment setup, finalization of most acceptable oviposition substrate,

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number of male and female pairs to be released for the experiment, application of compounds for uniform supply throughout the bioassay period for effective results and most important is duration of the bioassay. Therefore, in the present investigation, we tried to develop a reliable method for evaluation of oviposition deterrent compounds under laboratory condition against *H. armigera.*

Materials and Methods

The eggs and larvae of *H. armigera* were collected from fields of Indian Council of Agricultural Research- Central Institute for Cotton Research (ICAR-CICR), Nagpur (21°04'48.39"N 78°06'58.02"E) Maharashtra, India from cotton (*Gossypium hirsutum* L.) variety Suraj. Larvae were routinely reared on artificial diet under controlled environment conditions (65 ± 5% relative humidity (RH); 14L:10 D (L-Light, D- Dark) photoperiod 27 ± 1°C temperature) in insectary of ICAR-CICR, Nagpur.

Oviposition substrate

Total three experiments were designed to compare the relative suitability of oviposition substrate (natural and artificial) in terms of egg laying and hatching efficiency in female moth of *H. armigera*. In the first experiment (n=15), jars (transparent plastic container) were provided with cotton twig containing squares as a natural oviposition substrate (no choice experiment). In order to keep the twigs fresh for long time, a base of the cotton twigs was dipped in eppendorf tubes filled with water and covered with parafilm tape. In the second experiment, jars were provided with plain muslin cotton cloth as a top cover to the open end of the jar (no choice experiment) while in the third experiment, both muslin cloth as a top cover and cotton twig with square (dipped in Eppendorf tube) were provided to female of *H. armigera* (choice experiment). Numbers of pair (5 male & 5 female) were released in each jar and they were allowed to mate. Moths were provided with cotton swab dipped in 10% of honey solution every alternate day as moisture and energy source because adults feeding on honey solution required for egg maturation and mating. The preference index (PI)[22] for both substrates were calculated as PI= (B-A)/(A+B) * 100, where B = numbers of eggs deposited on muslin cloth and A= numbers of eggs deposited on cotton twig in choice experiment.

Number of male and female

Number of male (M) and female (F) pairs were standardized with series of experiments. Precise number of male and female (5:5, 4:4, 3:3, 2:2 and 1:1) of same age (1 day after emergence) were released separately in individual jars and were allowed to mate. Each jar was supplied with cotton swab dipped in 10% of honey solution, every alternate day as moisture and energy source. Cotton swab dipped in 10% honey solution was frequently changed on daily basis as honey tend to ferment, which may cause death of adult moth. Each plastic jar was closely observed for oviposition and hatching every day after scotophase of 48 hrs period. Since *H. armigera* females lay eggs singly at different intervals, the experiment was not terminated until the adult moth was alive.

Release of Oviposition deterrent compounds

During the experiment, different techniques were tried for appropriate release of synthetic oviposition deterrent compounds so that moths could get full exposure to the substrate. For example; cotton swab treated with compounds, glass vial (filled with compounds) with cotton wick, treated filter paper with compounds (below muslin cloth and above muslin cloth) [23] and treated muslin cloth were also used in the experiment.In treatments involving muslin cloth; test compound was supplied to the female moth in two different ways. An untreated muslin cloth was tied on jar containing pair of adults and compounds were directly smeared on muslin cloth, while in second setup, muslin cloth was dipped in compound for 20 seconds and was used to cover the open end of the jar. So, the treatments with seven replications for each under this experimental setup were as follows:T1, cotton swab treated with compounds; T2, glass vial (filled with compounds) with cotton wick; T3& T4, treated filter paper with compounds (below and above muslin cloth, respectively); T5& T6, treated muslin cloth (direct on jar and muslin cloth dipped for 20 s, respectively); and T7 control. Compound used in the experiment was Palmitic acid which was identified from the egg and larval fecal pellet of *H. armigera*[20] as an oviposition deterrent compound[24]. Numbers of eggs or hatching for control (C) and treatment (T) were counted. Statistical software SPSS Version 16.0 for windows was used to calculate mean. For comparison of mean values Tukey's HSD (honest significant difference) test at P=0.05 level of significance was used[25].

Bioassay protocol

After the finalization of oviposition substrate, number of male and female pair and method of

release of compounds; bioassay protocol was standardized. Mating of adult was allowed for the brief period of 48 h. After that, female was exposed to the treated oviposition substrate with identified compound (palmitic acid) with seven replications for each concentration (0.2, 0.4, 0.6, 0.8 and 1.0%). The oviposition substrate was dipped for 20 s to impregnate it completely.

The experiments were terminated on 5th day after the treated cloth was provided and numbers of eggs or hatching for control (C) and treatment (T) were counted.Moths were supplied with 10% of honey solution as described above. Moths were allowed to mate and lay eggs. The effectiveness of oviposition deterrent compounds was compared on the basis of the total number of eggs obtained at the end of an experimental period[26]. The data were further subjected to statistical analysis.The data collected on total number of eggs laid in each replication were summed up and compared with eggs laid for control (C) and treatment (T). The result is presented as: (a) Avoidance index (Ai): $Ai = (C - T)/(C + T)$[27] Ai = 1 indicates complete rejection of the test material; and(b) Per cent effective deterrence (PED) was calculated using the formula$PED\% = (NC - NT/NC) *100$ where, NC = Number of eggs in control, NT = Number of eggs in treatment

Statistical software SPSS Version 16.0 for windows was used to calculate mean and SEm. For comparison of mean values Tukey's HSD (honest significant difference) test at P=0.05 level of significance was used[25].

Results

Oviposition substrate

Oviposition preference was compared on cotton twig and muslin cloth under choice and no choice condition (Fig. 1). In the no-choice experiment, the numbers of eggs deposited on muslin cloth were 584.38±10.54 and on cotton twig it was only 18.38±2.28. In choice experiment, the mean numbers of eggs deposited on cotton twig were 79.75±1.74 and on muslin cloth the average was 674.13±13.18. The value of preference index in choice experiment clearly showed that muslin cloth was 78% more preferred substrate over cotton twig. Hence, muslin cloth was identified as better oviposition substrate.

Number of male and female

After finalization of oviposition substrate for egg laying number of male and female pairs were

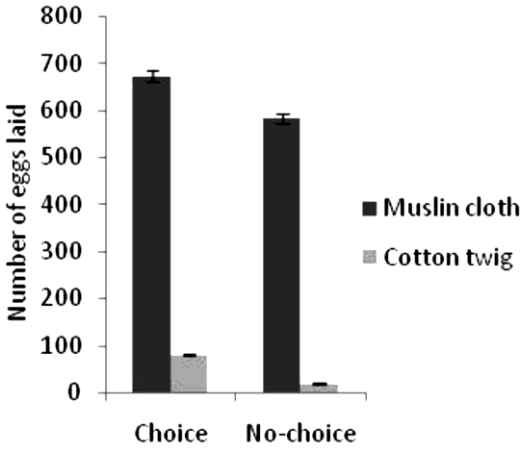

Fig. 1 — Number of eggs deposited by *Helicoverpaarmigera* under choice and no choice experiment

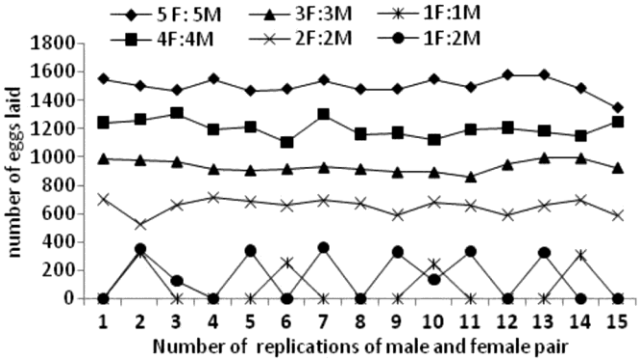

Fig. 2 — Egg laying pattern in different male (M) and female (F) pair of *H. armigera*

standardized for uniform egg laying by making different pairs (5:5, 4:4, 3:3, 2:2 and 1:1) that could be easily counted manually (Fig. 2). Using pair of 5 males and 5 females deposited approximately more than 1500±15.31 eggs which were not easy to count for number of treatments and replications hence increasing chances of manual error. Similar limitation also observed in a jar which has four pairs (1200 ±15.73 eggs) and three pairs (932 ±11.02 eggs) as well. Average number of eggs deposited in jar having two pairs was 649±13.65 which was more appropriate to count eggs. Oviposition pattern in a jar which has single pair was not consistent as in more than 50% jars female did not lay eggs. However, to increase the mating ratio number of male was increased to 2 males for 1 female but still some jars had no eggs. Therefore, it was concluded that 2 males and 2 females pairing was best for conducting bioassay.

Release of oviposition deterrent compounds

It is proved from the previous experiment (choice, no-choice) that female moth accepted the muslin cloth

as an oviposition substrate. Among different methods of treatment (T1 to T6) of oviposition deterrent compound *viz*., cotton swab treated with compounds, glass vial with cotton wick and treated filter paper with compounds (below muslin cloth and above muslin cloth), egg laying by female moth on treated jar was at par with control except in case of separate treatment of muslin cloth (Fig. 3). Even it was observed that muslin cloth treatment directly on prepared jar (with male female pairs), caused severe agitation and sometimes resulted in death of the adult moth due to pouring of some compound inside the jar. Among all, separate treatment of muslin cloth (for 20 s) was found the most acceptable method. The muslin cloths were completely dipped in experimental concentration for uniform coverage of the treatments on whole surface. Muslin cloth treatment was also promising as it caused full exposure of mated female to the oviposition deterrent compound treated surface because under laboratory condition female prefer to lay eggs on muslin cloth.

Bioassay protocol

Bioassays was conducted using identified compounds (with 99.99% purity) palmitic acid in order to evaluate their role as oviposition deterrents (ODs). Two pairs of newly-emerged male and female moths are allowed to mate in enclosed transparent plastic container (13.5 cm height and 11.5 cm diameter) during the scotophase for 48 h period. Transparent jars allowed the easy observation on activeness and mating of adult. Muslin cloth treated (separately) with experimental concentration for each identified compound can be used as oviposition substrate keeping diluents (methanol/water/hexane) as control with desired number of replications. The muslin cloths were completely dipped (for 20 s) for uniform coverage of the treatment on complete surface. The experiments were terminated on 5th day after the treatment and muslin cloth of each jar was stored in separate container under controlled conditions. In the present study number of eggs laid by the female decreased significantly with increasing concentration of palmitic acid compared with control (Table 1). Hence, value of Ai and PED were increased with the increasing concentration.

Discussion

In the present investigation, muslin cloth was found as a suitable oviposition substrate for *H. armigera*. Muslin cloth has been used widely in many studies for laboratrory rearing and bioassay studies in *H. armigera*[20,28,29]. Preference of *H. armigera* to the oviposition substrate depends on the other available substrate for oviposition. Preference to the muslin cloth over cotton twig might have been attributed due to roughness of muslin cloth and less preference to the cotton twig might be due to biochemical changes that have occurred in detached cotton twig[30]. Detached plant parts produce more ethylene than intact and promote abscission with high amounts of abscisic acid[31,32]. The present findings are in agreement with observation of Ramaswamy[33], who reported that majority of moth species preferred hairy or rough surfaces for oviposition. Surface texture of the substrate was also reported to be important factors for selection of an oviposition site than surface chemistry, presence of food, or humidity as in case of related species like *H. punctigera* (Wallengren)[34]. In contrast to our results, muslin cloth was also reported as least preferred substrate for *H. armigera*, in comparison to cotton wool, pigeon pea leaves and tissue paper[35].

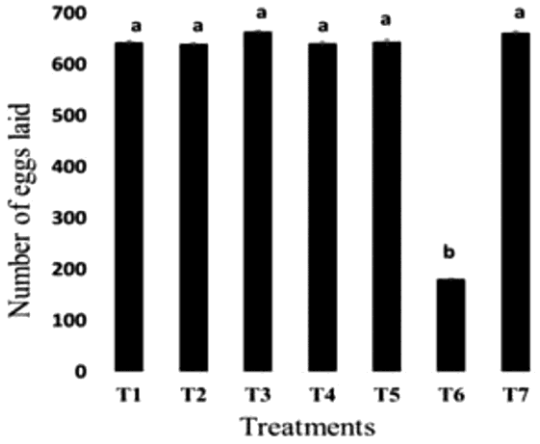

Fig. 3 — Egg laying pattern in different releasing method of oviposition deterrent compounds. Values followed by the same letters not significantly different at p 0.05 after Tukey's HSD test

Table 1 — Palmitic acid as oviposition deterrent against Old World bollworm

Treatments	No. of eggs laid	Avoidance index	PED
Control	564.71^{a}	0.00^{a}	0.00^{a}
Conc. of palmitic acid			
0.2%	471.29^{b}	0.09±0.05^{b}	16.44±5.09^{b}
0.4%	412.43^{bc}	0.15±0.04^{bc}	26.78±3.29^{bc}
0.6%	343.71^{c}	0.24±0.03^{c}	38.77±3.88^{c}
0.8%	223.14^{d}	0.43±0.03^{d}	60.41±1.52^{d}
1.0%	149.71^{e}	0.58±0.02^{e}	73.09±1.69^{d}

[Values followed by same letters are not significant at P= 0.05 Tukey's HSD (honest significant difference). *****Percentage effective deterrence (PED) values based on average number of eggs laid in different treatments]

Among different combinations of male and female pairs, 2 male and 2 female pairs were standardized for the release in bioassay studies. In oviposition deterrents (OD) bioassays the effective compounds can be compared on the basis of the total number of eggs obtained at the end of an experimental period[26]. Efficient counting of number of eggs and hatching is a most crucial step in the OD's bioassay which is mostly done manually. Hence, number of eggs deposited should be uniform and amenable for counting. In other pairing, either egg deposition was very high or egg laying pattern was not constant.

Separate treatment of muslin cloth for the evaluation of compounds as ODs was the best suited method. Similar methodologies were followed for the evaluation of leaf extract on oviposition study[28]. For ODs studies, it is compulsory that female come in complete contact of compounds. Muslin cloth was well accepted oviposition substrate in our study. Therefore, thorough treatment of the same was needed for the full exposure. Similarly, Thiery*et al*.[36], recommended use of different ovipositional substrate for different moths according to their acceptance of substrate in laboratory condition during evaluation of compounds on three other moth species of different families as an ODs, which was isolated from *Ostrinianubilalis*. Wax paper was used as a proven oviposition surface for mass production of European corn borer, *O. nubilalis* eggs[37] whereas, the cotton twig containing square was preferred for egg laying substrate by pink bollworm under laboratory conditions for evaluation of ODs[21].

In the present study, mating was allowed for the brief period of 48 h as 70% of *H. armigera* females commence calling to male within three nights of emergence[38] and first mating generally takes place between 2 to 4 nights[39].In fecundity test of *H. armigera* also, male and female were usually allowed to mate for 2 days *i.e.* 48 h after emergence[40]. The experiments were terminated on 5th day after the treatment as followed in the study conducted by Kathuria& Kaushik[35], because it is the high fecundity period for *H. armigera*[39,41] and finally numbers of eggs for control (C) and treatment (T) should be counted. In general, *Helicoverpa* sp. female moth commence oviposition once the proximal eggs attain full size and in laboratory-maintained adults pre-oviposition period ranged from 1-5 days[42] with its peak on 5th day[39].

The data can be further subjected to statistical analysis according to the need of result representation. The effective compounds can be compared on the basis of the total number of eggs obtained at the end of an experimental period. Avoidance index[27] and Per cent effective deterrence (PED) can be calculated for the interpretation. The relationship between increasing concentrations of fatty acids and numbers of eggs deposited by gravid female can be analyzed by regression analysis.

In the present study the bioassay method was designed keeping all the scientific reasoning as a base, so that researchers could replicate it in their laboratory conditions with slight modifications, if required. Use of muslin cloth as oviposition substrate and its direct treatment with compound ensure the constant and complete exposure of gravid female moth to the ODs compound. The number of female and male pair was reduced to only 2F:2M as a result large number of treatments could be attained because very small number of insect (adult moth) is required per replication. Number of eggs deposited is comparatively less therefore handling and counting is easy. No expensive equipment is needed for evaluating the efficacy of compounds right from treatment of muslin cloth to counting of eggs or larvae. Since, duration of the protocol is only five days, many experiments can be performed in a short period of time. Additionally, separation of muslin cloth after 5th day of treatment reduces the chance of escape of neonate larva. Continuous supply of honey as source of energy maintains the adequate food for adult so that any apparent loss in fecundity and longevity of moth could be avoided.

Conclusion

In the above study, bioassay method for evaluation of oviposition deterrents (ODs) was elaborated for exploring the new era of ethological management ofOld World bollworm, *Helicoverpaarmigera*. The efficacy of ODs compounds based on its significant exposure to the gravid female, finally concluded on the basis of reduction in egg laying and hatching efficiency of target insect. ODs compounds are the chemical which can create a defense line at very initial stage of infestation. Hence, bioassay of any compound should be adopted to ascertain the potency of particular test molecule before its release in the field condition. Till date, large number of compounds has been identified as ODs from various plant and

insect sources but none of them attained commercial importance. Therefore, intensive evaluation of the all identified compound is mandatory for further advancement in this direction.

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Conflict of Interest

Authors declare no competing interests.

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