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Role of fruit volatiles and sex pheromone components in mate recognition in fruit piercing moth *Eudocima materna* Linnaeus (Lepidoptera: Erebidae)

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Abstract
Specific mate recognition and host preference relies on the chemical cues in most animals especially in nocturnal insects. Host plant volatiles plays an important role in the perception of sex pheromone in males. Pheromone and plant volatiles are not perceived as independent messages and plant cues enhance discrimination of sex pheromone quality. This confirms the idea that specific mate recognition in noctuid moths - evolved in concert with adaptation to host plants. Shifts in either female host preference or sex pheromone biosynthesis give rise to new communication channels. The results of the present study indicated that *E. materna* demonstrated selective polyphagic feeding behavior and uses olfactory cues from preferred fruits to detect and locate potential food sources. Sex pheromone components of *E. materna* include (Z, E)-9, 11-Tetradecadienyl acetate (Major component) and Z-9-Tricosene and Z-9-Pentacosene (Minor components). To find an effective blend for field trapping, five blends of sex pheromone components were screened by Electroantennography (EAG). Blend that had (Z, E)-9, 11-Tetradecadienyl acetate (100 parts), Z-9-Tricosene (20 parts), Z-9- Pentacosene (20 parts) and 2-Ethyl Hexanol (20 parts) elicited maximum EAG response ($P<0.05$). Dose response experiments conducted at four doses *viz.*, 1, 10, 20 and 40µl through EAG, flight behaviour (wind tunnel) and field experiments revealed that, 40µl elicited higher electrophysiological and taxis responses and male moth catches compared to remaining blends. These findings hint at possible integral role of 2-Ethyl Hexanol with pheromone components in host finding.

Keywords: Blends, electroantennography, 2-Ethyl hexanol, field trapping, Z-9- Pentacosene (Z, E)-9, 11-Tetradecadienyl acetate

1. Introduction
Fruit piercing moths as pests are important economically in several countries. In view of the fast introduction and expansion of fruit cultivation, particularly of citrus and pomegranate, fruit sucking moths have gained significance recently. Thus, in India, fruit piercing moths were first recorded as serious pests [1] and later [2], in Tropical Africa [3] and [4]. Four major species *viz.*, *Eudocima fullonia* (Clerk), *Eudocima materna* (Linnaeus), *Eudocima homaena* (Hubner) and *Eudocima cajeta* (Cramer) and others were known to occur in India [5, 6, 7].
Moths of genus *Eudocima* have a highly specialized proboscis with hard spines capable of piercing hard, unripe fruits such as green citrus and tender pomegranates. Adult male and female moths penetrate the fruits at night. The damaged fruits become soft owing to secondary infections from different fungi, *Oospora* sp. [8]), *Fusarium* sp., *Colletotrichum* sp., and bacteria [9].These microbes gain entry through the hole pierced by the moths and/or inoculated by the infected proboscis [10] causing the fruits to rot and drop.The fruit-piercing moths attack many fruit and vegetable crops [11]. There are reports from Gujarat, India, that it also feeds on Cotton bolls [12].
Relative abundance of *E. fullonia*, *E. materna*, *E. homaena* and *A. janata* was 9%, 75%, 5% and 11%, respectively on pomegranate. The moths attack the Mrig bahar (August-November) crop of the pomegranate from 7.00 PM with peak activity at 11.00 PM and slight incidence until 2.00 AM with severe infestation on border row plants [13]. *E.* materna occurs in India, Africa, Southeast Asia, Australia and the South Pacific [14]. The fruit damage up to 57% was reported on pomegranate from Rahuri, Maharashtra [13].

Current suggested control measures for the management of fruit piercing moths are inefficient. The removal of all host plants in and around orchards is ineffective, because the moths are strong fliers and could fly long distances in search of food [15].

Though an ample number of parasitoid species documented to bout larvae of *E. materna* in India and Asia [16], they have not been exploited successfully in biological control programs due to the low level of parasitism. Different types of bagging material evaluated as protective measures against the fruit piercing moths are having their own limitation, as it is cumbersome and labor intensive [17].

Several fruit and non-fruit based baiting techniques screened for trapping the moths in the field but the desirable success is not achieved [14]. Insecticides are ineffective against *E. materna* owing to its typical piercing and feeding habits of adult moths. Use of chemical insecticides result in adverse effects on the export of fruits due to high residue levels in harvested fruits [13]. The partial accomplishment of these control approaches call for the development of alternative approaches like Semiochemical based pest management that is specific, effective, efficient, cheap and environmentally safe.

Non-chemical methods against the pest have not been attempted comprehensively. However, the sex pheromone compounds of the *E. materna* have not yet been identified. Considering these facts, a study was designed to isolate, identify and evaluate identified pheromone compounds and fruit volatiles against *E. materna.* In this regard, an attempt was made to utilize combination of pheromone and pomegranate fruit volatile (2-Ethyl Hexanol).

2. Material and Methods

2.1 Insect culture*: E. materna* insect culture was established in the laboratory in insect cages ($0.3m^3$) at Indian Council of Agricultural Research-National Research Center on Pomegranate (ICAR-NRCP), Solapur, Maharashtra and at rearing facility of Biocontrol Research Laboratories (BCRL) Pvt. Ltd. Sriramanahalli, Bengaluru, with field-collected adults from fruit bearing orchards at NRC on pomegranate, Solapur. Insect colonies were cultured in a 12 light (L)*:* 12 dark (D) regime with room temperature of 26±2 ºC and relative humidity of 70±10 percent. The field collected adult moths were sexed and five pair of males and females were transferred to the mass rearing cages of 60cm×40cm×80cm. The tissue culture trolley was covered with nylon mosquito net with mesh size of 1.2 mm. Adult moths were given 10 per cent honey solution and banana and/or pomegranate fruits were suspended in the cage. A bouquet (30-40 cm long) *of* fresh twig with leaves of larval host plant *Tinospora cardifolia was* put in cages for egg laying. Eggs were kept for hatching on nylon net and after 2-3 days of hatching of eggs, neonate larvae were picked by a fine camel hair brush (No. 000) and placed in plastic containers (7 cm dia, 12 cm height) and plastic tray {30×30×20cm} containing larval host plant leaves for individual and mass rearing and these were cultured in wooden rearing cages (0.35 × 0.35 × 0.60 m) provided with aeration on three sides. The first instar larvae were provided with tender host leaves (15-20 days) and food substrate was changed once in two days. From the second instar onwards the moderately matured (25-30 days) and matured leaves (>30 days) was provided. Food substrate was changed on daily basis and excreta and other extraneous substances were removed periodically. Once the larvae turned to pupae they were sexed and kept in separate cages (0.35 × 0.35 × 0.60 m) in small, medium and large petri plates measuring 9.0, 15.30 and 20.0 cm. Newly emerged adults were sexed on the venation of forewing and abdominal characters. In female moths, wings are more prominently striated with rufous; the silvery dots under and beyond cell very large and conjoined, crossed by white streaks above vein 2 and beyond cell is the most common differentiating character of the sexes externally **[18]**. Aforementioned procedure was adopted to get continuous culture of insects for further studies.

2.2 Blend preparation and bioassay

Three identified *E. materna* sex pheromone components *viz.*,(Z, E)-9,11-Tetradecadienyl acetate, Z-9-Tricosene, Z-9-Pentacosene with 2-Ethyl Hexanol were synthesized at the concentration of 20 mg/ml (2*%*) at chemical synthesis laboratory of BCRL, PCI Pvt. Ltd. and ICAR-NBAIR, Bengaluru. Following blends with their ratios were used with control solvent (Hexane) for further bioassays. Bioassays for pheromone blends were conducted using Electroantennography (EAG) and Wind tunnel at Insect Behavior Testing Laboratory (IBTL), BCRL and NBAIR, Bengaluru.

Table 1: Details of different blends used in the bioassay

Treatments	Blend No.	Blend components	Ratio of components
1	A	Z-9- Tricosene	20*:*
		Z-9- Pentacosene	20*:*
		(Z, E)-9,11-Tetradecadienyl acetate	100*:*
		2-Ethyl Hexanol	0*%*
2	B	Z-9- Tricosene	20*:*
		Z-9- Pentacosene	20*:*
		(Z, E)-9,11-Tetradecadienyl acetate	100*:*
		2-Ethyl Hexanol	20*%*
3	C	Z-9- Tricosene	20*:*
		Z-9- Pentacosene	20*:*
		(Z, E)-9,11-Tetradecadienyl acetate	100*:*
		2-Ethyl Hexanol	40*%*
	D	Z-9- Tricosene	20*:*
		Z-9- Pentacosene	20*:*
		(Z, E)-9,11-Tetradecadienyl acetate	100*:*
		2-Ethyl Hexanol	100*%*
4	E	2-Ethyl Hexanol	-
6	F	Hexane (Control)	-

	DF	5, 24
	F	5.132
	P	0.001

2.3 Electroantennography

Electrophysiological responses of male *E. materna* antennae to synthetic blends were recorded using Syntech EAG system. -Ten µl of blend was placed on the filter paper (6 cm length and 5 mm dia; Whatman No. 1) strip inside a glass Pasteur pipette (5.75, Length- Overall 145.0 mm; tip 47.0 mm) used for stimulus delivery. This was connected to the stimulus controller by silicone rubber tubing. After 10 seconds, the solvent was blown out with first puff. Another 60 seconds later, the stimulus was puffed on to the antenna by injecting the vapour phase of the chemical stimuli through a polyesterene tube along with the continuous air stream (pulse rate 0.5 s, continuous flow 25 ml s^{-1}, pulse flow 21 ml s^{-1}) to the test antenna. Before antennaectomy, moths were maintained at 6 ^{0}C for 1 min to make them inactive in plastella cups (dia 10cm). Antennae were incised using microscissors. Each recording was replicated five times. Best performed blend was tested at four different doses *viz.*, 1, 10, 20 and 40µl to find out the dose-variation response.

2.4 Wind tunnel

The blend having the best electrophysiological response from EAG was further tested to determine the behavioral effects in a wind tunnel in four above-mentioned doses to find out the dose-variation response. Flight behaviour of male *E. materna* to synthetic pheromone blends was recorded. A wind tunnel of 80 x 30 x 30 cm calibrated to 25 cm s^{-1}, 5 lux (light intensity), 26±1 ^{o}C and 65±5 *%* RH in a room separated from the *E. materna* culture was used in these studies. Observations on the number of moths taken to flight and number of moths reaching the zone where pheromone source kept was recorded.

2.5 Field trapping

Field trials were carried out to know the efficacy of different best performing blend at Kegaon (17° 43' 16.06" N; 75° 50' 39.31" E; 487 m Elevation 1.19 Km Alt) Solapur Maharashtra in pomegranate field during August-November, 2018. Lures used in the experiment were of polyethylene vial dispensers. Since the dose 1µl has not elicited better response in EAG and wind tunnel, so it was not considered for field trapping studies. So, doses *viz.*, 10, 20, 40µl were used in the field trapping experiment with control under Randomized Complete Block Design (RCBD). Experiment was replicated five times. Commercially available Ferro-T traps were used for trapping at 1.52 m height from the ground level. Within the field, individual traps were positioned 10-12 apart. Trap catches were counted daily and captured moths were removed. Data were pooled at weekly intervals for analysis. Mean data were compared using one-way ANOVA and the means were separated using Tukey's Honest Significant difference (HSD) test using IBM SPSS (version 25).

3. Results and Discussion

EAG responses (mV) of male *E. materna to* isomeric blends are summarized in Table 2. Among the blends of different sex pheromone components tested, blend of (Z, E)-9, 11-Tetradecadienyl acetate; Z-9-Tricosene; Z-9-Pentacosene and 2-Ethyl Hexanol (Blend B) elicited significantly higher EAG ($F_{5, 24}$=4.912; P=0.003) response than original components.

[19] Observed that *where host plant volatiles enhanaced responsiveness to pheromone blends* in European corn borer *moth, Ostrinia nubilalis* (Hubner) *but decreased attarction to single pheromone components. Volatiles from non host plants are less prefered* and *host plant red*uced *attraction to conspecific pheromones in S. littoralis and S. Frugiperda* [20, 21, 22, 23]. [24] Observed that *plant volatiles decreased male attraction to hetero specific or incomplete synthetic pheromones.* Different nutritional status might have led to altered pheromone production or responsiveness. The results of current study are in partial agreement with the above findings.

Table 2: EAG response **(**Mean ±SE**)** of ***E. materna*** male antennae to different blends of synthetic sex pheromone components and isomers with plant volatile, 2**-**Ethyl Hexanol

Treatments	Blend No.	EAG Response (mV) (Mean±SE)
1	A	1.84±0.17^{ab}
2	B	2.58±0.36^{a}
3	C	2.36±0.25 ^{ab}
4	D	2.28±0.24 ^{ab}
5	E	0.37±0.04^{c}
6	F	0.84±0.21^{ab}
DF		5, 24
F		5.132
P		0.001

Blend B revealed that addition of 2-Ethyl Hexanol, an identified pomegranate fruit volatile evoked higher response than other blends. Probably 2-Ethyl Hexanol enhanced the attraction activity of the pheromone components.

Recent studies conducted on synthetic blend of volatiles also suggested that there is a redundancy in the composition of host odor blends [25, 26 27, 24]). According [28], cetrain compounds in blend of volatiles used for host plant recognition play minor roles and can be *overlooked* without any significant loss in the level of attraction elicited. For example, attraction of grape vine moth females, *Lobesia botrana* [29] attracting to a 3-component blend was not significantly different from attraction to the full 10-component blend [30].

The EAD active compounds identified in the current study that is 2-Ethyl Hexanol is found in several other plants and this *is* in agreement with the report of [24].

Among different doses of best performed blend (Blend B) tested 1, 10, 20 and 40µl, the dose 40µl had significantly higher EAG response than remaining doses (R^2=0.955 y=0.358x+ 0.295) (Fig. 1). These results are in agreement with [24], who showed that dose-dependent increase in EAG response was observed with increasing doses of synthetic pheromone blend of *S. litura* (Fab.) (Noctuidae *:* Lepidoptera).

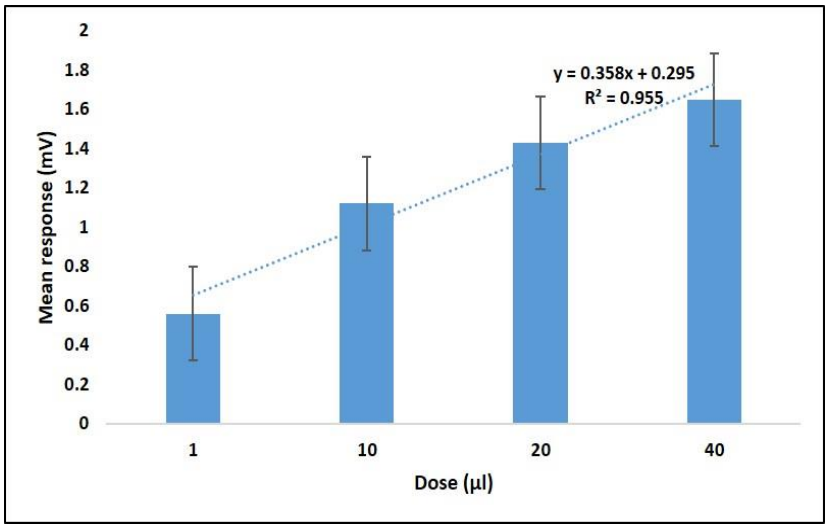

Fig 1*:* EAG dose response *E. materna* male antennae against increasing doses of synthetic blend B with 2-Ethyl Hexanol

3.1 Wind tunnel

EAG experiments clearly indicated that blend B had significantly higher response compared to other doses tested. Therefore, the behavioral assays in wind tunnel were conducted with this blend to determine the effect of different doses on male moth's flight. Results indicated that significant differences were noticed in male moths responding to doses compared to control except at 1µl (Table 3). Significantly higher taxis response was observed when 40 µl of synthetic blend was presented compared with the remaining doses ($F_{3, 16}$=5.124; P=0.015). The results of present investigations are in partial agreement with the results of Felipe *et al.* (2018) who demonstrated that when volatile compounds from cotton plant were tested one by one separately only α-farnesene elicited significant up -wind attraction in *S. littoralis* males. This may be due to the fact that in the present study, isomers of these components coupled with 1-Ethyl Hexanol, a possible synergist were used.

[24] Found that attraction to a combination of cotton volatile components and main pheromone compound significantly reduced compared to pheromone alone. While a combination of the same cotton blend with four pheromone-component did not reduce attraction. This indicated that 2-Ethyl Hexanol may serve as an integral part of the sex pheromone blend of the fruit piercing moths.

Table 3*:* EAG dose response (Mean ± SE) of *E. materna* male antennae to increasing doses of synthetic blend B

Treatments	Blend components	Dose (µl)	EAG Response (mV) (Mean±SE)
1	(Z, E)-9,11-Tetradecadienyl acetate Z9-Tricosene Z-Pentacosene 2-Ethyl Hexanol	1	0.56 ±0.11^{b}
2	(Z, E)-9,11-Tetradecadienyl acetate Z9-Tricosene Z-Pentacosene 2-Ethyl Hexanol	10	1.12±0.17^{ab}
3	(Z, E)-9,11-Tetradecadienyl acetate Z9-Tricosene Z-Pentacosene 2-Ethyl Hexanol	20	1.43±0.28^{ab}
4	(Z, E)-9,11-Tetradecadienyl acetate Z9-Tricosene Z-Pentacosene 2-Ethyl Hexanol	40	1.65±0.30^{a}
	DF		3, 16
	F		5.124
	P		0.015

Means followed by different letters in a column are significantly different by Tukey's Post-hoc test (P<0.05); * Mean of five replicates.

To determine the dose variation response, a series of bioassays were conducted with blends of different doses *viz.* 1, 10, 20 and 40µl separately were dispensed from filter paper. Antennal responses of males to different doses revealed that the blends elicited EAG response. The EAG activity increased with the dose of the synthetic pheromone blend. There were statistical significant differences among all the concentrations tested. All the concentrations tested were significantly different ($F_{3, 16}$=5.124; P=0.015). Stimuli elicited higher response at 40µl (1.65±0.30); 1µl had the lowest response (0.56±0.11) (Table 3).

3.2 Net house trapping

Using the Ferro-T traps in the net house cages, the tested blends and their doses were selected from EAG and wind tunnel tests. The effect of different pheromone blends at varying doses on male moth catches of *E. materna* were

determined by releasing known number of moths into the net house cages which were made of nylon mesh (50 moths for each dose of a blend; 10/day). *As* 1µl blends B did not elicit any behavioral response in wind tunnel compared to control. So these blends were not considered for further studies. Statistically significant higher number of male moths were trapped in the pheromone trap than in the control trap (Table 3). The mean trap catches was the highest for 40µl of Blend-B (9.25±1.13) compared to control (t=12.913; P=0.002; df=3) (Table 3). Thus the test used for evalaution of pheromones and results of net house cage studies confirmed that for *E. materna* male moths pheromone traps *and* pheromone blends proved effective. These were further taken to the field for evaluation.

Table 4*:* Mean catches of male *E. materna* to synthetic blend-B at different doses in net house cage

S No.	Doses (µl)	*Mean number of moths caught/day		*t*- test	P value
		Treatment	Control		
	10	6.50±0.40	0.5±0.0	16.15	0.001
2	20	7.25±0.91	0.5±0.0	7.156	0.007
3	40	9.25±1.13	1.0±0.0	12.913	0.002

* 10 moths were released per day (N= 5 days)

3.3 Field trapping

In the field experiment conducted with blend B at different doses, 40µl trapped 5.80 ±1.35 (Mean ± S.E) mean number of moths per trap significantly higher than remaining doses ($F_{3, 16}$= 3.453, P=0.042) (Fig. 2). Similar study conducted for attraction to cotton plant volatiles significantly reduced male attraction when added to the principle pheromone component Z9, E11-14Ac. Similar study conducted for attraction to cotton plant volatiles significantly reduced male attraction when added to the principle pheromone component Z9, E11-14Ac.While a combination of the same cotton blend with four-component pheromone did not reduce attraction (Felipe *et al.*, 2018). This indicated that 2-Ethyl Hexanol may serve, as an integral part of the sex pheromone blend. Moth catches in this study appears to be less, possibly due to experiment being conducted in regular experimental plots/block where the plant protection measures are taken as per IDIPM schedule. Because of this *E. materna* population could have been low.

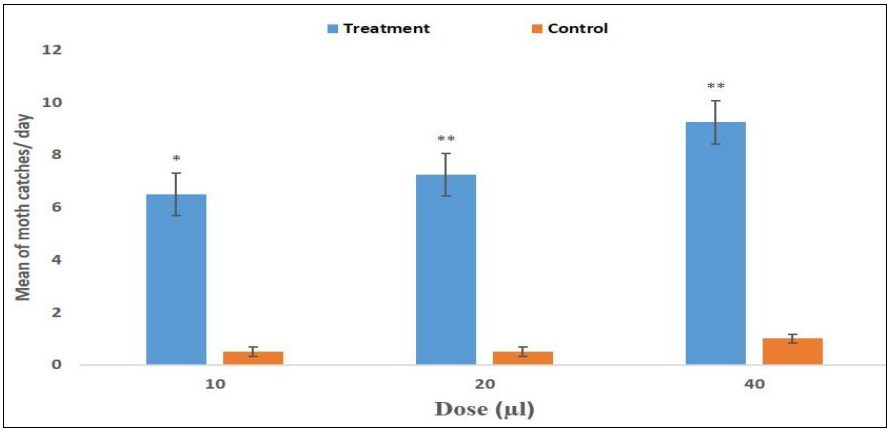

Fig 2*:* Catches of male *E. materna* to synthetic blend-B in field

A field experiment was conducted at Kegaon, Solapur during August 2018 where different dose of synthetic blend B were field evaluated. The results of the field experiment presented in Table 3. Traps were kept in the field for 15 days during August 2018 in and the trap catches were monitored daily under field conditions. In all 20 traps were placed in 1.2 acre pomegranate field at 15 meter apart. Statistical significant higher moth catches were obtained among the different doses compared to the control ($F_{3, 16}$=6.164; P=0.007). The average number of male moths trapped per trap was in blend having 20 µl (5.4±1.36) was on par with 40µl (5.1±2.48) and no significant difference in trapping of female moths was found (Table 4).

Table 4: Mean trap catches of male and female *E. materna* in traps baited blend-B with different doses in experimental field at Kegaon, Solapur.

S. No.	Doses (µl)	*Number of male moths caught/trap (Mean±SE)	*Number of female moths caught/trap (Mean±SE)
1	10	2.1±0.24^{ab}	1.30±0.24
2	20	5.4±1.36^{a}	1.70±0.37
3	40	5.1±2.48^{a}	1.98±0.24
4	Control	0.25±0.16^{b}	0.20±0.0
Df		3,16	3,16
F		6.164	2.821
P		0.007	0.184

Means followed by different letters in a column are significantly different by Tukey's Post-hoc test (P<0.05); * Mean of five replicates.

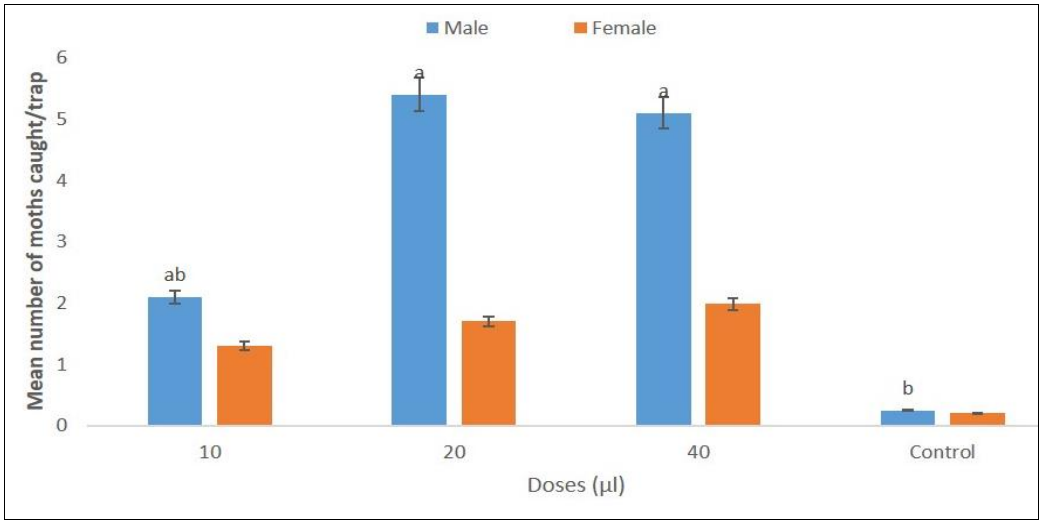

Fig 3*:* Mean trap catches of male and female ***E. materna*** in traps baited blend-B in experimental field at Kegaon, Solapur

Biological laboratory assays (EAG and wind tunnel) and field tests, revealed that the blend consisting of a (Z, E)-9, 11-Tetradecadienyl acetate; Z-9-Tricosene; Z-9-Pentacosene and 2-Ethyl Hexanol proved to be the most effective for catching moths. This study reported the possible synergism of 2-Ethyl Hexanol with the pheromone components/isomers of ***E. materna***. Based on moth trapping it would be important to use a pheromone concentration of 40µl in order to optimize attraction of moths under field conditions.

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