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The sex pheromone of Legume Pod Borer, Maruca vitrata (Lepidoptera: Crambidae) Revisited

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Abstract: Female produced sex pheromone of *Maruca vitrata* containing three components had been reported from countries in tropical Asia and sub-Saharan Africa. (*E*,*E*)-10,12-hexadecadienal as a major component, (*E*,*E*)-10,12-hexadecadienol and *E*-10-hexadecenal as minor components blended in the ratio of 100:5:5 was found to attract *M. vitrata* male moths in few countries in West Africa. However, this blend was found to have less or no effectiveness in trapping *M. vitrata* population in Asia, which triggered the need to refine the *M. vitrata* pheromone formulation. The isomers of major and minor pheromone compounds of *M. vitrata* were evaluated in this study. When subjected to Electroantennogram (EAG) analysis against male antennae, the isomer (*Z*,*E*)-10,12-hexadecadienal as a major component blended with the minor components (*E*,*E*)-10,12-hexadecadienol and (*E*)-10-hexadecenol in 100:10:5 was found to elicit better antennal response than the standard three component blend. GC-EAD profiles of the individual synthetic isomer components supported the EAG results. Additional experiments also confirmed the attraction of *M. vitrata* pheromone compounds and their isomers along with host plant volatiles in EAG and wind-tunnel experiments. Field experiments in India and Cambodia confirmed the effectiveness of improved *M. vitrata* pheromone lures in attracting the adult male moths in legume fields.

Keywords: *Maruca vitrata*; EAG profile; isomers of female sex pheromone; EAG/GC-EAD profiling; field trials.

1. Introduction

The Legume pod borer, *Maruca vitrata* Fab. (Lepidoptera: Crambidae; Syn: Maruca testulalis) is a pantropical insect pest of vegetable and grain legumes including cowpea, yard-long bean, pigeon pea, field bean and chickpea in the tropics and subtropics. It is widely distributed in Asia, Africa, Oceania and Americas (Sharma, 1998) [1]. *M. vitrata* can feed on at least 45 different host plant species in tropical Asia and sub-Saharan Africa (Malini et al., 2014) [2]. *M. vitrata* larvae create webs on floral buds, flowers and pods, and thus internally feeding on these plant parts. First instar larvae prefer flowers rather than pods or leaves. The mature larvae, especially from the third instar, are capable of damaging pods (Srinivasan et al., 2015) [3]. Hence, up to 80% yield losses

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have been reported in several vegetable and grain legumes due to *M. vitrata* damage in Asia and Africa (Singh et al., 1990 [4]; Afun et al., 1991 [5]; Dreyer et al., 1994 [6]; Ulrichs and Mewis, 2001 [7]).

Because of the extensive damage caused to the reproductive parts of legume crops, farmers apply pesticides indiscriminately against this pest. In Bangladesh, the country bean (Lablab purpureus) was sprayed at weekly or biweekly intervals – sometimes every day – to control *M. vitrata* (Hoque et al., 2002) [8]. A study in Thailand and Vietnam has confirmed that farmers heavily rely on synthetic pesticides to manage *M. vitrata* because no other methods are generally used (Schreinemachers et al., 2014) [9]. A recent study found that Cambodian farmers mixed an average of 3.7 pesticides together in a single spray to reduce the pest incidences in crops including yard-long bean (Schreinemachers et al., 2017) [10]. Hence, alternative pest management strategies are warranted to reduce the pesticide misuse.

Sex pheromones are an important component in integrated pest management programs, especially for monitoring, mass-trapping and/or mating disruption. Sex pheromone components of *M. vitrata* were already identified. The major compound is (*E*,*E*)-10,12-hexadecadienal (*EE*10,12-16:Ald) (Adati and Tatsuki, 1999) [11], whereas the minor components are (*E*,*E*)-10,12-hexadecadienol (*EE*10,12-16:OH) and (*E*)-10-hexadecenal (*E*10-16:Ald) (Downham et al., 2003) [12]. A synthetic pheromone lure for *M. vitrata* consisting of *EE*10,12-16:Ald, *EE*10,12-16:OH, and *E*10-16:Ald in 100:5:5 was attractive to male moths in Benin and Ghana, while *EE*10,12-16:Ald alone was most effective in Burkina Faso (Downham et al., 2004) [13]. Neither of the pheromone components was effective against *M. vitrata* in Southeast Asia, although it attracted significantly higher male moths of S. litura (Schläger et al., 2012 [14]; Srinivasan et al., 2015 [3]). Hence, it has become imperative to improve these blends and the current study has evaluated the isomers of major and minor components of *M. vitrata* pheromone in laboratory conditions in India, and field experiments in both India and Cambodia.

2. Materials and methods

***Insects*.** The larvae of *M. vitrata* were collected from the infested fields in Hesaraghatta Hobli, Bengaluru, Karnataka, India (13°11’26 20’’N: 77°31’10 50’’E) and reared in laboratory of Bio-Control Research Laboratories (BCRL), Bengaluru. Larvae were maintained individually in culture boxes to avoid larval cannibalism and periodically provided with bean (*Phaseolus vulgaris* or *Lablab purpureus*) pods as food. No artificial diet or vitamin supplements were used. Larvae fed for about 3-6 d (depending on the instar collected) entered into pupation for a period of 5-6 d at 27±2°C and 60±10% RH. The adults emerged were identified for its gender and fed with 10% honey solution through a swab of sterilized cotton. One to two days old adults were used for the Electroantennogram (EAG) studies.

***Chemical compounds*.** *M. vitrata* pheromone blend is a combination of three components, (*E*,*E*)-10,12-hexadecadienal, (*E*,*E*)-10,12-hexadecadienol, and (*E*)-10-hexadecenal. All the chemical compounds were synthesized at BCRL, Bengaluru and the isomeric purities (Table 1) were determined by GC system 7890A from Agilent Technologies. All the synthetic compounds were confirmed by GC-MS analysis on Agilent 7820A GC system interfaced to a 5977E mass selective detector (MSD) fitted with a HP-5 column (both 30-m X 0.25-mm id, 0.25 μm film; J&W Scientific, Folsom, CA, USA) and compared with the standard commercial pheromone blends from Pest Control (India) Pvt. Ltd.

Table 1. List of synthetic isomers used in the Electroantennogram study

Set numbers in EAG profiling 1	Isomers*	Isomeric Purity
Set 1	*(E,Z)*-10,12-hexadecadienal	90.61%
	(Z,Z)-10,12-hexadecadienal	93.56%
	(E,E)-10,12-hexadecadienal	100%
	(Z,E)-10,12-hexadecadienal	97.76%
Set 2	*(E,Z)*-10,12-hexadecadienol	90.3%
	(Z,Z)-10,12-hexadecadienol	94.76%
	(E,E)-10,12-hexadecadienol	99.9%
	(Z,E)-10,12-hexadecadienol	98.1%
Set 3	*(E)*-10-hexadecenal	99%
	(E)-10-hexadecenol	96.2%
	(Z)-10-hexadecenal	93.1%
	(Z)-10-hexadecenol	97.2%

For the follow up experiments, selected pheromone components/isomers and plant volatiles (Table 2) were synthesized and used at the concentration of 20 µg/mL (2%). The concentrated stock solution of pheromone compound / plant volatile was diluted in hexane to prepare the required concentrations. Different blends were prepared using different pheromone components/isomers and plant volatiles at different ratios for testing bio-efficacy.

Table 2. Purity of synthetic pheromone components/isomers and plant volatiles

S. No.	Pheromone component/isomer	Purity (%)
1	(*E, E*)-10, 12-hexadecadienal	100.00
2	(*E, E*)-10, 12-hexadecadienol	100.00
3	(*E*)-10-hexadecenal	99.00
4	(*Z,E*)-10,12-hexadecadienal	97.76
5	(*Z,E*)-10,12-hexadecadienol	98.10
6	(*Z*)-10-hexadecenal	93.10
7	1-octen-3-ol	97.00
8	(*Z*)-3-hexenol (*Z*3-6:OH)	98.50

Electroantennogram (EAG). The EAG studies were carried out with an EAG Combi Probe (Syntech, NL) supported by a Signal acquisition controller which records the elicited signal and a Stimulus controller which helps to manage the stimulus delivery parameters. EAG records a small

voltage fluctuation generated across the two ends of the antenna when stimulated with a pheromone/stimulant. The antenna of sexually matured adult moths is excised from the head and placed across a positive and negative electrode of EAG combi-probe using a highly conductive electrolyte gel (Signa gel, Parker Laboratories, Inc.). The DC potential was recorded on a computer using an IDAC -2 (Intelligent Data Acquisition Controller) A/D converter and software (EAG Pro v. 2, Syntech, Hilversum, The Netherlands). In each set of experiment, four to six isomers or their combinations were used to perform the analysis with five different antennae as five replications. Each replication of the treatment is a direct puff of stimulant which exposes about 2µg (approximately) compound on to the antenna. Besides isomers or isomer combinations, hexane and honey were used as control treatments. Treatments were prepared by inserting thin strips of filter paper (Whatman^{TM}) into glass Pasteur pipettes measuring about 10 cm and incorporating about 20 µL of sample which contain 2 µg compound after drying the solvent to get exposed on the antennae. Each treatment was puffed on to the antennae and the response was recorded in mV. The treatment(s), which elicited maximum antennal response was/were carried forward for comparison with other formulations for further set of studies.

EAG profiling 1. Entire experiment was grouped into three sets (Table 1), each of which containing six treatments including four isomers of (*E,E*)-10,12-hexadecadienal (major component) in set 1 and four isomers of (*E,E*)-10,12-hexadecadienol (minor component) in set 2, with honey as a standard and hexane (solvent) as a control, whereas for the minor component (*E*)-10-hexadecenal in set 3, their geometrical isomers and the 'aldehyde' and 'alcohol' components were also considered. This grouping was essential for avoiding the antennal saturation due to numerous exposures.

EAG profiling 2. The single, two and three component pheromone systems as available in literature were analyzed against the male *M. vitrata* antennae for comparison. Single component system is the major component (*E,E*)-10,12-hexadecadienal alone (Adati and Tatsuki, 1999) [11], two components system being the combination of (*E,E*)-10,12-hexadecadienal and (*E*)-10-hexadecenol in the ratio 90:10 (Hassan, 2007) [19] and three-component system consists of (*E,E*)-10,12-hexadecadienal, (*E,E*)-10,12-hexadecadienol and (*E*)-10-hexadecenal formulated in 100:5:5 (Downham et al., 2003) [12].

EAG profiling 3. Comparison was made between the Electroantennogram responses of *M. vitrata* male adults to different variants of two and three-component pheromone systems. The treatments were designed by formulating different isomers of major and minor components in 100:5 ratio in case of the variants of two blends (Downham et al., 2002) [18] and 100:5:5 in case of three component system, and the response to each of those combinations by the male *M. vitrata* antenna was recorded.

EAG profiling 4. Keeping the two-component system to which the insect has shown highest response [(*Z,E*)-10,12-hexadecadienal: (*E,E*)-10,12-hexadecadienol in the ratio 100:5] as a standard model, the isomers of aldehyde and alcohol variants of the minor component was added to formulate all the three components in a 100:5:5 ratio, and tested against male *M. vitrata* antenna.

EAG profiling 5. The treatments which elicited higher responses from each study were considered for a final round of comparison to determine the best formulation and compared with Benin blend (*E,E*-10,12-hexadecadienal: *E,E*-10,12-hexadecadienol: E-10-hexadecenal in 100:5:5 ratio), honey and hexane as control.

GC-EAD. For the identification of active components out of the twelve synthetic isomers, the entire EAG with its accessories, coupled to a 7890A GC System from Agilent Technologies was used. A capillary non-polar HP-5 column of 30-m X 0.320-mm and 0.25µ film thickness was used. The entire study was done in an oven program of 150°C initial temperature with a hold time of 2

min and increased at the rate of 10°C to reach a final temperature of 220°C. Recordings were made by the GC-EAD software from Syntech, NL. All the synthetic isomers used for the study were subjected to EAD profiling.

EAG profiling 6. Based on the results of earlier EAG and GC-EAD experiments, the treatments in this EAG profiling included the combination of pheromone compounds / isomers and/or plant volatiles to identify the best blend.

Wind tunnel bioassay. Behavioral assays were conducted in Insect Behavior Testing Laboratory (IBTL) of BCRL at 25±1°C and 65±5% RH. The tunnel was constructed using transparent Perspex acrylic sheet with 210cm x 45cm x 45cm dimensions. On one side, the wind tunnel was provided with service windows (15x15 cm), 20 cm away from both ends and one in the middle to introduce the source and insects. The pheromone blends having the better electrophysiological response from EAG 6 were further tested to determine the behavioral effects in wind-tunnel in a room separated from the *M. vitrata* colony. The control or blank (hexane) treatment was tested first to ensure that the wind-tunnel was not contaminated. Male moths were allowed to naturally leave the cage just after setting a stimulus source at the upwind end. Behavioral categories recorded as responses of males to the sources were scored as follows: NR: no response, moths did not respond (did not leave the release box); TF: moth took flight but did not fly upwind; UF: upwind flight by moth, but it did not reach within 20 cm of the source; SA: source approach, moth clearly followed plume and hovered in front of the source within 20 cm, but failed to contact the source; SC: source contact, made contact with or landed on the source (Hassan, 2007) [19]. Each experiment was terminated 2 h later and repeated four times with different set of moths.

Field Validation of most suitable pheromone blends on legume crops

(i) ***India***

Field experiments were carried out to know the efficacy of different blends at farmer's field in Kakol (13°11'16.59" N; 77°30'48.75" E; 882 m amsl) village of Bengaluru rural district, Karnataka, India. For each blend, separate *Dolichos* bean fields were used to evaluate the trap catches to varying doses of different blends. Lures were loaded with 5, 25 and 50 µLof pheromones with control (hexane). Each treatment was replicated for five times. Commercially available Wota-T water traps were used for trapping. Synthetic pheromone lures used in the experiment were prepared at BCRL using polyethylene vial dispensers (23 mm x 9 m x 1.5 mm thick; Just Plastics, London, E10 7PY, U.K). The experimental design used was a randomized complete-block design (RCBD).

(ii) ***Cambodia***

Three different field trials were conducted in Kandal Province, Cambodia during June – September 2015, August – November 2016 and October – December 2017 to evaluate the sex pheromone lures against *M. vitrata* on yard-long bean. The trials in 2015 and 2016 tested four different lures (codes H, I, J & K, Table 3) along with an untreated control, each being replicated for three times, following the RCBD. The third trial in 2017 used only one improved lure (code K) with an untreated control and each treatment was replicated for eight times. The pheromone lures were prepared at BCRL using polyethylene vial dispensers (23 mm x 9 m x 1.5 mm thick; Just Plastics, London, E10 7PY, U.K). The weekly trap catches of *M. vitrata* as well as *Spodoptera litura* male moths and yield at every harvest were recorded.

Table 3. Composition of lures from pheromone components of *M. vitrata* and the host plant volatile.

Sample code	Composition	Ratio	loading
H	*Z*,*E*-10,12-hexadecadienal (*E*,*E*)-10,12-hexadecadienol (*Z*)-10-hexadecenol 1-octen-3-ol	100 parts 5 parts 5 parts 10 %	0.2 mg
I	*Z*,*E*-10,12-hexadecadienal (*E*,*E*)-10,12-hexadecadienol (*Z*)-10-hexadecenol 1-octen-3-ol	100 parts 5 parts 5 parts 10 %	0.5 mg
J	*Z*,*E*-10,12 hexadecadienol (*E*,*E*)-10,12-hexadecadienol (*Z*)-10-hexadecenol 1-octen-3-ol *Z*,3-hexenyl acetate	100 5 5 10% 10 %	0.5 mg
K	*Z*,*E*-10,12-hexadecadienal (*E*,*E*)-10,12-hexadecadienol *E*-10-16 OH 1-octen-3-ol	100 10 5 50%	0.5 mg & 1 mg

Statistical analysis. Statistical analyses were carried out on the electrophysiological responses (mV) of various blends by Analysis of Variance (ANOVA) and treatment means were separated by Tukey's post hoc test. But for wind tunnel data, chi-square (χ^2) cross-tabulation was first carried out to determine whether significant difference existed among different behavioral categories. Once this was validated, a non-parametric multiple regression approach (Beasley and Schumacker, 1995) [15] was used to determine whether the proportion of insects making a particular response differed significantly between an individual treatment and the control. Data were analyzed using the IBM-SPSS (version 21). Moth trap catches of different experiments were compared separately using one-way ANOVA and the means were compared using Tukey's post hoc test. The analysis of data from India was carried out using IBM SPSS (version 21), whereas the data from Cambodia was analyzed using SAS version 9.1 (SAS Institute, Cary, USA).

3. Results and Discussion

EAG profiling 1. The results of the EAG profiling of the three sets containing individual pheromone components (major & minor) and their isomers had shown interestingly different response patterns (Tables 4-6). The result showed that significant response of the male *M. vitrata* moths was recorded for the (*Z, E*)- isomer of the major component, when compared to the standard (honey) (Table 4). There is a maximum response by adult male *M. vitrata* antenna to elicit a response magnitude of 2.72mV to the (*Z*,*E*)- isomer of major component, *viz.*, (*Z*,*E*)-10,12-hexadecadienal, followed by the isomers (*Z*,*Z*) (2.23mV), (*E*,*E*), and (*E*,*Z*) (1.82mV), respectively. The individual EAG analysis of minor components showed the responses to (*Z*,*E*)-10,12-hexadecadienol (1.92mV) (Table 5) and *E*-10-hexadecenal (1.20mV) (Table 6), but the third component analysis showed non-significance. Thus, the responses of male *M. vitrata* moths to one of the minor components were not significantly different. Hence, only those treatments which showed maximum response were considered for further formulations.

Table 4. EAG responses of male *M. vitrata* moths to Set. 1: *E* & *Z* isomers of 10,12-hexadecadienal.

Pheromone isomer	Mean (±SEM) EAG response (mV)
(*E*,*Z*)-10,12-hexadecadienal	1.82±0.33 ab
(*Z*,*Z*)-10,12-hexadecadienal	2.23±0.35 a
(*E*,*E*)-10,12-hexadecadienal	1.82±0.30 ab
(*Z*,*E*)-10,12-hexadecadienal	2.72±0.55 a
Honey	1.48±0.17 ab
Hexane	0.49±0.06 b
Df	5, 24
F value	5.200**
P value	0.002

Values followed by same letter(s) in the column are not significantly different, (Tukey's HSD).
**- highly significant difference at P<0.01, N=5.

Table 5. EAG responses of male *M. vitrata* moths to Set. 2: isomers of 10,12-hexadecadienol.

Pheromone isomer	Mean (±SEM) EAG response (mV)
(*E*,*Z*)-10,12-hexadecadienol	1.07±0.13 b
(*Z*,*Z*)-10,12-hexadecadienol	1.46 ±0.20 ab
(*E*,*E*)-10,12-hexadecadienol	1.27±0.31 ab
(*Z*,*E*)-10,12-hexadecadienol	1.92±0.33 a
Honey	1.91±0.29 a
Hexane	0.96±0.16 a
Df	5, 24
F value	2.779*
P value	0.041

Values followed by same letter(s) in the column are not significantly different, (Tukey's HSD).
**- highly significant difference at P<0.01, N=5.

Table 6. EAG responses of male *M. vitrata* moths to Set. 3: *E* & *Z* isomers of 10-hexadecenal and 10-hexadecenol.

Pheromone isomer	Mean (±SEM) EAG response (mV)
(*E*)-10-hexadecenal	1.20±0.24 a
(*E*)-10-hexadecenol	0.78±0.12 a
(*Z*)-10-hexadecenal	1.15±0.31 a
(*Z*)-10-hexadecenol	0.91±0.19 a
Honey	1.04±0.22 a
Hexane	0.51±0.12 a
Df	5, 24
F value	1.480 ^{NS}
P value	0.233

Values followed by same letter(s) in the column are not significantly different (Tukey's HSD). NS-Non significant difference at P<0.05, N=5.

EAG profiling 2. The single [(*E,E*)- 10,12-hexadecadienal], two [(*E,E*)- 10,12-hexadecadienal and (*E*)-10-hexadecenol] and three [(*E,E*)-10,12-hexadecadienal, (*E,E*)-10,12-hexadecadienol and (*E*)-10-hexadecenal]-component pheromone systems had shown significantly higher responses of male *M. vitrata* moths, followed by single component [(*E,E*)-10,12-hexadecadienal] (Table 7). Hence the two component system reported by Hassan (2007) [19], which has only one minor component blended to the major component (*E,E*)-10,12-hexadecadienal in high ratio (90:10) was replaced with another combination where (*E,E*)-10,12-hexadecadienal and (*E,E*)-10,12-hexadecadienol served as a new two component system.

Table 7. EAG responses of male *M. vitrata* moths to different pheromone blends.

Pheromone blend	Ratio	Mean (±SEM) EAG response (mV)
(*E,E*)-10,12-hexadecadienal:(*E,E*)-10,12-hexadecadienol:(*E*)-10-hexadecenal	100:5:5	1.36±0.18 a
(*E,E*)-10,12-hexadecadienal	100	1.08±0.15 ab
(*E,E*)-10,12-hexadecadienal:(*E*)-10-hexadecenal	90:10	1.04±0.10 abc
Honey (Standard)	-	0.71±0.12 bc
Hexane (control)	-	0.51±0.07 c
Df		4, 20
F value		6.351**
P value		0.002

Values followed by same letter(s) in the column are not significantly different, (Tukey's HSD). **- highly significant difference at P<0.01, N=5

EAG profiling 3. The EAG results showed that the formulations of two component system [(*Z*,*E*)-10,12-hexadecadienal, and (*E*,*E*)-10,12-hexadecadienol], and a three-component system [(*Z*,*E*)-10,12-hexadecadienal, (*E*,*E*)-10,12-hexadecadienol and (*E*)-10-hexadecenal] were found to elicit the highest significant response, followed by another three component system [(*Z*,*E*)-10,12-hexadecadienal, (*Z*,*E*)-10,12-hexadecadienol and (*E*)-10-hexadecenal], and two other two component systems [(*E*,*E*)-10,12-hexadecadienal + (*E*,*E*)-10,12-hexadecadienol) and (*Z*,*E*)-10,12-hexadecadienal + (*Z*,*E*)-10,12-hexadecadienol)] (Table 8).

Table 8. EAG responses of male *M. vitrata* moths to variants of 2 & 3-component pheromone systems.

Pheromone blend	Ratio	Mean (±SEM) EAG response (mV)
(*E*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12hexadecadienol: (*E*)-10-hexadecenal	100:5:5	1.5062±0.1089 abc
(*E*,*E*)-10,12hexadecadienal : (*E*,*E*)-10,12-hexadecadienol	100:5	2.0710±0.3875 ab
(*Z*,*E*)-10,12-hexadecadienal: (*Z*,*E*)-10,12-hexadecadienol: (*E*)-10-hexadecenal	100:5:5	2.1404±0.2662 ab
(*Z*,*E*)-10,12hexadecadienal : (*Z*,*E*)-10,12-hexadecadienol	100:5	2.0052±0.3746 ab
(*Z*,*E*)-10,12-hexadecadienal : (*E*,*E*)-10,12-hexadecadienol : (*E*)-10-hexadecenal	100:5:5	2.2692±0.1999 a
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol	100:5	2.2670±0.2315 a
Honey (Standard)		1.1116±0.1172 bc
Hexane (control)		0.4976±0.1415 c
F test		7, 32
SEm(±)		6.819**
P value		0.0001

Values followed by same letter(s) in the column are not significantly different, (Tukey's HSD). **- highly significant difference at P<0.01, N=5

EAG profiling 4. Keeping the two-component system to which the insect showed highest response [(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol in the ratio 100:5] as a standard model, the isomers of aldehyde and alcohol variants of the minor component was added to formulate all the three components in a ratio of 100:5:5. The aim was to screen most efficient formulation and compare it against the so far proven-to-be-efficient blends (based on the EAG response by the male antenna). However, all the blend combinations elicited responses similarly,

because there is no significant difference among the pheromone blends (Table 9). Nevertheless, individual treatments were found to be highly significant when compared with honey as standard and hexane as control.

Table 9. EAG responses of male *M. vitrata* moths to different formulations.

Pheromone blend	Ratio	Mean (±SEM) EAG response (mV)
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenal	100:5:5	3.0944±0.2743 a
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenol	100:5:5	3.2148±0.2282 a
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*Z)*-10-hexadecenal	100:5:5	2.9972±0.2476 a
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*Z)*-10-hexadecenol	100:5:5	2.7626±0.1962 a
Honey (Standard)		0.8516±0.1928 b
Hexane (control)		0.4240±0.0584 b
F test		5, 24
SEm(±)		34.740**
P value		0.0001

Values followed by same letter(s) in the column are not significantly different, (Tukey's HSD). **- highly significant difference at P<0.01, N=5

EAG profiling 5. The treatments which elicited higher responses from each study and also the Benin blend were considered for a final round of comparison to determine the final best formulation in the entire study. The results showed that the blend (*Z,E*)-10,12-hexadecadienal, (*E,E*)-10,12-hexadecadienol and (*E*)-10-hexadecenol in 100:10:5 elicited significantly higher responses, followed by (*Z,E*)-10,12-hexadecadienal: (*E,E*)-10,12-hexadecadienol (100:5), (*Z,E*)-10,12-hexadecadienal: (*E,E*)-10,12-hexadecadienol: (*E)*-10-hexadecenol (100:5:5) and (*Z,E*)-10,12-hexadecadienal: (*E,E*)-10,12-hexadecadienol: (*E)*-10-hexadecenal (100:5:5). However, the two treatments were statistically on par and higher than Benin blend. (Table 10).

Table 10. EAG responses of male *M. vitrata* moths to different formulations which elicited higher responses.

Pheromone blend	Ratio	Mean (±SEM) EAG response (mV)
(*E*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenal	100:5:5	2.0522±0.1869 b
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenal	100:5:5	2.6560±0.2656 ab
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol	100:5	3.0030±0.3411 ab
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenol	100:5:5	2.9290±0.3170 ab
(*Z*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenol	100:10:5	3.4916±0.2477 a
Honey (Standard)		0.7320±0.0839 c
Hexane (control)		0.5066±0.1092 c
F test		6, 28
SEm(±)		23.459**
P value		0.0001

Values followed by same letter(s) in the column are not significantly different, (Tukey's HSD). **- highly significant difference at P<0.01, N=5

The need for improving the existing three-component pheromone blend for mass trapping *M. vitrata* adults became necessary, since the traps baited with the lures containing the standard pheromone blend (Benin blend) (Downham et al., 2004) [13], trapped adults of *M. vitrata* only in Benin, but failed in Asia (Schläger et al. 2012 [14]; Srinivasan et al. 2015 [3]). This could be due to the presence of possible pheromone polymorphism (Schläger et al. 2015) [16] or due to differences in pheromone composition between Asian and African *M. vitrata* populations or geographically distinct *Maruca* species or sub-species (Malini et al. 2015) [17].

The standard three component system containing the Benin blend was showing higher EAG response than single and two component systems which confirmed that three components are present in *M. vitrata* pheromone but the ratio should be fine-tuned to be more attractive. During the elaborate field studies in Benin (Downham et al., 2002) [18], different combinations of three components of Benin blend including 100:0:0, 100:5:0; 100:0:5 and 100:5:5 ratio of (*E*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol: (*E)*-10-hexadecenal were considered. Hence, two component blend of (*E*,*E*)-10,12-hexadecadienal: (*E*,*E*)-10,12-hexadecadienol in 100: 5 ratio, without the presence of third component was considered for one of the EAG profiling experiments in the current study. Between the two and three component systems, surprisingly the response magnitude was found to be higher in two component system than the three component

system which may be due to the geographical variation in *Maruca* species or sub-species as confirmed by Malini et al. (2015) [17]. It has to be noted that *M. vitrata* populations from Benin, Taiwan, Thailand and Vietnam were found to produce only (*E*,*E*)-10,12-hexadecadienal and (*E*,*E*)-10,12-hexadecadienol, but (*E*)-10-hexadecenal was absent in all the four populations, according to a study by Schläger et al. (2015) [16]. Hence, these two compounds, *viz.*, (*E*,*E*)-10,12-hexadecadienal and (*E*,*E*)-10,12-hexadecadienol are believed to play a vital role in the pheromone communication of *M. vitrata* in Asia.

It is interesting to note that EAG profiles of all single component systems have clearly demonstrated the highest response for (*Z*,*E*)-10,12-hexadecadienal. In addition, wherever compared with (*E*,*E*)- isomer in the modified two component as well as three component systems, (*Z*,*E*)- isomer was showing higher response indicating that (*Z*,*E*)- 10,12-hexadecadienal would be essential as the major component in the *M. vitrata* pheromone blend to increase the attraction of male moths. Incidentally, (*Z*,*E*)-10,12-hexadecadienal is found to play a significant role in the chemical communication systems of several Sphingid moths including broad-bordered bee hawk-moth (*Hemaris fuciformis*), the Snowberry clearwing (*H. diffinis*), one-eyed Sphinx or Cerisy's Sphinx(*Smerinthus cerisyi*), twin-spotted Sphinx (*S. jamaicensis*), eyed hawk-moth (*S. ocellatus*) and *Gracilaria elongella* (Reed et al., 1987 [20]; Paczkowska et al., 2012 [21]).

***EAD Profiling*.** All the synthetic isomers used in the study were subjected to EAD profiling. All the compounds in the final formulation, *viz*., (*Z*,*E*)-10,12-hexadecadienal, (*E*,*E*)-10,12-hexadecadienol, and (*E*)-10-hexadecenol were found to elicit a significant positive response on the mounted insect antenna once in three consecutive replications performed (Fig 1). Within the number of trials, except (*Z*)-10-hexadecenol which induced a significant response, Benin blend components elicited minimal response and all other isomers failed to elicit any positive response on the mounted antenna and hence they were not considered for further studies.

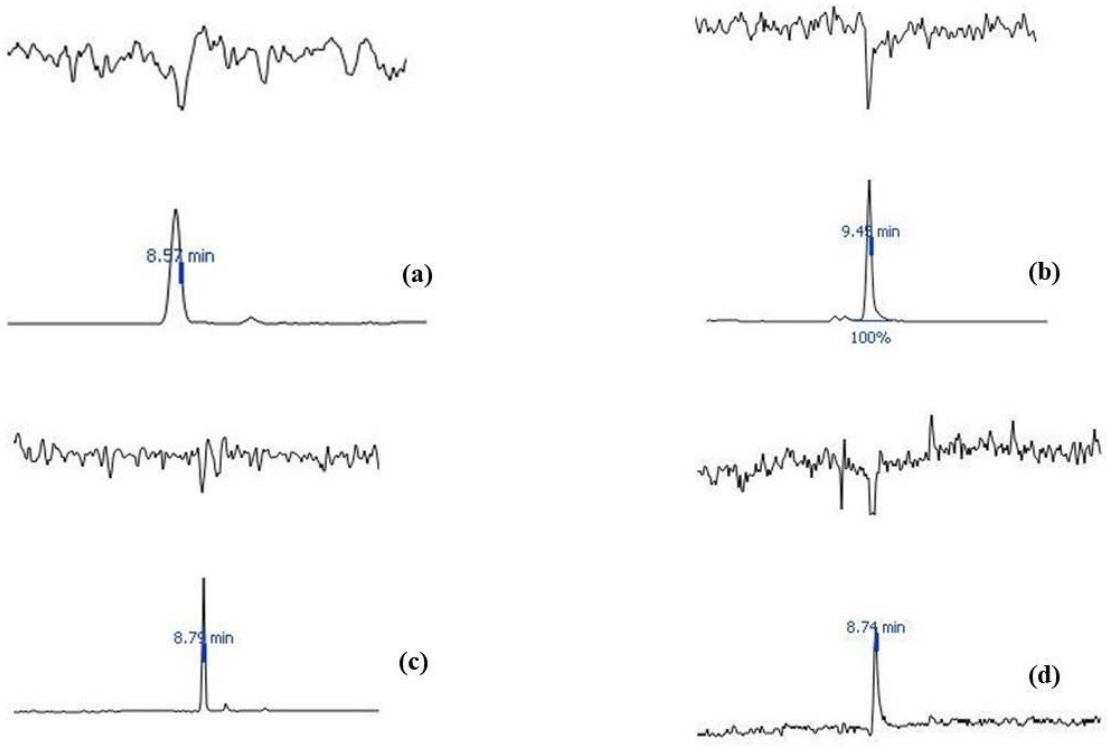

Fig 1. EAD profile showing the response of *M. vitrata* male antenna to **(a)** (*Z,E*)-10,12-hexadecadienal; **(b)** (*E,E*)-10,12-hexadecadienol; **(c)** *E*-10-hexadecenol and **(d)** *Z*-10-hexadecenol.

EAG profiling 6. Based on earlier results, three superior blends *viz*., blends-M, P and T were identified. These three blends were compared with the existing standard Benin blend (S). Results indicated that all the blends elicited significantly high responses ($F_{3,16}$=7.663; P=0.002) (Table 11). Among the different blends compared, blend-M elicited higher EAG response (1.51±0.15) that was on par with blend-P (1.39±0.13) and blend-T (1.38±0.15). Benin blend had the lowest EAG response (0.70 ±0.11). Results indicated that addition of host plant volatiles to sex pheromone blends significantly increased EAG response than when presented pheromone blends alone indicating synergism between pheromone components and plant volatile.

Table 11. Comparison of EAG response (Mean ±SE) of *M. vitrata* male antennae to different blends with Benin blend.

Sl. No.	Blend components	Ratio of components	Blend code	*EAG Response (mV) (mean±SE)
1	(*E*,*E*)-10,12-hexadecadienal (*E*,*E*)-10,12-hexadecadienol (*E*)-10-hexadecenal	100 5 5	S	0.70 ±0.11 b
2	(*Z*,*E*)-10,12-hexadecadienal† (*E*,*E*)-10,12-hexadecadienol (*Z*)-10-hexadecenal† 1-octen-3-ol	100 5 5 10%	M	1.51±0.15 a
3	(*Z*,*E*)-10,12-hexadecadienal† (*E*,*E*)-10,12-hexadecadienol (*Z*)-10-hexadecenal† 1-octen-3-ol *Z*3-6:OH	100 5 5 10% 10%	P	1.39±0.13 a
4	(*Z*,*E*)-10,12-hexadecadienal† (*E*,*E*)-10,12-hexadecadienol (*E*)-10-hexadecenal 1-octen-3-ol	100 5 5 50%	T	1.38±0.15 a
	Df			3, 16
	F			7.663
	P			0.002

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

Results of this study are consistent with earlier findings that in Lepidoptera, several cases of synergism were observed in both laboratory and field experiments (Giblin-Davis et al., 1996; Dowd and Bartlet, 1991; Phillips et al., 1984; Conner et al., 1981; Dickens, 1989) [22~26]. For instance, mixtures of green leaf volatiles (GLVs) from cabbage (*Brassica oleracea* L.) and the pheromone [a mixture of (*Z*)-11-hexadecenal, (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenol] induced higher attraction and arresting behavior in unmated males of the diamondback moth (*Plutella xylostella*) than the pheromone alone (Reddy and Guerrero, 2004) [27]. It may be concluded that there is a likely synergistic action of 1-octen-3-ol and *Z*3-6:OH with the pheromone components or its isomers, since it was already demonstrated that cowpea volatile (1-octen-3-ol) can possibly augment responses in both sexes of *M. vitrata* (Bendera et al., 2015) [28]. Hence,

these types of synergistic interactions used by male or female insects were probably aimed to optimize mating opportunities (Reddy and Guerrero, 2004) [27].

Wind-tunnel bioassay. Behavioral responses of *M. vitrata* moths in wind tunnel showed that there were no significant differences among the blends tested for 'TF' (χ^2=4.705, df=3, P=0.51) (Table 12). But, significantly highest number of moths responded to blend-M under 'UF' category and it was on par with blend-P (χ^2=15.012, df=3, P=0.039). Blend-M stimulated significantly more moths to approach sources (SA) than remaining blends, which was followed by Blend-P and Blend-T (χ^2=10.30, df=3, P=0.024). However, none of the synthetic pheromone blends were able to elicit source contact (SC) responses.

Table 12. Taxis response of *M. vitrata* male moths to different synthetic blends at 0.02 µL dose (20 µL dilute solution in hexane) in wind tunnel.

Blends	Replications	(TF)	(UF)	(SA)	(SC)
M	20	16	14[a]	12[a]	0
P	20	14	11[a]	10[ab]	0
T	20	17	10[ab]	9[ab]	0
S	20	14	5[b]	7[b]	0
Control (Solvent)	20	13	3[b]	3[b]	
Chi-Square value		4.705	15.012	10.30	-
P value		0.512	0.039	0.024	-
df		3	3	3	-

Values within a column followed by a common letter were not significantly different (Beasley and Schumaker, 1995[15]; post-hoc test).
TF- Took flight; UF- Upwind flight; SA- Source approach and SC- Source contact

A three component *M. vitrata* pheromone blend (*EE*10,12-16:Ald+*EE*10,12-16:OH+*E*10-16:Ald) was not highly attractive to male moths in previous studies (Mondhe, 2001[29]; Downham et al., 2003 [12]). But, in the present study behavioral responses have been observed to synthetic blends possibly due to the fact that these are isomeric blends and may be having better responses than original components which have been tested in previous studies. Mondhe (2001) [29] reported that with 3-component blend, most individual male moths were non-responsive (> 80%) but few flew upwind and hovered in front of the pheromone source (< 10% each). On the contrary, the study found that female ovipositor washings resulted in 57% of tested male moths making source contact or landing on the source. Whereas, Downham et al. (2003) [12] showed that standard 3-component blend was non-responsive to males compared either of the two binary blends of each minor component (*EE*10,12-16:OH and *E*10-16:Ald) with the major component, *EE*10,12-16:Ald. So, the current study contradicts the results of Mondhe (2001) [29] and Downham et al. (2003) [12]. Possible explanation for differences between the present results and those of Downham et al. (2003) [12] could also be due to the fact that different diets were used in these two studies. Downham et al. (2003) [12] employed a vitamin-enhanced artificial diet based on soybean and wheat germ fresh diet, whereas the present study used natural diet (*Dolichos* bean). It is possible that different nutritional status might have led to altered pheromone production or responsiveness, since diet is one of the most significant environmental factors shaping chemical signals in animals (Henneken et al., 2017) [30], although other factors especially the genetic differences among the *M. vitrata* populations in Asia and Africa cannot be ruled out (Malini et al., 2015) [17].

Lure optimization in field trials in India. There were significant differences among the blends compared ($F_{4,15}$= 5.915; P= 0.005) with respect to *M. vitrata* male moths attracted. Whereas, no significant differences were observed for female *M. vitrata* moths ($F_{4,5}$=0.850; P=0.515). Among the different blends, 1mg of blend-M caught significantly more male moths than others (Table 13). Control traps had the least number of male moths. Overall trap catches throughout the trapping period indicated that 87.8% of the total catches were males and 12.2% were females.

Table 13. Mean trap catches of male and female *M. vitrata* moths in traps baited with different blends in farmer field at Bengaluru rural district.

Blend	Number of male moths caught (Mean ± SE)	Number of female moths caught (Mean ± SE)
Blend-M	8.25±0.85 a	1.25±0.47
Blend-P	6.75±1.65 a	0.75±0.25
Blend-T	5.5±0.95 ab	0.5±0.28
Blend-S	1.67±0.88 b	0.67±0.33
Control	1.00±0.40 b	0.5±0.28
df	4, 15	4, 15
F	14.42	3.69
P	0.0004	0.07

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

Effectiveness of pheromone lures in field trials in Cambodia. In 2015 trial, all the *M. vitrata* pheromone lures (H to K) attracted significantly higher *M. vitrata* male moths compared to untreated control (F=11.39; P=0.002) (Table 14). However, the number of *S. litura* adults attracted by various lures or the untreated control did not differ significantly recording 2-11 adults/trap. The yield was significantly higher in lure J (57t/ha), followed by lures H (47t/ha) and K (44t/ha) (F=7.20; P=0.007). In 2016 trial, all the *M. vitrata* pheromone lures (H to K) attracted significantly higher *M. vitrata* male moths compared to untreated control (F=10.78; P=0.002). However, the number of *S. litura* adults attracted by various lures or the untreated control did not differ significantly recording 26-36 adults/trap as in the previous year. The yield also did not differ significantly among the treatments (11-13 t/ha). In the final trial in 2017, lure K attracted significantly higher *M. vitrata* moths than the untreated control (F=31.97; P=0.0008). The pod damage also significantly lower in lure K treated plots than the untreated control (F=8.36; P=0.02). Similarly, the yield was also significantly higher in pheromone treated plots than the untreated plots (F=24.49; P=0.002). Hence, the pheromone lure was found to attract *M. vitrata* moths in yard-long bean fields in Cambodia.

Table 14. Evaluation of sex pheromone against *M. vitrata* on yard-long bean in Kandal Province,Cambodia.

Treatment	2015			2016		
	Total no. of *M. vitrata*/trap*	**Total no. of *S. litura*/trap**	**Yield (t/ha)***	**Total no. of *M. vitrata*/trap***	**Total no. of *S. litura*/trap**	**Yield (t/ha)***
Lure H	24.33 (4.96) a	10.67	46.74 (6.85) ab	44.00 ab (6.67)	35.00	13.04 a (3.68)
Lure I	30.00 (5.52) a	5.67	35.56 (6.00) b	42.33 b (6.54)	36.00	10.89 b (3.37)
Lure J	26.00 (5.14) a	7.33	56.74 (7.56) a	53.33 a (7.31)	32.33	10.89 b (3.37)
Lure K	27.33 (5.25) a	9.33	44.30 (6.68) ab	48.00 ab (6.96)	26.67	11.63 ab (3.48)
Check	6.00 (2.53) b	2.33	34.52 (5.90) b	26.33 c (5.17)	26.33	11.78 ab (3.50)
F value	11.39	1.69	7.20	10.78	0.69	2.86
P	0.002	0.24	0.007	0.002	0.66	0.09

Means followed by the same letter(s) in a column are not significantly different ($p<0.05$) by Tukey's HSD

Table 15. Evaluation of sex pheromone against *M. vitrata* on yard-long bean in Kandal Province, Cambodia (October – December 2017).

Treatment	Mean pod damage (%)†	Total no. of *M. vitrata*/trap*	Yield (t/ha)*
Lure K	16.30 (23.80) b	20.63 (4.58) a	15.00 a
Check	20.35 (26.70) a	9.88 (3.16) b	12.70 b
F value	8.36	31.97	24.49
P	0.02	0.0008	0.002

Means followed by the same letter(s) in a column are not significantly different ($p<0.05$) by Tukey's HSD

†Figures in parentheses are *arc-sine* transformed values

*Figures in parentheses are square-root transformed values

There are several explanations for the low number of moths trapped in both India and Cambodia. It is possible that experimental sites were mostly farmer fields, which received the regular agronomic and plant protection measures including chemical pesticides and hence the fluctuation in infestation levels of the legume pod borer. Secondly, there were consistent raining during the experimental period, and this might have overflowed the trapped moths in the water traps, especially in India. Despite these environmental influences, Blend-M containing pheromone isomer components and plant volatile was found to be superior in attraction at a dose of 1mg in India. As indicated earlier, it is possible that the plant volatiles may have enhanced the attraction of *M. vitrata* moths due to possible synergism between the pheromone compounds and host plant

volatiles (Reddy and Guerrero, 2004 [27]; Bendera et al., 2015 [28]).

Attraction of female *M. vitrata* to the synthetic pheromone blend in Benin was also reported by Downham et al. (2003) [12]. They reported that significant numbers of female moths, up to 50% of male catches, were trapped by the pheromone. But we recorded maximum of 12% of the total catches that were females in India, which was consistent with the observations of Downham et al. (2004) [13]. They found that captures of female moths made up 14% of the total moths in their experiment. Hassan (2007) [19] found that a majority of pheromone catches in India were males. There was only one moth species for which captures of females in traps baited with the synthetic version of the female-produced pheromone have been reported, *i.e.*, the noctuid, *Trichoplusia ni* Hubner (Mitchell et al., 1972 [31]; Birch, 1977 [32]). In laboratory work at NRI, the possibility of female attraction to pheromone was not supported by the wind-tunnel results of Mondhe (2001) [29]. In general, the presence of female moths in pheromone traps that used lures based on a female sex pheromone was observed in India and the trapped adults were not identified for their sex in Cambodia. It may be due to the presence of 1-octen-3-ol, an identified cowpea volatile and *Z*3-6:OH, a green leaf volatile in the lures. But, males clearly outnumbered females among pheromone trap catches. Interestingly, another noctuid moth, *Spodoptera litura* was attracted by the *M. vitrata* pheromone lures in Cambodia. Although it was not clear why *S. litura* moths were attracted by the *M. vitrata* pheromone lures, we obtained similar results in Taiwan, Thailand and Vietnam in earlier studies (Srinivasan et al., 2015) [3]. Hence, additional studies are suggested to understand the cross attraction of *S. litura* to *M. vitrata* pheromone lures.

Since the EAG responses to *E*& *Z* isomers of 10-hexadecenal & 10-hexadecenol were on par with each other, *E*10-hexadecenal which was the minor component in Benin blend was considered for the field studies in India. Since the anticipated result was not obtained due to several reasons mentioned above, the counterpart *E*10-hexadecenol which is alcohol component of the same was used as minor component in field trials in Cambodia. Our experiments demonstrated that blends M and P in India and lure K in Cambodia have the potential in trapping *M. vitrata* moths under field conditions. Hence, these lures should be evaluated in different legume crops in different geographical regions in South- and Southeast Asia before they are explored for practical pest management. However, from the wind tunnel studies, none of the blend combinations including standard Benin blend had source contact, which showed that there are still possibilities for further improvement in *M. vitrata* blend formulation.

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