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Electrophysiological and behavioral response of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae) to fermented coconut sap neera

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Abstract

Red palm weevil (RPW) (Coleoptera: Curculionidae) is a lethal pest of coconut in India and various palms across the world. Fermenting toddy has been traditionally used for trapping RPW. The traditional method of collecting neera, the coconut inflorescent sap, in an open earthen pot emanates volatiles that attract these insects. In this study, the volatile compounds released from fermenting neera were characterized and the compounds that cause physiological and behavioral response to RPW were established using electrophysiological and behavioural assays. Acetoin, which caused the neuronal response in adult RPW antennae, was present in head space volatiles of fermenting neera from day one onwards. Fermenting neera, when used in tandem with aggregation pheromone, trapped a high number of weevils (53.2 per trap) suggesting possibilities of its use in RPW management.

Keywords: EAD, GC-MS, neera, pheromone synergist, *Rhynchophorus ferrugineus,* wind tunnel

Introduction

Red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae) is a key pest of *Cocos nucifera* L. (Faleiro, 2006). With its origins in South East Asia, RPW has today spread to Middle Eastern countries, parts of Europe, Africa and western coast of the United States of America (Fiaboae *et al.,* 2012; EPPO, 2014; Yan *et al.,* 2015; CABI/EPPO, 2016). The RPW host ranges from coconut, arecanut, canary palms, date palm and sago palm (Esteban-Durán *et al.,* 1998; Longo *et al.,* 2011).

These weevils are active flyers (Murphy and Briscoe, 1999) that are attracted to volatiles emanating from the wounds of palm trees (Gunatilake and Gunawardane, 1986). Adult male weevils feed on damaged areas of the palm secreting aggregation pheromone (4-methyl-5 nonanol) to attract their female counterparts (Giblin-Davis *et al.,* 1996). Upon mating, female insects deposit their eggs in injured base of the fronds or in cracks and crevices of the palm. On hatching, immature larvae gregariously feeds on the internal contents of the palm trunk, resulting in their decay and ultimate death of the palm. Adult weevils, on emergence, damage the neighbouring susceptible palms (Jaffe *et al.,* 1993; Murphy and Briscoe, 1999; Giblin- Davis *et al.,* 2013). This obscure nature of the pest makes it hard to spot the initial symptoms in order to adopt control measures (Rajamanickam *et al*., 1995; Avand-Faghih, 1996; FAO, 2017).

Products derived from coconut have nutritional and medicinal properties (Faole, 2003; Perera *et al.,* 2008). Coconut sap or neera tapped from inflorescence is a healthy and nutritious drink having minerals and vitamins (Bipasha Mishra, 2016). Neera tapped in an unorganized manner in India is consumed largely by rural population. Popularizing neera as a nutritive drink has facilitated in getting additional income to coconut farmers (Hebbar *et al.,* 2015) in a

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situation when the market prices of coconut are fluctuating across the globe (Dhara *et al.,* 2016; CACP, 2018). A device 'coco-sap chiller' developed by ICAR-Central Plantation Crop Research Institute (ICAR-CPCRI), Kasaragod, facilitates collection of neera in a hygienic manner (Hebbar *et al.,* 2013). The controlled temperature maintained in the device, coupled with airtight sealing, prevents the volatiles from being released from neera. On contrary to this, majority of the farmers follow traditional method to tap neera in open earthen pots (Hebbar *et al.,* 2013). Improper tapping leads to fermenting neera that attracts the RPW.

Neera is immensely prone to fermentation (initially alcoholic and later becoming acidic), a process accelerated by sunlight (Odunfa*,* 1985; Iwuoha *et al.*, 1996). Fresh sap starts fermenting within five hours of placing it in ambient conditions and the fermentation continues slowly for few more days (Hebbar *et al.,* 2015). The fermented neera is known as toddy and it is reported that palms subjected to toddy tapping have more incidence of RPW infestation (OPC, 2012; Justin *et al.,* 2008). Coconut petioles smeared with fermented toddy have been used as baits to trap the weevils (Justin *et al.,* 2008).

Chemical communication is well developed in palm weevils, especially for identifying food, mates and oviposition sites (Giblin-Davis *et al.,* 1996). Combining food/host odour along with aggregation pheromone increases the trapping of RPW. Identifying the compounds that attract adult weevils is essential to develop pheromone kairomone synergists, which would help the coconut farmers to do away with the use of food materials in traps (Vacas *et al.,* 2014).

Hence, we have made an attempt to study the head space volatiles isolated from fresh and fermented neera causing physiological and behavioral response in weevils. Compounds isolated from fresh, refined and fermented neera, via solvent extraction, were characterized according to Borse *et al.* (2007). The compounds listed have value for natural product chemistry, but the volatiles trapped by head space sampling would help to decipher the compounds that cause olfactory response in RPW. Therefore, the aim of the study was to characterize the compounds from fermenting neera that could be used as a pheromone kairomone synergist.

Materials and methods

Collection of neera

Coconut sap, neera was collected from 10 palms drawn from the unopened spadix of the palm from ICAR-CPCRI, Kasargod, Kerala, India. Fresh neera was collected in 'coco-sap chiller' following the protocol of Hebbar *et al.* (2013). Collection was made under sterile conditions to avoid any microbial or insect contamination. Two hundred fifty milliliters of collected sample was transferred to one-liter Erlenmeyer flask, with its mouth covered by muslin cloth until head space sampling.

Mass culturing of *R. ferrugineus*

Adults of RPW collected from infested palms from farmers' garden located in, Kasaragod, Kerala, India were mass cultured in laboratory for further investigations. RPW colony was maintained on coconut petioles/sugarcane bits. Coconut petioles (8 cm length by 4 cm width) were placed in a polypropylene box (21 x 14 x 13 cm L x W x H) to which five pairs of adult weevils were released for egg laying (for 24 hrs at 26 ± 0.2 ºC and 0L: 24D). The coconut petioles were replaced once in three days. On hatching, the larvae were fed on the petioles. The cocoons were placed individually in a plastic container (100 mL) for eclosion. After two generations in the laboratory, progeny was used for the physiological and behavioral assay.

Head space collection of volatiles from neera

After tapping neera, the volatile organic compounds' (fresh and fermenting) (250 mL) were collected using the dynamic head space collection technique on 0, 1, 3, 5 and 7 days. An air entrainment apparatus fabricated at Entomology section in ICAR-CPCRI, Kasaragod was used. The matrix to be sampled was placed in one-liter Erlenmeyer flask. Gentle stream of air was sucked through activated charcoal cartridge @ 30 mL per min. It was allowed to pass over the sample held in a glass chamber. The odour laden air was trapped in glass tube containing absorbent Tenex 30 mg with glass wool on either side as stoppers. The volatile organic compounds were trapped for six hours. The trapped volatiles in the adsorbent were eluted with HPLC grade dichloromethane (400 µL) and condensed to 200 µL by passing gentle stream of ultra-high pure nitrogen. The vials with extracts were stored at - 20 ºC until further analysis.

Characterization of head space volatile from neera

The sample (1 µL) was injected into gas chromatography (Agilent) GC-7890A (G3440A) and MS- G3171A.HP 5 MS phenyl methyl silox capillary column (30 m × 250 µm i.d. × 0.25 µm film thickness, Agilent Technologies, USA column was used. The temperature of column and oven were maintained at 40 ºC for 1 minute and then increased at the rate of 20 ºC per minute to 280 ºC and held at 300 ºC for 10 min. The injector and column temperature were 250 ºC. The total run was for 23 min. The compounds were identified by comparison of mass spectra with the NIST library.

Assessing electrophysiological response of antennae using GC-MS-EAD gas chromatography-mass spectrometry-electroantennographic detector (GC-MS-EAD).

Coupled analysis of head space volatiles of fermented neera to RPW adult antennae was done with chemical detector [Agilent 7820A GC equipped with a flame ionization detector (FID) and G3171MS] attached to a biological detector (Electroantennograph, Syntech). HP 5 MS Phenyl methyl silox capillary column (30 m × 250 µm i.d. × 0.25 µm film thickness, Agilent Technologies, USA) was used with a 3-way splitter. At GC column effluent, carrier gas was split 1:1:1 for simultaneous detection between the MS - FID and EAD apparatus. The sample (1 µL) was injected (splitless, inlet temperature = 250 °C) while the temperature of column and oven were maintained at 40 ºC for 1 minute and then increased at the rate of 20 ºC per minute to 280 ºC and held at 300 ºC for 10 min. The injector and column temperature were 250^{0} C. The total run was for 23 min. Antennal depolarization was detected with a high impendence EAD probe, a signal interface box IDAC-4, and a stimulus controller (CS-55) (Syntech, Germany). The antenna was fixed between the two electrodes using Spectra 360 conductive gel (Parker, Orange, New Jersey). The proximal tip was fixed to one electrode and scape to another electrode as suggested by Reinecke *et al.* (2005). The antenna was flushed continuously with a flow of activated charcoal filtered air.

In an attempt to pin point the compound eliciting response from the head space effluent of fresh and fermenting neera, the volatiles trapped were injected in GC-MS. Effluent to the antenna was passed through a heated transfer line (Syntech, Germany) set at ±10 ºC above the maximum program temperature that entered a 16-mm diameter glass tube via a small hole in the wall of the glass tube. Finally, the GC trace was over laid with EAD response using the GC-EAD software of Syntech. A compound was considered electroantennographically active when it elicited an antennal response.

Behavioral assay of RPW

To assess the behavioral response of RPW to odorants in neera, the olfactory response of weevils were estimated through wind tunnel assay. Purified air was allowed to flow in the tunnel by a suction motor with variable speed. The insects were released against the air current and the odorant to be evaluated was kept at the end from which the air flow started. The positive response was scored by the number of weevils orienting to the source, when an adult weevil took flight, it was scored positive for flight, as the weevil moved from the release end towards the source end, it was scored positive for activation, as the weevil crossed midline of the wind tunnel path it was scored positive for midline, when the weevil stayed at the odour for more than a minute it was scored positive for upwind, lastly if an adult weevil came in contact with the lure source it was scored positive for source contact. Twenty insects with ten replications was carried out. The data was subjected to one-way ANOVA and the means were subjected to Tukeys post hoc test to determine significant difference.

Field evaluation of fermenting neera in trapping RPW adults

To assess the field efficacy of pheromone when used in tandem with fermenting neera, bucket traps were installed with pheromone lure (400 mg) and 250 mL of fermented neera in a farmers' garden planted with WCT (West Coast Tall) at Chittarikal, Kasaragod, India. Traps were also placed with pheromone and neera alone. The trap density was maintained at one trap $hectare^{-1}$. Each treatment had five replicates. The design of the experiment was randomized block design (RBD). Observations were done at 10 days intervals to count the number of weevils trapped and fresh neera was added to the bucket. The collected weevils were removed from the trap. The data was analysed by one-way ANOVA and the means were subjected to Tukeys Post hoc test using SPSS 16.0.

Results and discussion

Characterisation of volatile organic compounds of neera

The compounds present in fresh and fermented neera was isolated by head space sampling on 0, 1, 3, 5 and 7 days after trapping are presented in Table 1. A total of 51 compounds were characterized from fresh (day zero) and fermented.

Table 1. Volatile profile of fermenting neera

Retention index	Compound	Day 0	Day 1	Day 3	Day 5	Day 7
4.1	Acetic acid					*
4.1	Acetoin		*	*	*	*
4.28	2-Methyl butanol		*	*	*	*
4.86	Isobutyric acid			*		*
5.0	Isobutanoic acid				*	*
5.02	1-Isopropoxyacetone				*	*
5.07	Propyl glycol					*
5.42	3-Methylvaleric acid				*	
5.47	n-Heptanal	*				
5.49	n-Hexanoic acid		*			
5.53	α-Methylcaproic acid			*		
5.63	Valeric acid			*		
5.68	Isovaleric acid					*
5.7	2-Methylbutanoic acid				*	*
5.84	α-Methyl butyric acid				*	
6.4	ß-Pinene	*				
6.5	Caproic acid			*	*	
6.6	6-Methyl-5-heptene-2-one				*	*
6.7	Dihydro-2-methyl-3(2H)-thiophenone	*				*
6.71	n-Octoic acid		*			
6.72	(E)-2-Decenol	*				
7.0	Ethyl hexanol					*
7.2	ß-cis-ocimene			*		
7.6	2-Nonanone	*			*	
7.67	Hendecane		*			
7.7	Linalool				*	
7.74	Nonanal				*	
8.13	Actinobolin	*				
8.2	Octanoic acid				*	
8.3	n-Octanoic acid			*		
8.34	n-Caprylic acid					*
8.53	Ethyl octanoate				*	
8.54	Ethyl decanoate		*			
8.57	Dodecane		*			
9.07	Chavicol			*		
9.16	Phenethyl acetate		*			
9.25	Acetophenone, 2',4'-dimethyl				*	
9.9	Decanoic acid					*
10.15	Ethyl caprylate		*		*	
10.15	6-Methyloctadecane					*
10.16	Ethyl laurate			*		
10.24	Copaene			*		
11.3	(+)-ä-Cadinene			*		
11.47	Nerolidol					*
11.61	Ethyl laurate			*		
13.64	2-Nonadecanone					*
13.79	Homosalate					*
16.2	Heptacosane			*	*	
17.83	1-Octacosanol				*	
18.1	Octacosane			*	*	*
18.4	Nonacosane			*	*	

* Indicates presence of compound

There were seven compounds in day 0 followed by 9 compounds on day 1. Acetoin and 2-methyl butanol were present from day 1 to day 7. Acetoin was present at very high level in the samples on all the days of sampling except day zero. Presence of acids like isobutyric, butanoic, isovaleric, methyl butanoic acid and octanoic acid were present in fermented neera sample drawn on day 3, 5 and 7. Twenty-one compounds were isolated from fresh neera subjected to simultaneous distillation and solvent extraction method (Borse *et al.,* 2007). Uncovered fresh sap is subjected to fermentation enhancing the growth of microbes (Borse *et al.,* 2007). This is confirmed by the presence of acetic acid in neera fermented for seven days.

Acetoin (3-hydoxy-2-butanone) a four-carbon hydroxy-keto compound, is a microbial by product of diverse microbe when grown in an environment rich in glucose and various other fermentable carbon sources (Xiao and Xu, 2007; Zhang *et al.,* 2011).

Identification of physiologically relevant compound in fermenting neera to RPW using chemical and biological detectors.

The physiologically relevant odour to red weevil antennae released by fermenting neera were identified by GC-MS-EAD. Fermenting neera caused antennal response in adult weevils. Simultaneous detection of the compound present in fermenting neera and the antennal response to it was recorded in GCMS coupled with electroantennographic detector (Fig. 1). As the weevil's antennal response to volatile did not exhibit sexual dimorphism, both male and female were pooled to identify the compounds in the host volatiles that caused physiological response.

The volatiles trapped from neera fermenting for three days injected to GC-EAD revealed that the antennal neurons of the RPW responded to acetoin. The volatile organic compounds from the fermenting neera that caused physiological response in red palm weevil antennae were confirmed by injecting the synthetic compounds *viz*., acetoin. Vacas *et al*. (2014) reported that 3-hydroxy-2-butanone (acetoin) elicited significant responses in *R. ferrugineus* that fed on the Canary Islands date palms, *Phoenix canariensis*.

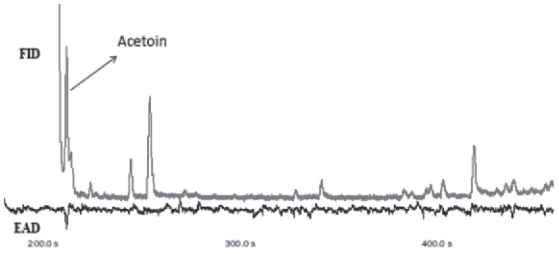

Fig. 1. Electrophysiological response of *R. ferrugineus* antennae to volatiles from fermenting neera. FID-GCMS peaks from fermented neera sample. EAD-antennal response of adult weevil

Behavioral response of RPW to fermenting neera

Wind tunnel assay was carried out to confirm the orientation of the weevil to the odor source. Neera fermenting over the period of seven days from day zero were assessed for their behavioural response in wind tunnel. Fermented neera for seven days caused only over 20 per cent of RPW adult to cross the mid line in wind tunnel and the move towards the source (Fig. 2), fresh neera elicited least response in the adult weevil. Maximum response was recorded on day three of fermented neera with 90 per cent response of RPW adult crossing the midline in wind tunnel. Shift in volatile profile in fermenting neera is due to the production of acetoin as a result of microbial activity attracting a greater number of the weevils to the source (Saïd *et al.,* 2005; Vacas, *et al.,* 2014). In the wind tunnel assay, the highest behavior response of the weevils was observed on day three followed by day five and day one to the odor of fermenting neera.

The response of RPW, to neera and pheromone in isolation and in combination was assessed in the wind tunnel (Fig. 3). The response of weevils to fermenting neera in combination with pheromone resulted in 8 times higher in both flight and source contact response when compared to RPW's response to pheromone alone. Neera in isolation was capable of eliciting 94 per cent of midline response in weevils. This could be due to plant volatiles translating varied behavioral response in insects, acting as cue for food and reproduction (Light *et al.,* 1993; Deng *et al.,* 2004).

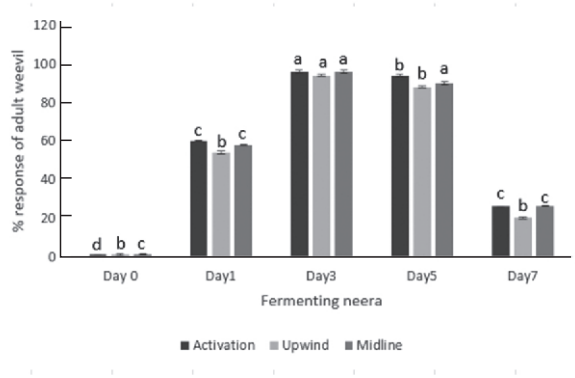

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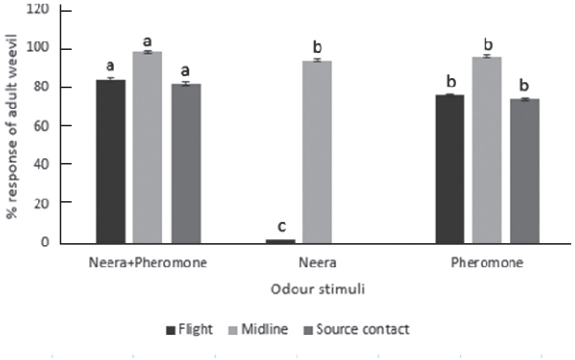
Fig. 2. Behavioural activity of the adult weevils in wind tunnel to fermenting neera

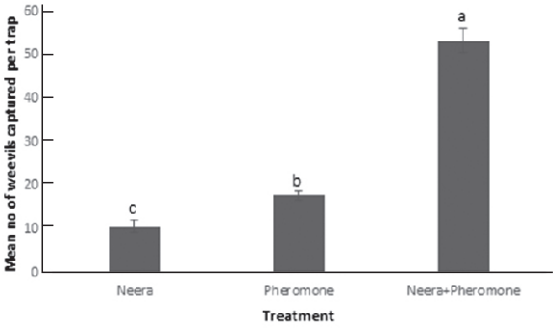


Bars labeled with the same alphabet are not significantly different P<0.05 by Tukeys HSD test

Fig. 3. Behavioural activity of the adult weevils in wind tunnel to odour stimuli

Field evaluation of fermenting neera with pheromone

Field trial was laid to assess the effect of fermenting neera volatiles along with RPW pheromone at farmers plot in Chittarikal, Kasaragod. The mean number of weevils captured per trap (Fig. 4) was estimated to assess the efficacy. Pheromone when used in tandem with neera, trapped over 52 weevils per trap. When pheromone was used in isolation it trapped over 15 weevils, but it was superior to fermenting neera when used in isolation.



Bars labeled with the same alphabet are not significantly different P<0.05 by Tukeys HSD test

Fig. 4. Assessing the field efficacy of neera and pheromone to trap adult weevils

Toddy (Fermented neera) smeared on coconut petiole has been used as an attractant for adult weevil (Kurian *et al*., 1984; Abraham, 1987). The volatiles from the food bait acts synergistically with pheromone to attract RPW adults into the traps (Hallett *et al*., 1999). Addition of 5 g yeast or 5 mL acetic acid to 1000 mL toddy was used for attraction of red palm weevil (Justin *et al*., 2008).

Our study shows that there is over 35-fold increase in the trapping of the adult weevil in the pheromone trap along with neera on contrary to two to five-fold increase in the trapping of weevils by adding fermenting host materials as reported earlier (Jaffe *et al*., 1993; Oehlschlager *et al* 1993; 1995; 2016; Gries et al., 1994; Guarino *et al*., 2011). Our observations revealed that fermenting neera caused physiological and behavioural response in weevils. The volatile summary undergoes a drastic change when a healthy palm afflicted to pest or physical damage, initially releasing esters, alcohols later on releasing acetic acid, propanoic acid and, butanoic acid as fermentation progresses as a result of microbial activities. Pheromone along with acetoin and esters trapped higher number of weevils. Apart from Rhynchophorus sp (Saïd *et al*., 2005), acetoin has been reported as an attractant of cockroach (Sreng, 1993, Vlasáková *et al*., 2008) and Amphimallon solstitiale (Tolasch *et al*., 2003).

Conclusion

Volatile profiling of fermenting neera revealed the presence of acetoin, an attractant for RPW that was confirmed by electrophysiological and behavioural assay. This indicates that fermenting

neera act as a synergist in combination of aggregation pheromone. This could be exploited for use in management of RPW as the fermenting volatiles could help to replace the dependence on placing food baits that are laborious as they need to be serviced at frequent intervals adding to the cost in RPW management.

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