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In vitro evaluation of antennal olfactory sensillum response and chemo-detection ability of Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier) for pheromone blends by Electroantennography (EAG)

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Abstract
A comparative study of chemo-detection ability to stimulate the pheromone-responsive neurons (PRNs) was conducted to evaluate the red palm wee vil (RPW) antennal olfactory sensillum response to elicit depolarization of olfactory sensory neurons (OSNs) producing optimal neuronal action potential. Electroantennography (EAG) was carried out using insect antenna and corresponding electroantennograms were recorded for selected different aggregation pheromone blends of variable ratios, results revealed that aggregation pheromones 4-methyl-5-nonanol and 4-methyl-5-nonanone, in combination produced distinct electroantennograms establishing chemo-detection ability by the insect. The experimental results demonstrated chemo-detection ability by the antennal olfactory sensillum of RPW, producing optimal neuronal response by weevil for the blend of 4-methyl-5-nonanol and 4-methyl-5-nonanone at 7:1 blend ratio and was found to be the optimum blend ratio to produce good action potential and depolarization effect.

Keywords: Aggregation pheromone, red palm weevil, electroantennography (EAG)

Introduction
Red palm weevil (RPW) (*Rhynchophorus ferrugineus* Oliver Coleoptera: Curculionidae.) a key pest, causes huge economic loss to the farmers of ccoconut (*Cocos nucifera* L.), date palm (*Phoenix dactylifera*) and oil palm (*Elaeis guineensis*) across the globe. The infestation of RPW is widespread, mainly from south and Southeast Asia, reported as a major pest of coconut palm [1, 2]. The pest is known to attack over 40 different species of palms of various genera. Palm cultivators including coconut farmers in India are badly affected by the infestation of RPW resulting in huge economic losses, affecting lives of millions of people. Globally, these palm plantations accounts to about 12 million ha, cultivated and spread across 90 countries. In India, infestation in Tamil Nadu is estimated at 11.65%, including young coconut trees (5- to 10-year-old saplings) resulting in huge economic loss [3]. In Kerala, infestation accounts to 6.9% of coconut plantations and other palms affecting the livelihood of farmers [4]. Farmers are baffled because of silent infestations and devastating damage caused by this pest. The pest silently infests and take over the plantations and the farmer will never know its infestation until the whole tree collapses, ruining the years of farmer's effort in growing and maintaining the plantations. At present there are no effective methods available to control this economically high-risk pest. In this context, the promising solution is pheromone traps to control and minimize the economic losses to the farmers. Thus, there is a need for the development of safe, effective, and affordable pheromone-based trapping and control methods to support our farmers for controlling this silent pest. Pheromone based products, not only help to control infestation also reduce the use of pesticides and there by resulting a healthy harvest. Male aggregation pheromones of *R. ferrugineus* were identified and chemically established as 4-methyl-5-nonanol and 4-methyl-5-nonanone.

Number of synthetic palm esters in various combinations along with aggregation pheromones were tested for the effective attraction of the insect. These chemical agents are likely to be used in red palm weevil management as it attracts both male and female insects [5, 6]. The main objective of the current research is to identify and evaluate the perfect blend ratio of aggregation pheromones to increase the chemo-detection sensitivity and its ability and to elicit optimal influence on insect olfactory sensillum to stimulate pheromone-responsive neurons (PRNs) of red palm weevil. The chemo-detection ability of the insect were recorded as electroantennograms and interpreted. The study data can be further used in developing the suitable formulation for effectively attracting and trapping of RPW.

Materials and Methods

Source of Red palm weevil insects

Different stages of adult male and female used in this study were meticulously collected from infested palm trees of coconut fields located at Naranahalli of Kunigal Taluk, Tumkur, Karnataka, India (13.1098° N, 76.9570° E). Collected insects were gently brought to the laboratory and quarantined in the naturally simulated environment to acclimatize the insects to the laboratory conditions. Adult male and female insects were identified and were separated based on the sex. Each group of insect (male and female) were placed separately in rectangular plastic boxes of dimensions 30 cm (l) x 25 cm (b) x 15 cm (h) with tight-fitting lids with pin holes to provided adequate aeration. Insects were reared in laboratory by providing nature simulated environmental conditions (25 °C ± 2 °C; R.H-75% - 90%; L13:D11) and fed with 10% w/v sucrose solution with the help of cotton swabs to facilitate egg laying. The first instar larvae were carefully transferred to Cleaned and conditioned fresh sugarcane internode stems for larval and pupal development. After the adult emergence, male and female insects were isolated and quarantined in clean semi-transparent rectangular plastic boxes of dimensions 20 cm (l) x 15 cm (b) x 10 cm (h) with tight-fitting lids with pin holes to provided adequate aeration.

EAG Experiment

Adult RPW were selected, insect antenna was cut off using sterile lancet, antenna was carefully transferred to a container with Ringer's solution, Iimmediately insect antenna was carefully mounted onto the tip of the recording silver electrode. Both silver electrodes were previously applied with Spectra 360 electrode gel (Parker, Fairfield, New Jersey) to support the mounting of the antenna. Short cables connect the electrodes to a high impedance (1012 ohm) DC amplifier, to record the amplified signal (100 x). The antenna was subjected to a steady flow of filtered and purified air (produced by passing the air through activated carbon and zeolites) to accommodate the antenna for the experimental setup. Insects were grouped into six different groups, in each experiment a minimum of five puffs of different concentrations of the blends of pheromones were used to stimulate the mounted antenna and respective electroantennograms were recorded [7]. Experiments were conducted on different insect's antenna (sextuplicate) and respective electroantennograms were recorded.

Electrophysiological response of adult RPW

Antennal olfactory sensilla response and chemo-detection ability of the insect to stimulate pheromone-responsive neurons (PRNs) of both male and female adult insects were recorded as electroantennograms (EAGs), using dual electrode probe and an amplification unit. A chemo-stimulated pheromone-responsive neuronal signal was indicated by a sharp drop in the baseline followed by the decay of the signal was noted every time when the specific blend of pheromone is puffed on to the mounted antenna of the insect during the EAG experiment.

Time of response (TOR) of adult RPW insect

To determine the time of response (TOR) of RPW insect in EAG, the major component of aggregation pheromone was used as a stimulus. 10 mcg of pheromone blend was impregnated onto to a small Whatman filter paper, Grade 40 (ca. 0.8 cm^2), which was placed into a Pasteur pipette. The test was performed using five different puffs of pheromone blends at an interval of 5 min during 40 min and the resultant electroantennograms (EAGs) were recorded against purified air mixture containing hexane as blank. In each electroantennogram recorded, a typical wave form with rapid depolarization were observed. The puffs were delivered by an electro valve driven membrane pump, which opened for 5000 ms. The air fed into another independent stream of air, producing uniform mixture of pheromone blended air and glided continuously over the isolated and mounted antenna of the insect. Six different insect responses were recorded, with an average five applications per antenna in each trial to record the response of the insect to the specific pheromone blend and average response was computed.

Pheromone blend screening for EAG

Evaluation of the EAG responses of the insect to the indigenously synthesized aggregation pheromone blends was carried out as per our earlier RPW response based on prior experimental results for optimization of pheromone responses were identified as 7:1; 8:1 and 9:1 ratio [7]. Pheromone blends (7:1; 8:1 and 9:1) were prepared by mixing required quantity of both the pheromones (dissolved in analytical grade hexane) in specified ratios. On application of 100 µl of each of the prepared solutions (for each blend) deposited 25 mcg, 50 mcg, 75 mcg, 100 mcg and 125 mcg of the specific blend onto different small pieces of Whatman filter paper, Grade 40 (ca. 0.8 cm^2) respectively. At room temperature, the filter paper impregnated with the pheromone blend were placed inside a Pasteur pipette. EAG responses produced by isolated antenna of adult weevil for each of the blends were recorded by applying five different puffs of every blend of pheromones and an average response of minimum of six antennal response was considered. A blank of purified air mixture containing hexane was insufflated before and after every puff of pheromone blend. The resultant EAG response data was subjected to statistical analysis to evaluate its significance.

Results and Discussion

The EAG results clearly demonstrated weevil's ability to detect and respond to aggregation pheromone blends (different chemical moieties) in their specific ratios. The chemical nature and concentration of these molecules had a significant influence on intensity of the electroantennogram signal produced (EAG responses), aggregation pheromone blend ratio of 7:1 (4-methyl-5-nonanol and 4-methyl-5-nonanone) stimulated the weevil's antenna with a amplitude generating optimum EAG signal with a concentration of 100mcg, with 4.1mV (Δ Potential (mV) /EAG signal) and at concentration of 125mcg, with 4.0mV (Δ Potential (mV) /EAG signal) the amplitude produced was almost the same as 100 mcg concentration. In the blend ratios of 8:1 and 9:1 (125 mcg) the EAG signals recorded 3.2mV and 3.8mV respectively. The experimental results clearly demonstrated the greater sensitivity of the insect's ability to detect and stimulate chemo responsive signal for the blend ratio 7:1, which is significantly greater than the blend ratio 9:1, while that of blend ratio 8:1 gave intermediate EAG response. These experimental results indicate that different chemical moieties in different concentration blends have a variable influence on chemo-detection ability of the RPW antennal olfactory

sensillum to stimulate the PRNs. Antennal response of adult RPW to aggregation pheromone (4 methyl 5 nonanol and 4 methyl 5-nonanone) in 7:1 and 9:1 ratios resulted maximum antennal response of 4 and 3.8 mV respectively. These findings are in compliance with earlier findings [8].

Fig 1: Antennal response of adult RPW to indigenous synthesized aggregation pheromone molecules at 7:1 ratio

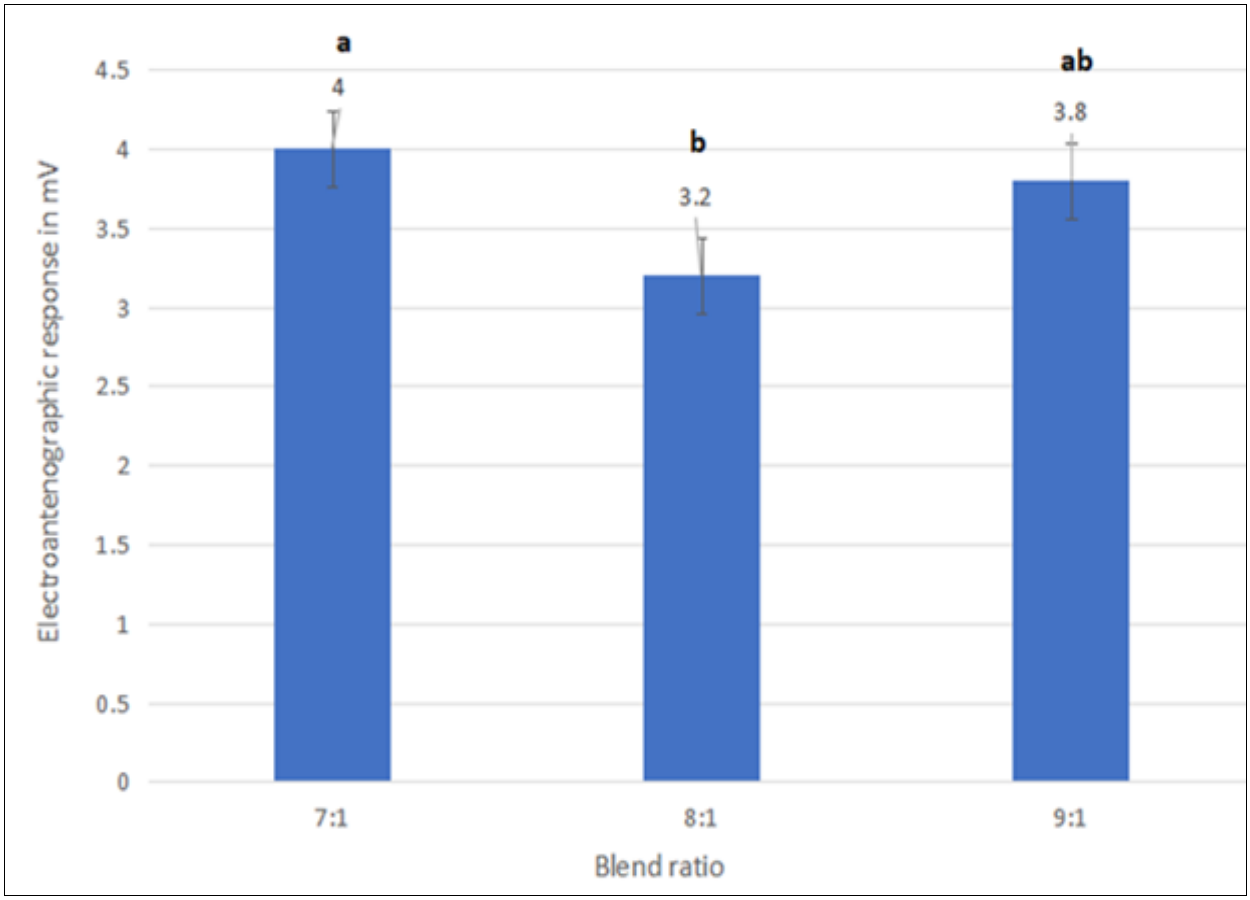

Fig 2: RPW response on different pheromone blend using EAG

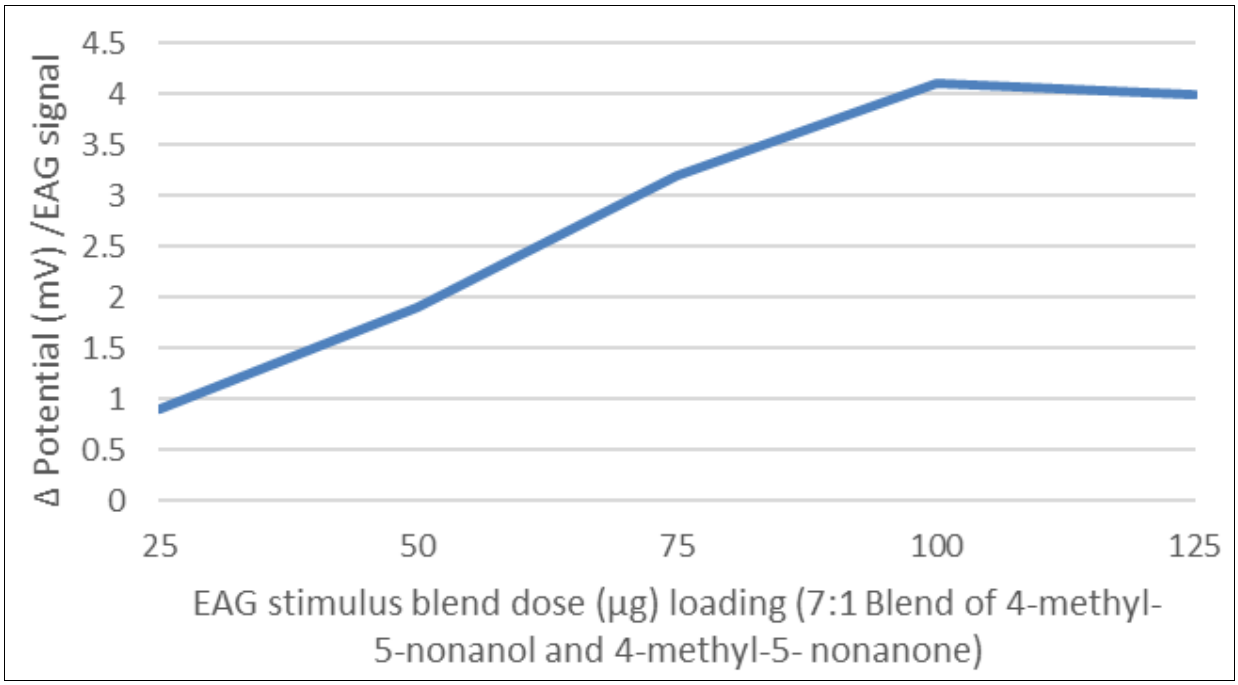

Fig 3: △ Potential (mV)/EAG signal by EAG stimulus blend dose (µg) loading (7:1 blend of 4-methyl-5-nonanol and 4-methyl-5-nonanone)

Conclusion

Experimental results demonstrated that specific blend ratios of aggregation pheromones were liable to elicit EAG responses on antenna of the RPW. Present experimental results significantly demonstrate the variable chemo-detection ability of the insect's antenna for different pheromone blends and demonstrates its sensitivity to the specific blend ratios of aggregation pheromones. Thus, we can conclude that specific blend ratios of the pheromones have a greater influence on chemo-detection ability of red palm weevil's antennal olfactory sensillum for significant chemo-detection and to stimulate pheromone-responsive neurons (PRNs). This in turn validates the relation between the attractiveness and intensity of electroantennogram signal produced by the specific pheromone blend ratios. The experimental results clearly demonstrate that specific blend of 4-methyl-5-nonanol and 4-methyl-5-nonanone at 7:1 is the best ratio in producing the optimal electroantennography (EAG) signal which significantly influences antennal olfactory sensillum to stimulate pheromone-responsive neurons (PRNs) of RPW.

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