TECHNICAL PROGRESS REPORT 2011

"Resolution of Cryptic Species Complexes of Tephritid Pests to Overcome Constraints to SIT Application and International Trade"
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

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Title : Male rectal gland volatile constituents of five economically important cryptic species within the Oriental fruit fly, Bactrocera dorsalis complex (Diptera: Tephritidae)
Chief Scientific Investigator : Alvin Kah-Wei Hee
Research Institute : UNIVERSITI PUTRA MALAYSIA (UPM)
Co-investigators : Suk-Ling Wee, Ritsuo Nishida, Keng-Hong Tan
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I. PROGRESS REPORT FOR THE FIRST YEAR

A. Collection of wild fruit flies, *Bactrocera carambolae* and *B. papayae* for establishment of laboratory colony

Several attempts were made to collect wild fruit fly larvae from infested fruits from various locations in Peninsular Malaysia to obtain ‘good species’ of *Bactrocera carambolae* and *B. papayae* for pheromone work. Table 1 summarized the collection and emergence of *Bactrocera* flies of various rotten fruits from several locations in peninsular Malaysia:

<table>
<thead>
<tr>
<th>Locations</th>
<th>Fruit type</th>
<th>Typical <em>B. carambolae</em></th>
<th>Typical <em>B. papayae</em></th>
<th>Morphological hybrids*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mantin, Negeri</td>
<td>Guava</td>
<td>0</td>
<td>47 (65.3%)</td>
<td>25 (34.7%)</td>
<td>71 (100%)</td>
</tr>
<tr>
<td>Sembilan (Southern region)</td>
<td>Mango</td>
<td>0</td>
<td>5 (71.4%)</td>
<td>2 (28.6%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Raub, Pahang (Central region)</td>
<td>Starfruit</td>
<td>20* (100%)</td>
<td>0</td>
<td>0</td>
<td>20 (100%)</td>
</tr>
<tr>
<td></td>
<td>Starfruit</td>
<td>89 (42.6%)</td>
<td>89 (42.6%)</td>
<td>31 (14.8%)</td>
<td>209 (100%)</td>
</tr>
<tr>
<td>Serdang, Selangor (Central region)</td>
<td>Starfruit</td>
<td>5 (4.4%)</td>
<td>52 (45.6%)</td>
<td>57 (50.0%)</td>
<td>114 (100%)</td>
</tr>
<tr>
<td>Gertak Sanggul, Penang (Northern region)</td>
<td>Starfruit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Morphological hybrids referred to *Bactrocera* fruit flies bearing the combinations of morphological traits of a typical *B. carambolae* and *B. papayae*.

Most of the wild flies that emerged from the larvae collections from almost all regions contains morphi-hybrids, i.e. the morphological hybrids between the typical of *B. carambolae* and *B. papayae* based on the original description of Drew & Hancock (1994), i.e. (a) the present of a dark spot on front
femora in *B. carambolae* (absent in *B. papayae*), (b) the subcosta band exceeding R4+5 and forming a recurved pattern at tip in *B. carambolae* (subcostal band non-exceeding R4+5 in *B. papayae*) and (c) abdominal bands on terga III-IV appeared to be bar-shaped in *B. carambolae* (wedge-shape in *B. papayae*). As a precautionary step of avoiding true hybrids from these collections which may have different pheromone composition from that of the two species (Wee & Tan 2005a), all flies collected from sites with morpho-hybrids were not used for culture establishment. These flies will be sent to Dr Sujinda Thanapum (Mahidol University, Thailand) for molecular analysis to ascertain species status before further culture activities.

Except for a colony collected from infested starfruits from a village near to the forest reserve in Raub, Pahang, the emerged fruit flies were all typical *B. carambolae* based on the above mentioned characters. As a precautionary measure, the colony was raised in our laboratory for at least three generations to ascertain their progenies contain no morphological hybrids. After confirmation to having only morphological traits belonging to a typical *B. carambolae*, the wildish colony is now ready for further experimentation. However, it is worth noting that *B. carambolae* has a longer sexual maturation period (25 to 30 days after adult eclosion) as well as a lower fecundity plus fertility than its sibling species, *B. papayae* (Wee & Tan 2000). Therefore, it has taken a long time to establish in the *B. carambolae* culture in our laboratory and to raise enough flies for further behavioural assay or headspace collection of pheromone volatiles. At present, mature flies are for pheromone gland extraction and GC-MS analysis only.

B. **Methyl Eugenol Treatment and Extraction of pheromone rectal gland**

Sexually mature *B. carambolae* males at 28 – 30 day-old (Wee & Tan 2000) were divided into two groups, i.e. ME-fed and ME-deprived. For ME-fed group, individual male was allowed to feed on 1-μl of pure ME (99.8%; Merck®) for 1 hour, between 08:00 and 10:00 hr (dawn ca 07:00 hr), which corresponds to the peak period of ME response. The treated flies were then left in a screened cage (30 x 30 x 30 cm) provided with food and water *ad libitum*. One-day after ME treatment, the treated flies were individually cold immobilized and the rectal gland excised using a pair of fine forceps. Extracted glands were each soaked in 20 μl of absolute ethanol and stored at −20°C for further analysis. For chemical analysis, each extracted rectal gland was homogenised with a fine glass rod followed by 5-min sonication. Similar procedures were conducted for ME-deprived males of the same cohort.
Sexually mature laboratory strain *B. philippinensis* were obtained from IAEA Entomology Unit in Sibersdorf, Austria. The flies were divided into two groups, ME-deprived and ME-fed, and processed as mentioned above.

C. **GC-MS analysis of male rectal glands**

For GC-MS analysis, sample aliquot (1 µl) of a rectal gland extract was injected into a Varian 450-GC connected to a Varian 240-MS IT mass spectrometer (electron impact at 70 eV), equipped with a FactorFour column (non-polar; 30 m x 0.25 mm x 0.25 µm, VF-5ms, fused with silica column coated with cross-linked bonded dimethyl polysiloxane). The carrier gas was helium and the oven temperature was programmed from 40 °C (5 min holding) to 200 °C at a rate of 5 °C/min thereafter to 220°C at 10°C/min (10°C holding). Injection mode was splitless.

Chemical identification was performed by comparison with the retention times of known authentic standards. Spectra were compared with those of the authentic compounds and MS fragmentation pattern of authentic chemical standards, published spectra and NIST library. In addition, kovats indices of the identified compounds were obtained by injecting commercially available alkane series (C8 to C20; Fluka, Switzerland) under similar condition as mentioned above; and confirmed with published data whenever possible.

**RESULTS & DISCUSSION**

(a) **Bactrocera carambolae**

Results showed the male rectal glands of untreated and ME-fed *B. carambolae* were almost identical except for the presence of a phenylpropanoid compound, *trans*-coniferyl alcohol (CF), in the ME-fed males. The rectal gland of a sexually-mature male (ME-deprived) consists of the naturally occurring components, 2-hydroxy-3-methyl, ethyl butanoate, 3-ethyl-2,5-dimethyl pyrazine, *N*-3-methylbutyl acetamide, 6-oxo-1-nonanol and 1,6-nonanediol (Figure 1). Of these, mass-spectra of compounds *N*-3-methylbutyl acetamide, 6-oxo-1-nonanol were consistent with those of authentic standards and previously published records (Perkins et al. 1990, Wee & Tan 2005a); each with kovats indices of 1143.6 and 1352.9, respectively (Table 2). Mass-spectra fragmentation pattern of compound CF was also matched with those of Nishida et al. (1988) and NIST library; with a kovats index of 1745 consistent with the published data of Lalel et al. (2003). The kovats indices of other compounds, i.e., 2-hydroxy-3-
methyl, ethyl butanoate and 3-ethyl-2,5-dimethyl pyrazine identified by the NIST library were 970.0 and 1079.5, respectively of which the latter were consistent with the data published by Schnermann & Schieberle (1997) and Rychlik et al. (1998).

Figure 1. Gas chromatographic-mass spectra profile of the major volatile compounds in the rectal gland of a sexually mature (i) untreated, and (ii) methyl eugenol-fed male of Bactrocera carambolae. Compounds 2 = 2-hydroxy-3-methyl, ethyl butanoate; 3 = 3-ethyl-2,5-dimethyl pyrazine; 4 = N-3methylbutyl acetamide; 6 = 6-oxo-1-nonanol; 7 = 1,6-nonanediol, and 11 = trans-coniferyl alcohol. Compounds 1, 5, 8, 9 and 10 are unknown (Table 2).
Table 2. Volatile constituents of male rectal gland contents identified by GC-MS in the chromatogram peaks shown in Figure 1.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time</th>
<th>Compound</th>
<th>Kovats index</th>
<th>Kovats index (Lit.)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.357</td>
<td>Unknown</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.622</td>
<td>Unknown</td>
<td>922.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13.325</td>
<td>2-hydroxy-3-methyl, ethyl butanoate</td>
<td>970.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>17.047</td>
<td>3-ethyl-2,5-dimethyl pyrazine</td>
<td>1079.48</td>
<td>1079</td>
<td>(i), (ii)</td>
</tr>
<tr>
<td>5</td>
<td>19.065</td>
<td>N-3-methylbutyl acetamide</td>
<td>1143.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20.717</td>
<td>Unknown</td>
<td>1197.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>25.050</td>
<td>6-oxo-1-nonanol</td>
<td>1352.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25.494</td>
<td>1,6-nonanediol</td>
<td>1369.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>26.169</td>
<td>Unknown</td>
<td>1394.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>28.952</td>
<td>Unknown</td>
<td>1505.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>34.396</td>
<td>(E)-coniferyl alcohol</td>
<td>1745.01</td>
<td>1745</td>
<td>(iii)</td>
</tr>
</tbody>
</table>

(i) Schnermann & Schieberle 1997;  
(ii) Rychlik et al. 1998;  
(iii) Lalel et al. 2003

(b) *Bactrocera philippinensis*

Result showed that ME-fed males of *B. philippinensis* produced trans-coniferyl alcohol (CF) and 2-allyldimethoxyphenol (DMP) in the rectal gland compared to ME-deprived conspecific males. This shows that after feeding on ME, *B. philippinensis* males also produced similar phenylpropanoid compounds as in *B. dorsalis* and *B. papayae*. Fletcher & Kitching (1995) showed all the three species of untreated *B. dorsalis*, *B. philippinensis* and *B. papayae* were having similar rectal contents which are low in volatile content with fatty acids dominating. We are in the process of getting wild *B. philippinensis* from the Philippines for chemical analysis and comparison in the near future. The details of the results will be included in the next report.
II. CONCLUSION

The male rectal gland contents of *B. carambolae* found in this study was consistent with those previously published data (Nishida et al. 1988, Perkins et al. 1990, Fletcher & Kitching 1995, Tan & Nishida 1996, Wee & Tan 2005b, Wee et al. 2007). ME-fed males produced only CF along with its endogenous rectal volatiles which are identified as 2-hydroxy-3-methyl, ethyl butanoate, 3-ethyl-2,5-dimethyl pyrazine, N-3-methylbutyl acetamide, 6-oxo-1-nonanol, and 1,6-nonanediol. Through this present study, kovats indices of the major compounds were also determined of which some were new data while some were consistent with published data done by other researchers.

Males of *B. philippinensis* produced both CF and DMP similar to that of *B. papayae* (Nishida et al. 1988, Tan & Nishida 1996).

III. WORK PLAN FOR THE COMING YEARS

1. Due to the presence of morphological hybrids in the Peninsular Malaysia, precautions in and more careful selection of wild colony is a pre-requisite to kick start the establishment of a wild/wildish colony of *B. papayae* for future pheromone work. Continued efforts will be made to search for a more ‘reliable source of *B. papayae* colony for use in future study.

2. Once the establishments of wildish colony of *B. carambolae* (and hopefully *B. papayae*) produce good numbers of flies, headspace volatiles collection will be conducted to collect the volatiles emitted from the males (ME-deprived and ME-fed) during their courtship period for further chemical analysis and comparison with the rectal gland contents.

3. Quantitative analysis of the major compound(s) in the male rectal gland for ME-deprived and ME-fed males. Authentic compounds will be purchased or synthesized (by Dr. Ritsuo Nishida) for quantitative analyses to determine the relative ratios of the various volatile chemicals found in the rectal gland.

4. Wild/wildish *B. philippinensis* will be obtained for chemical analysis and comparison with the present results on laboratory strain from IAEA Entomology Unit.
IV. REFERENCES


Rychlik, M., Schieberle, P. & Grosch, W. 1998. In compilation of odour thresholds, odour qualities and retention indices of key food odorants. Deutsche Forschungsanstalt für Lebensmittelchemie and Institut für Lebensmittelchemie der Technischen Universität München.


