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Comparative sensitivity to and consumption of methyl eugenol in three *Bactrocera dorsalis* (Diptera: Tephritidae) complex sibling species

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Summary. Sensitivities to methyl eugenol of three sibling species in the *Bactrocera dorsalis* complex were compared. The degree of species sensitivity to methyl eugenol, i.e. *B. dorsalis* > *B. papayae* > *B. carambolae* (in decreasing order), was concomitant with the species age-related response to methyl eugenol as previously reported. The ability to consume methyl eugenol by the three sibling species showed similar trend - the average ME consumption per male was 0.70 µl for *B. dorsalis*, 0.58 µl *B. papayae* and 0.18 µl *B. carambolae.* Results obtained were discussed in relation to area-wide control of fruit fly.

Key words. *Bactrocera dorsalis* sibling species – *B. carambolae* – *B. dorsalis* – *B. papayae* – methyl eugenol sensitivity – consumption

Introduction

To date, methyl eugenol [1,2-dimethoxy-4-(2-propenyl) benzene; ME] is the most powerful Tephritid male fruit fly attractant compared with other naturally existing male attractants, such as raspberry ketone. Following extensive studies, fruit fly's behavior in relation to their strong attraction and voracious feeding on ME was exploited and has emerged as the key factor of success in the implementation of male annihilation program in the control of the Oriental fruit fly, *B. dorsalis* (Steiner *et al.* 1965; Koyama *et al.* 1984). The recent eradication of *B. papayae*, a sibling species of the *dorsalis* complex, from Queensland, Australia further demonstrated the potential use of ME in the fruit fly eradication programs (Fay *et al.* 1997).

Another sibling species of the *dorsalis* complex, *B. carambolae,* is posing an increasing threat to many commercially grown fruits in Malaysia, Indonesia and Thailand. It was ranked the third most economically important fruit pest with 75 host fruit species after *B. papayae* (193 species) and *B. dorsalis* (117 species) in the region (Allwood *et al.* 1999). It is also a fruit pest in the Latin America since its establishment in 1975 (Malavasi *et al.* 2000). A Regional *B. carambolae* Eradication Program launched in 1993 by the Guyana Government has successfully eradicated this pest from the Republic of Guyana in 2000 (Malavasi *et al.* 2000).

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The attraction to and consumption of ME in the *Bactrocera* spp. have an ecological significance in the fruit fly's intra- and inter-specific communication system. Generally, their attraction to ME corresponded with sexual maturity (Tan *et al.* 1987; Wong *et al.* 1989; Wee & Tan 2000a). Mature males after ME consumption produced additional and more potent sex pheromone components such as *trans*-coniferyl alcohol (CF), 2-allyl-4,5-dimethoxyphenol (allyl-DMP) and *cis*-3,4-dimethoxycinnamyl alcohol (*cis*-DCA) (Nishida *et al.* 1988a, 1988b; Tan & Nishida 1996). ME-acquired males have an earlier onset of courtship behavior and were sexually more competitive than ME-deprived males (Tan & Nishida 1996, 1988; Hee & Tan 1998; Shelly 2000). With the consumption of ME, the fly also developed an anti-predation mechanism to deter potential vertebrate predators (Tan & Nishida 1998; Wee & Tan 2001). The discovery of ME metabolites in wild *B. carambolae* and *B. papayae* male rectal glands further marked the importance of ME foraging in the fruit fly community (Wee & Tan 2001).

Both *B. dorsalis* and *B. papayae* males responded to ME optimally at 14 day-old or earlier (Tan *et al.* 1987; Wong *et al.* 1989). However, even though *B. carambolae* males responded to ME as early as 10 days after adult eclosion (< 5%), but optimum response (> 80%) was recorded only at an older age (Wee & Tan 2000a). Furthermore, this species consumed less amount of ME when compared with its sibling species (Wee 2000). Therefore, these studies aim to compare the sensitivity to and consumption of ME between *B. dorsalis*, *B. carambolae* and *B. papayae* males to aid in the fruit fly control strategy and to provide more information on the species' closeness between the *B. dorsalis* complex sibling species.

Materials and methods

Insects

The *B. carambolae* (10 generations per year) and *B. papayae* (12 generations per year) strains were collected from field-infested starfruits, *Averrhoa carambola* L. since 1995. *B. dorsalis* is not endemic in Malaysia. *B. dorsalis* pupae (laboratory strain 40th generation) was supplied by Taiwan Agricultural Research Institute, Taiwan in 2000. The original *B. dorsalis* colony in Taiwan was established since 1997 (12 generations per year) from field-infested guava in Wufeng, Taichung and was with invigoration once in every six months.

Subsequent colonies of the three species were bred, each in separate insectary room. All three sibling species were cultured in the insectary using standard diet and procedures as previously described (Wee & Tan 2000a). Virgin female and male were

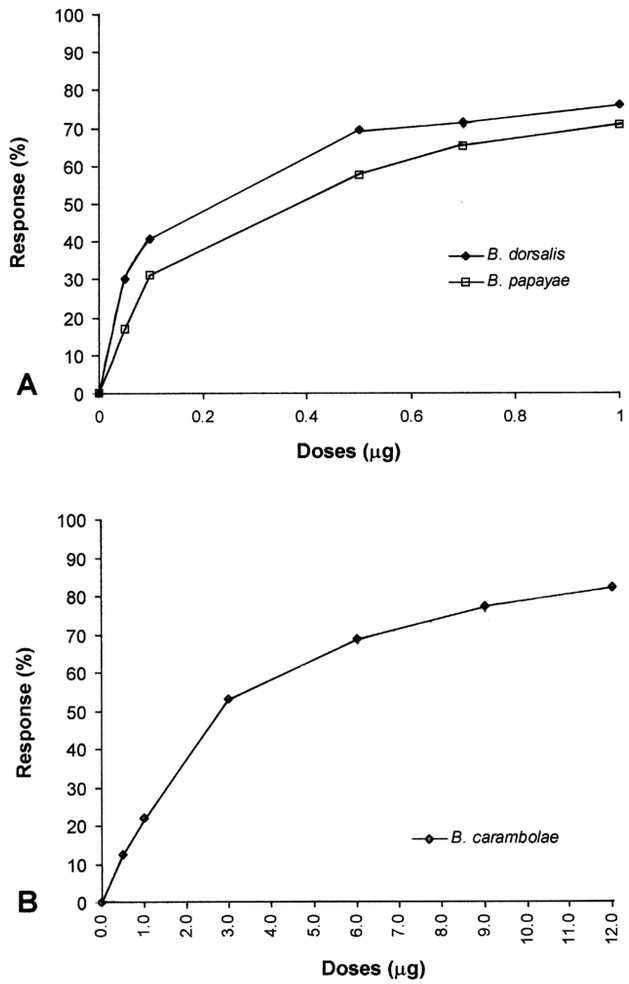

Fig. 1 Dose-sensitivity response curves of three *dorsalis* sibling species to male attractant, methyl eugenol at different concentrations. **(A)** *Bactrocera dorsalis* and *B. papayae*; **(B)** *B. carambolae* males

separated within 4 days after emergence (DAE) and held at 25-29 °C, 83-90% RH, and a 12 L :12 D photoperiod. Mature males (*B. papayae*: 14-19 DAE, *B. carambolae*: 30-35 DAE [maturity period; Wee & Tan 2000a], *B. dorsalis*: 14–19 DAE) were used in all experiments.

Methyl eugenol sensitivity

Bioassay was conducted in a research laboratory between 08:00–10:00 h during the peak fruit fly response to ME (Tan 1985) at 28–30°C with relative humidity, 83–91%. The bioassay site, which received natural sunlight from the north, was with minimum air movement. The bioassay was conducted indoor in small cages (30 × 30 × 30 cm; with screen sides) than in a large field cage in order to minimize the fluctuation of external factors (such as temperature, wind speed, light intensity and humidity) which may contribute to the differences in the males' attraction to ME, especially when three different but closely related fruit fly populations were tested. Sexually mature males [60 – 100 per replicate per cage] were allowed to acclimatize in the experimental area for at least 24 h.

The stock solution of ME (> 99.8 % purity) was serially diluted into different concentrations ranging from 12 µg to 0.05 µg per 5 µl absolute ethanol. Starting with the lowest concentration (i.e., 0.05 µg per 5 µl), 5-µl diluted ME was dispensed using a 10 µl Hamilton® syringe onto a filter paper disc (Whatman® No.1; 4.5 cm diameter) which was placed on an inverted glass petri dish (8 cm diameter) supported by a plastic cup.

A positive response was recorded when a male landed and fed on the filter paper containing the ME. An attracted male was immediately removed from the cage using an aspirator. Preliminary study has shown that under such controlled condition, the optimal duration for bioassay was 10 min. No significant changes in the number of males attracted to ME were observed beyond the 10-min bioassay period. Hence, the total number of flies responded was noted for 10 min before a new batch of males in a separate cage was tested for a higher dosage of ME. Five minutes was used for preparation of subsequent assay. In each assay, new glass petri dish and filter paper were used. Used filter paper was discarded into an enclosed container (for disposal) and the petri dishes were soaked in chromic acid for 24 h and cleaned to remove traces of ME. Inside of cages was cleaned with absolute ethanol before introducing any experimental flies for next series of bioassays on the following day. As controls, the tests were repeated with absolute ethanol in place of the ME solutions.

There were five replicates of each dosage (0.05 – 1.0 µg/5 µl) and five of the control for *B. dorsalis* males, while there were six replicates of each dosage and the control for *B. papayae* males. Preliminary tests showed a much lower ME sensitivity in *B. carambolae.* Therefore, bioassays at higher ME concentrations (i.e. from 0.5 to 12.0 µg/5 µl) and four replicates of each dosage and the control were conducted for *B. carambolae*.

Results obtained were pooled and analyzed using Probit analysis (Finney 1971). The probit of male response was plotted against the logarithm of ME dosage (ng) and the line of best fit was obtained. ED_{50} (effective median dose) is the dose required to elicit response in 50% of the population tested. 285 – 600 males of each species (per dose) were used in order to obtain a reliable estimate of ED_{50}. As three different species of fruit flies were studied, hence different fly populations were involved; the data was not subjected to parallelism test.

Methyl eugenol consumption

For each species, sexually mature virgin males were lightly anaesthetized with carbon dioxide and placed ventrally with plasticine fixed on the wings to reduce struggling. A 1-µl microcapillary pipette (32 mm long) (Drummond®) containing pure ME (1-µl contains 1.05 mg pure ME) was mounted on a micromanipulator and placed 3–5 mm away from a male. Upon sensing the ME, the male would reach for the lure by stretching out its proboscis. The fly was then allowed to consume the ME from the microcapillary pipette. The ME source was removed when the male stopped stretching out its proboscis or when it moved its proboscis away. The amount of ME consumed per fly was then calculated with the help of a micrometer calibrating glass slide.

Most males stopped feeding on ME within 5 minutes. For each species, 20 males were used on each day of feeding. A total of 5 replicates were performed, each using flies from a different cohort. Feeding results were subjected to one-way variance analysis (ANOVA) ($P = 0.05$) and means consumption of ME were compared between the species using Tukey's test (Kirk 1968) at $P = 0.05$.

Results

At the lowest concentration of ME i.e. 0.05 µg/5 µl, *B. dorsalis* males already showed an average of 30.3% response and the response increased for higher dosages of ME (Fig. 1A). The dose of 171 ng was required for ED_{50} in *B. dorsalis* males. However, at 0.05 µg/5µl, 17% *B. papayae* males responded and a dose of 318 ng was required for ED_{50}

Table 1 Probit analysis of males' attractancy to methyl eugenol for *Bactrocera carambolae, B. dorsalis* and *B. papayae*

Species	*n*	Regressions equation	$\chi 2$	*df*	ED_{50} (ng)	95% fiducial limits (ng)	
						Upper	Lower
B. carambolae	325	Y = 1.5313 X – 0.3028	1.673	4	2898	2635	3179
B. dorsalis	280 – 500**	Y = 0.9527 X + 2.8679	1.329	3	171	148	196
B. papayae	600	Y = 1.0996 X + 2.2359	2.995	3	318	288	351

*ED50 (effective median dose) is the dose required to elicit response in 50% of the fruit fly population tested.
**depending on the availability of insects.
n number of insects tested per dose.

in *B. papayae* (Fig. 1A). Approximately 12% of *B. carambolae* responded to 0.5 µg/5 µl, the lowest concentration of ME tested for this species (Fig. 1B). The male response increased gradually with the increasing ME concentrations. A dose of 2898 ng was needed for ED_{50} in *B. carambolae* males. In all the controls, no males were attracted to the filter paper disc containing absolute ethanol.

Based on the individual species response, the ED_{50} of *B. carambolae* (~2.90 µg) was approximately 17 and 9 times higher than those of *B. dorsalis* (~0.17 µg) and *B. papayae* (~0.32 µg), respectively while ED_{50} of *B. papayae* was two times higher than that of *B. dorsalis* (Table 1). Hence, *B. carambolae* males are least sensitive to ME among the three species.

There were differences in the ME consumption among the three species studied ($F_{2,12} = 57.69$, $P < 0.01$). *B. dorsalis* (0.70 ± 0.05 µ1) and *B. papayae* (0.58 ± 0.02 µl) males consumed significantly higher quantity of ME than *B. carambolae* males (0.18 ± 0.03 µl) ($P < 0.01$). Nevertheless, there was no significant difference in the quantity of ME consumed per male fly between *B. dorsalis* and *B. papayae* ($P > 0.05$). Most of the *B. dorsalis* (97%) males could individually consume ≥ 0.2 µl of ME, followed by 78% in *B. papayae* and 22% in *B. carambolae*. The highest recorded ME consumption per male fly in *B. dorsalis*, *B. papayae* and *B. carambolae* was 2.2, 2.7 and 1.3 µl, respectively.

Discussion

Here, we showed that within the ME-attracted group, three closely related *Bactrocera* spp. of the same complex demonstrated different sensitivity to ME. The trend of species sensitivity to ME, i.e. *B. dorsalis* > *B. papayae* > *B. carambolae* (in decreasing order), was concomitant with the species age-related response to ME as previously reported. *B. dorsalis* males attracted optimally to ME as early as 10 day-old (Wong *et al.* 1989), most *B. papayae* males responded to ME by 14 day-old (Tan *et al.* 1987) while *B. carambolae*, at 28 day-old (Wee & Tan 2000a).

The fact that *B. dorsalis* and *B. papayae* males are more-sensitive to ME and responded at a younger age before mating may have contributed largely to the success of previous eradication programs using male annihilation technique (Steiner *et al.* 1965; Koyama *et al.* 1984; Fay *et al.* 1997). This is because the essential pre-requisite for the successful application of the male annihilation technique lies in the fact that males respond to the lure before they are able to mate and subsequently produce offspring. By mass-killing the males before they can mate with females will help to remove large numbers of potential mates from the population and hence reduce greatly the numbers of gravid females from causing fruit damage.

The discovery of a much lower sensitivity to ME in *B. carambolae* males may explain the low trapping number of this species in most of the ecological work conducted in Malaysia (Wee 2000). Hence, the distance between ME-baited traps, thus trap distribution, in an area-wide control program may affect the trapping of *B. carambolae.* This result plus the older age at which *B. carambolae* responded to ME, show that *B. carambolae* eradication program should be carefully planned (i.e. the increase of the number traps/affected area) and integrated with other control measures to achieve satisfactory results. The latter include killing the immature stages by increasing the predation and parasitism rates, removal of their breeding source (i.e. infested fruits), as well as killing both adult males and females with protein and insecticide baited traps in order to provide a better control coverage.

A successful *B. carambolae* eradication program by incorporating the above measures into a male annihilation program was the Regional *B. carambolae* Eradication Program in Guyana as not a single *B. carambolae* male was trapped for 2 consecutive years by the end of the program (1999-2000) (Malavasi *et al.* 2000). It is important to stress that the monitoring program of this species using ME-baited traps should be extended for a longer period than its sibling due to lower sensitivity to and consumption of ME, besides having an older age response to ME (Wee & Tan 2000a).

ME, although an extraordinarily attractive and very widely used lure for the Oriental fruit fly and related species, has been shown to be an animal carcinogen (Miller *et al.* 1983; Wee & Tan 2001). Fruit flies fed on ME appeared to be sluggish in the beginning (Shelly & Dewire 1994; Wee 2000). *In vitro* study has shown that conversion of ME into other phenylpropanoids in *B. papayae* males involved an enzyme, i.e. mixed-function monooxygenase which is based on the cytochrome P-450 system (Lim *et al.* 1998). Using a simple oxidative process, the insect detoxifies and converts ME into less harmful by-product(s) to be stored in the rectal gland prior to release. In nature, ME, as a secondary plant compound, promotes mutualistic relationship with the fruit fly, e.g. in pollination or protection against phytophagous insects. In return, the male fruit fly is rewarded with

improved sexual attraction and protection from potential predator following pharmacophagy of ME (Tan & Nishida 1996, 1998; Wee & Tan 2001; Tan *et al.* 2002).

In the wild, availability of pure ME has not been discovered so far. ME is usually found in varying quantities in components of plants. Therefore, we suggest that the amount of ME that a male *Bactrocera* species could consume will indirectly reflect the ability/efficiency of their ME detoxification system and thus, having great evolutionary significance in terms of species advancement in relation to their co-evolutionary relationship with plants. In this case, while there was no significant difference between *B. dorsalis* and *B. papayae*, the ability of *B. carambolae* to consume ME was significantly lower than the other two sibling species. Furthermore, previous behavioral studies demonstrated that males of *B. dorsalis* and *B. papayae* 1-day after feeding on ME were sexually more competitive than ME-deprived conspecific males (Tan & Nishida 1996). However, 1-day post ME-feeding *B. carambolae* males appeared to be sluggish, and significant mating competitiveness against the ME-deprived males was only obtained on the third day post ME-feeding (Wee 2000).

Similar trend of closeness between *B. dorsalis* and *B. papayae* compared with *B. carambolae* were also reported in their glandular chemistry (Perkins *et al.* 1990), tissue iso-enzyme analysis (Yong 1995), genitalia length (Iwaizumi *et al.* 1997) and ribosomal DNA analysis (Armstrong & Cameron 2000). Therefore, the ability to respond to (i.e., sensitivity and age-related response) and consumption of ME (detoxification efficiency) may be of evolutionary and systematic significance to these sibling species. However, the ability of the three sibling species to interbreed with one and the other as well as producing viable offspring (Wee & Tan 2000b) suggest that they are probably sub-species belonging to a single species.

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