

# Synergists isolated from cade oil for the parapheromone $\alpha$ -ionol for male *Bactrocera latifrons* (Diptera: Tephritidae)

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## Abstract

The solanaceous fruit fly, *Bactrocera latifrons* (Hendel), is a tephritid fruit fly of the subfamily Dacinae which does not respond to male attractants attractive to most other members of this subfamily (methyl eugenol or cuelure). Male *B. latifrons* have been found to be attractive to  $\alpha$ -ionol, with the attractancy synergistically enhanced by the addition of cade oil, a destructive distillation tar of *Juniperus oxycedrus* L. twigs. Solvent extracts and chromatographic fractions of cade oil were tested for attractancy enhancement for  $\alpha$ -ionol to sexually mature *B. latifrons* in an outdoor olfactometer and macadamia nut orchard, and chemicals were analyzed by gas chromatography–mass spectrometry (GC–MS). Approximately 220 chemicals were found in the hexane and base extracts. Profile comparison of GC–MS chromatograms and bioactivities between fractions indicated seven chemicals in cade oil likely to enhance the attractancy of  $\alpha$ -ionol. These chemicals are 4-allyl-2-methoxyphenol (eugenol), 2-methoxy-4-propenylphenol (isoeugenol), 2-allyl-6-methoxyphenol (*o*-eugenol), 2-methoxy-4-propylphenol (dihydroeugenol), 3-hydroxy-4-methoxybenzaldehyde (isovanillin), 2-butenal (crotonaldehyde), and 4-ethyl-2-methoxyphenol. Bioassays in an outdoor olfactometer and preliminary field trials of the authentic chemicals showed that eugenol, isoeugenol, dihydroeugenol, and 4-ethyl-2-methoxyphenol enhanced  $\alpha$ -ionol attractancy up to 1.3–2.1-fold (olfactometer) or 2.0–2.4-fold (field) compared to  $\alpha$ -ionol alone when each of them was presented together with  $\alpha$ -ionol. The identification of eugenol as a synergist for the attractiveness of  $\alpha$ -ionol to *B. latifrons* helps to better place *B. latifrons* in the overall Dacinae male lure response pattern.

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**Keywords:** *Bactrocera latifrons*; Cade oil;  $\alpha$ -Ionol; Eugenol; Isoeugenol; Dihydroeugenol; Parapheromone

## 1. Introduction

The solanaceous fruit fly, *Bactrocera latifrons* (Hendel), is a tephritid fruit fly native to South and Southeast Asia (White and Elson-Harris, 1992). It was first detected in Hawaii in 1983 (Vargas and Nishida, 1985) where it has been found to infest fruits of solanaceous and cucurbitaceous plants (Liquido et al., 1994). As has been discovered for many other tephritid fruit fly species, *B. latifrons* males have been found to respond to a “parapheromone” which is not naturally used in intraspecific communication but which elicits a similar response to that of a true pheromone

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(Cunningham, 1989). However, whereas most Dacinae tephritid fruit fly males respond to either methyl eugenol or cuelure (Metcalf, 1990; Cunningham, 1989; Drew and Hooper, 1981; Drew, 1974), *B. latifrons* males have been found to be one of the tephritid fruit fly species of the subfamily Dacinae that has failed to fit into this male lure dichotomy, responding instead to  $\alpha$ -ionol [4-(2,6,6-trimethylcyclohex-2-enyl)-but-3-en-2-ol (**1**, Fig. 1)] (Flath et al., 1994; McGovern et al., 1989), with attraction synergistically enhanced by the addition of cade oil (McQuate and Peck, 2001; Liquido et al., 2000). Cade oil, destructive distillation tar of *Juniperus oxycedrus* L. twigs, is composed of a large number of chemicals (Chalchat et al., 1988, 1990; Markku et al., 1996; Ucar and Balanban, 2002), making it difficult to discern why its addition to  $\alpha$ -ionol enhances attraction to male *B. latifrons*. Because male tephritid fruit fly response to attractants has been found to partly conform to taxonomic divisions (Metcalf, 1990; Drew and Hooper, 1981; Drew, 1974), improved understanding of the atypical male lure response of *B. latifrons* has the potential to contribute significantly to tephritid fruit fly systematics.

In this study,  $\alpha$ -ionol synergists in cade oil for *B. latifrons* were isolated and identified with column chromatography and high performance liquid chromatography (HPLC). Bioassays were initially conducted in outdoor olfactometers, followed by field trials with the authentic chemicals to test synergistic effects on  $\alpha$ -ionol attractancy. Results of chemical isolation and identification steps and olfactometer and preliminary field trials are reported here while the results of further field trials are reported elsewhere (McQuate et al., 2004). Additionally, using one of the identified active ingredients in cade oil, field tests were conducted to better assess the relation of the male lure response of *B. latifrons* to the predominant male lure dichotomy of methyl eugenol-responding and cuelure (4-(3-oxobutyl)phenyl acetate)-responding tephritid fruit fly species.

## 2. Materials and methods

### 2.1. Chemicals

$\alpha$ -Ionol was obtained from Bedoukian Research Inc. (Dansbury, CT). Rectified cade oil was from Penta Manufacturing (West Caldwell, NJ). 2-Methoxy-4-propylphenol (dihydroeugenol, **2**), 4-allyl-2-methoxyphenol (eugenol, **3**), 4-ethyl-2-methoxyphenol (**4**), 2-methoxy-4-propenylphenol (isoeugenol, **5**), 2-butenal (crotonaldehyde, **6**), 3-hydroxy-4-methoxybenzaldehyde (isovanillin, **7**), and 2-allyl-6-methoxyphenol (*o*-eugenol, **8**) were purchased from Sigma Chemical Co. (St. Louis, MO) and TCI America (Portland, OR).

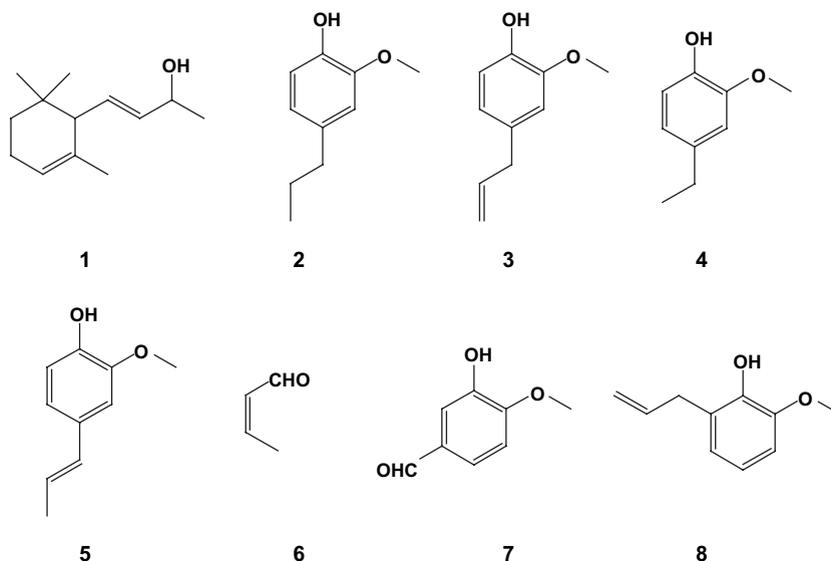


Fig. 1. Chemical structures of  $\alpha$ -ionol and potential synergists isolated from and identified in cade oil, and tested for synergistic effects on  $\alpha$ -ionol attractancy to *B. latifrons*.  $\alpha$ -Ionol (**1**), dihydroeugenol (**2**), eugenol (**3**), 4-ethyl-2-methoxyphenol (**4**), isoeugenol (**5**), crotonaldehyde (**6**), isovanillin (**7**), and *o*-eugenol (**8**).

## 2.2. Extraction and fractionation of cade oil

Cade oil extraction scheme is shown in Fig. 2. Cade oil (100 g) was extracted with 1 N sodium hydroxide (200 ml  $\times$  2). After the base extract was neutralized with aqueous HCl, it was extracted with dichloromethane (300 ml  $\times$  3). The remaining residues after base extraction were extracted with hexane (400 ml  $\times$  6) and diethyl ether/hexane mixture (1/1, v/v, 200 ml  $\times$  3). The organic solvent extracts were combined and fractionated with vacuum liquid chromatography (VLC). To a silica gel column (3 cm i.d.  $\times$  20 cm, 200 g of silica gel, Aldrich, TLC grade), extracts (1 ml) were applied and eluted sequentially with 100 ml of hexane, 100 ml each of hexane–diethyl ether mixtures at volume ratios 9:1, 8:2, 7:3, 200 ml of hexane–diethyl ether (5:5), and 200 ml of diethyl ether. Each fraction was 80 ml. After removal of the solvents, residues were re-dissolved in chloroform and an aliquot of samples was tested for bioactivity. Residues having bioactivity were combined and further purified with HPLC.

The HPLC system was a Perkin Elmer Series 10 liquid chromatograph with an Applied Biosystems 1000S diode array detector. The mobile phase was 50% aqueous methanol at a flow rate of 0.6 ml/min. The detection wavelength was 210 nm. An Econosil C18 column (250 mm length  $\times$  10 mm i.d., 10  $\mu$ m) was purchased from Alltech (Deerfield, IL). Each fraction was 0.6 ml. Bioactivity was tested for all HPLC fractions, and the bioactive fractions were extracted with chloroform and analyzed with gas chromatography–mass spectrometry (GC–MS).

## 2.3. Chemical identification

The GC–MS used was a Hewlett Packard (HP) Series II 5890 GC equipped with a HP 5989A MS, and a HP-1 column (30 m  $\times$  0.25 mm film thickness). It was operated in electron impact mode (70 eV). The column temperature was started at 50  $^{\circ}$ C for 5 min, raised up to 280  $^{\circ}$ C at a rate of 2.5  $^{\circ}$ C/min, and then held at 280  $^{\circ}$ C for 10 min. The injector and ion source temperatures were 250  $^{\circ}$ C. The carrier gas was helium at a flow rate of 1 ml/min.

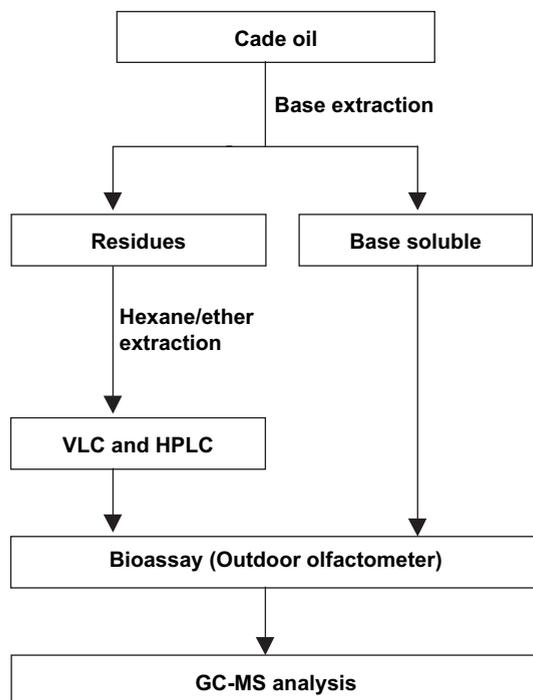


Fig. 2. Procedures of isolation and identification of synergists in cade oil.

## 2.4. Bioassay

*B. latifrons* individuals were obtained as pupae from the USDA-ARS – Pacific Basin Agricultural Research Center (Honolulu, HI) and held in 5.0-L plastic screen-topped buckets for adult emergence and aging to sexual maturity. Synergistic activities were tested with sexually mature *B. latifrons* in an outdoor 2.7 m<sup>2</sup> by 2.4 m high screened Gow olfactometer (Beroza and Green, 1963; Flath et al., 1994; Gow, 1954; Liquido et al., 2000; McQuate and Peck, 2001). In the olfactometer, 10 Jackson traps with sticky inserts were hung at the ends of 10, 0.61-m spokes of a horizontally mounted motor-driven wheel suspended from the top of the cage. Eight of the traps included a cotton wick treated with 0.2 ml of a fraction and a separate cotton wick treated with 1.0 ml of **1**. The 9th trap had only a cotton wick treated with 1.0 ml, of **1** and the 10th trap had two wicks treated with 1.0 ml and 0.2 ml water, respectively. During the bioassay, the wheel rotated slowly (28 s/revolution) to minimize positional effects on trap response. Traps were retrieved after 4 h and flies counted for each treatment. Multiple runs were necessary to complete testing of all fractions. Attractancy tests with the authentic chemicals as controls were conducted without  $\alpha$ -ionol according to the same experimental procedures.

To test the synergistic activity in the field, a 5000 ppm solution of each of the seven identified compounds (**2**–**8**) was prepared in methanol. The field trial was conducted using a 1:4 volume ratio of potential synergist solution to **1**. For each synergist solution, 0.1 ml of synergist solution and 0.4 ml of **1** (giving an effective ratio of 1:800) were added to separate wicks and then placed in a Jackson trap equipped with a sticky insert to catch attracted flies. Also included were the following treatments: 0.1 ml cade oil and 0.4 ml of **1** (on separate wicks), 0.4 ml of **1** alone, and 0.4 ml water alone. Five traps of each treatment were set out in a random complete block design in a macadamia nut orchard (a non-host environment). Traps were placed in every tree down a row (4.6 m spacing) with replicate blocks in adjacent rows (9.2 m spacing). Approximately 22,000 sexually mature *B. latifrons* adults were uniformly released from holding containers in the aisles between tree rows and beyond the outer tree rows with traps. Traps were retrieved 24 h after the fly release. There were a total of three repetitions for this test. To assess the relation of the male lure response of *B. latifrons* to the predominant male lure dichotomy of methyl eugenol – responding and cuelure – responding tephritid fruit fly species, a field test was conducted with Jackson traps baited with cotton wicks treated with either 1.0 ml methyl eugenol, 1.0 ml cuelure, 1.0 ml  $\alpha$ -ionol, 1.0 ml eugenol (one of the active ingredients identified in cade oil), or 1.0 ml  $\alpha$ -ionol + 1.0 ml eugenol. Eight traps of each treatment were set out in a random complete block design in a macadamia nut orchard. Traps were placed in every tree down a row (4.6 m spacing) with replicate blocks in adjacent rows (9.2 m spacing). Approximately 22,000 sterile, sexually mature *B. latifrons* adults and 13,000 sterile, sexually mature *Bactrocera cucurbitae* (a cuelure – responding species) adults were uniformly released from holding containers in the aisles between tree rows and beyond the outer tree rows with traps. Higher numbers of *B. latifrons* were released than *B. cucurbitae* to compensate for known lower responsiveness of *B. latifrons*. Wild *Bactrocera dorsalis* (a methyl eugenol – responding species) adults were naturally present in this field so no additional release of this species was necessary. Traps were retrieved 24 h after the fly release. There were a total of three repetitions for this test.

For the olfactometer and field trials, all trap catch results were square-root transformed prior to statistical analysis with SAS (olfactometer and initial field trial) (SAS Institute, 1998) or SYSTAT 10 (three species field trial) (SPSS Inc., 2000) statistical software. The difference in catch among treatments was tested using an analysis of variance on the transformed values followed by a Waller–Duncan *K*-ratio *t* test (olfactometer and initial field trial) or a Bonferroni pairwise procedure (three species field trial) for separation of means. Untransformed trap catch results are presented together with statistical results based on transformed values.

## 3. Results and discussion

Two hundred and twenty chemicals in cade oil were found with GC–MS. A major class of constituents in cade oil was mono-, sesqui- or di-terpenes (Table 1), which were also found in the other parts of the same plants (Adams, 1998; Barrero et al., 1991, 1995; Chalchat et al., 1988, 1990; Hernandez et al., 1987; Hinkley et al., 1994; Markku et al., 1996; Ucar and Balanban, 2002). Substituted phenols, alkyl- and alkoxy-benzenes and polyaromatic hydrocarbons (PAHs) were also found in cade oil. Among the phenolic compounds, methyl- and

Table 1  
Chemicals found in hexane and base extracts of cade oil

Chemical class	Representative chemical	General substitution pattern	No. of chemicals
Phenol and alkylphenol	2,3-Dimethylphenol	Mono-, di- or tri-methyl or ethyl	20
Alkoxy or alkyl alkoxy phenol	2-Methoxy-4-methylphenol Eugenol (870) <sup>a</sup> Isoeugenol (730) <sup>a</sup> 4-Propyl-2-methoxyphenol (1200) <sup>a</sup> 4-Ethyl-2-methoxyphenol (2000) <sup>a</sup> <i>o</i> -Eugenol (2500) <sup>a</sup>	Mono- or di-methoxy, or methyl-, ethyl- or propyl-ethoxy	14
Alkyl and alkoxybenzene	1-Methyl-3-propylbenzene	Mono-, di- or tri-methyl, or ethyl or propyl- methoxy	18
PAHs, alkyl or alkoxy PAHs	Naphthalene	Mono- or di-methyl or ethyl-anthracenes, benzofurans, indenenes, naphthalenes, phenanthrenes	53
Terpenoids	Widdrene	Mono-, sesqui- or di-terpenes	71
Others	Isovanilin (430) <sup>a</sup> , crotonaldehyde (6300) <sup>a</sup>	Aromatic aldehyde and ester, fatty acids, isoprenoid	44

<sup>a</sup> Numbers in parenthesis are concentrations (mg/kg cade oil).

methoxy-phenols were the most abundant phenols. Small quantities (<0.05%) of non-substituted indenenes and anthracenes were found, but most of the PAHs were polyalkyl (di- or tri-methyl or ethyl) substituted naphthalene and phenanthrene.

Several VLC fractions presented together with  $\alpha$ -ionol showed 1.8–4.0-fold enhancement of male catch compared with  $\alpha$ -ionol alone (3.0, 1.8, 1.8, 1.9, 1.8, and 4.0-fold enhancement by the fractions 2–5, 8, 11–12, 17, 20–23, and 28–32, respectively). Because of chemical complexity, synergistic VLC fractions were further fractionated with HPLC. Thirty-six HPLC fractions (3.0, 2.8–4.16, 3.8, 2.8, 2.2–2.6, 2.2-fold enhancement by the fraction nos. 4–6, 16–30, 49–51, 55–57, 67–75 and 85–87, respectively), which showed twofold or more synergistic activity, were analyzed for chemical compositions. The chemical profiles and concentrations of bioactive fractions were compared with those of little or no activity (Fig. 3). Characteristic and differentiated chemicals, found only in bioactive fractions, were 2–8 (Fig. 1).

The seven pure chemicals were bioassayed with *B. latifrons* for synergistic effects on the attractancy of 1 (Fig. 4). In the outdoor olfactometer study, there was a significant difference in catch ( $F = 6.81$ ,  $df = 9,30$ ,  $P < 0.001$ ), but only the catch at the water control was significantly different from other treatments. However, there was a significant difference in catch among treatments in field trials performed in a macadamia nut orchard ( $F = 31.93$ ,  $df = 9,140$ ,  $P < 0.001$ ). Though the catch was significantly less than that at the trap baited with 1/cade oil, four of the seven chemicals showed a moderate synergistic activity (Fig. 4). The four chemicals were 2, 3, 4, and 5, which are structurally similar. These four compounds, when presented at higher concentrations relative to  $\alpha$ -ionol, all showed good synergistic enhancement of the attractiveness of  $\alpha$ -ionol to male *B. latifrons*, though wild fly catch at traps baited with eugenol (3) +  $\alpha$ -ionol was significantly less than catch at traps baited with  $\alpha$ -ionol + cade oil (McQuate et al., 2004).

The results of the test of comparative response of three fruit fly species (Fig. 5) show that there is little overlap of attractiveness of the three male lures. McQuate et al. (2004), though, reported a relatively higher response of *B. dorsalis* to eugenol, but that response was still low relative to the attraction to methyl eugenol. Nishida et al. (2004) also reported marginal attraction of *Bactrocera papayae* Drew & Hancock (a methyl eugenol – responding species not considered to be genetically different than *B. dorsalis*) to eugenol. The identification of eugenol, however, as a synergist for the attractiveness of  $\alpha$ -ionol to *B. latifrons*, helps to better place *B. latifrons* in the overall Dacinae male lure response pattern. Eugenol is considered to be a precursor in the evolutionary development of methyl eugenol as a Dacinae male attractant (Metcalf, 1990). Although the *B. latifrons* antennal kairomone receptor has evolved to respond differently than those species responding to methyl eugenol or to cuelure (Metcalf, 1990), there remains some influence of a methyl eugenol evolutionary precursor.

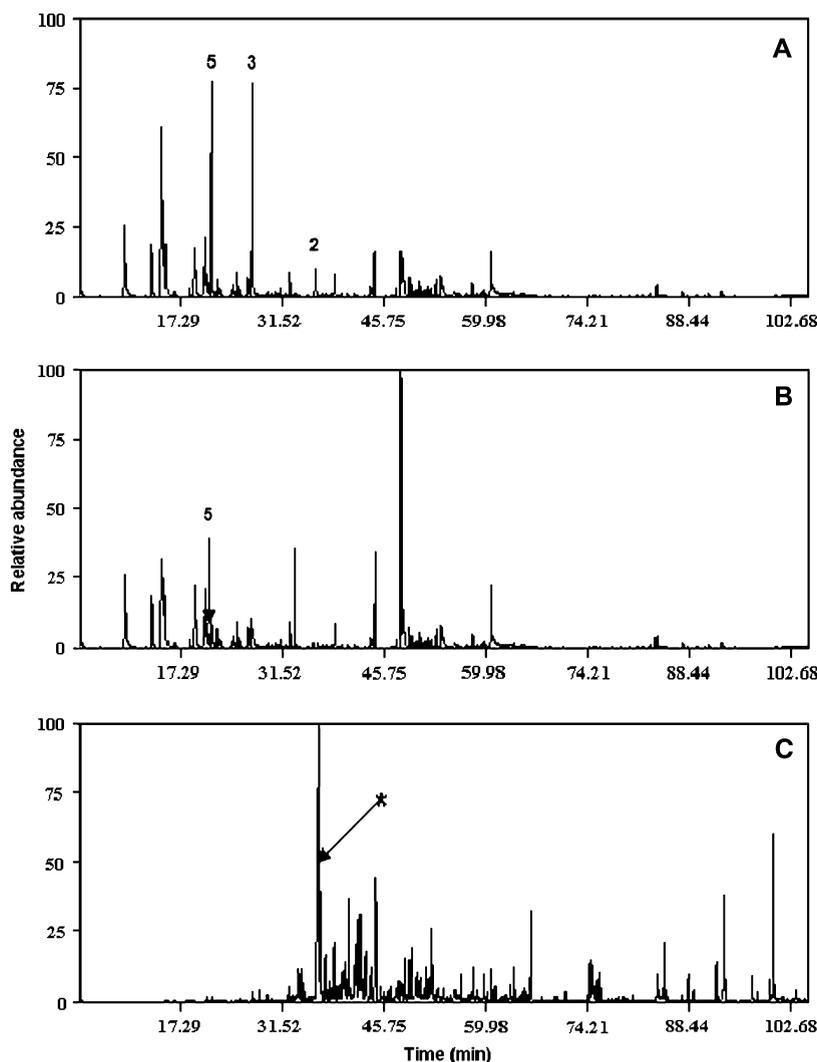


Fig. 3. Representative GC–MS chromatograms of strongly synergistic (A), weak synergistic (B) and non-active HPLC fractions (C). Dihydroeugenol (2), eugenol (3), and isoeugenol (5), the \* denotes that it was not dihydroeugenol, but was 2,4,5-trimethylphenol.

It is noted that all VLC fractions were tested for attractive activity to male and female fruit flies (data not shown). No fractions had appreciable attraction to female flies while VLC fractions 3–8, 15, and 33, presented without **1**, showed approximately 10–20% of the attractancy of **1** to male flies. The characteristic compounds in these fractions were alkyl- and alkoxy-benzenes. Attractive activities of alkyl- or alkoxy-benzenes were known for *Anomala schonfeldti* (Coleoptera: Scarabaeidae) (Maekawa and Imai, 2001). Male specific attractants for *B. latifrons* may exist in cade oil, in addition to the  $\alpha$ -ionol synergists identified here, which needs further investigation.

Many insect attractant synergists have been reported (Coracini et al., 2001; Czokajlo and Teale, 1999; Dowd and Bartelt, 1991; Osborne and Boyd, 1975; Warner, 1991). In general, chemicals having structural resemblance with an attractant showed strong synergistic or inhibitory effects (Coracini et al., 2001). However, some simple alcohols, heterocycles, and phenols having very different structures from corresponding attractants enhance the activities of terpenoid and alkenyl attractants (Czokajlo and Teale, 1999; Warner, 1991). The chemicals reported here are structurally very different from **1**, and have *ortho*-methoxyphenol as a common moiety except crotonaldehyde. It appears that a structural similarity to **1** is not a strict requirement for synergistic effects.

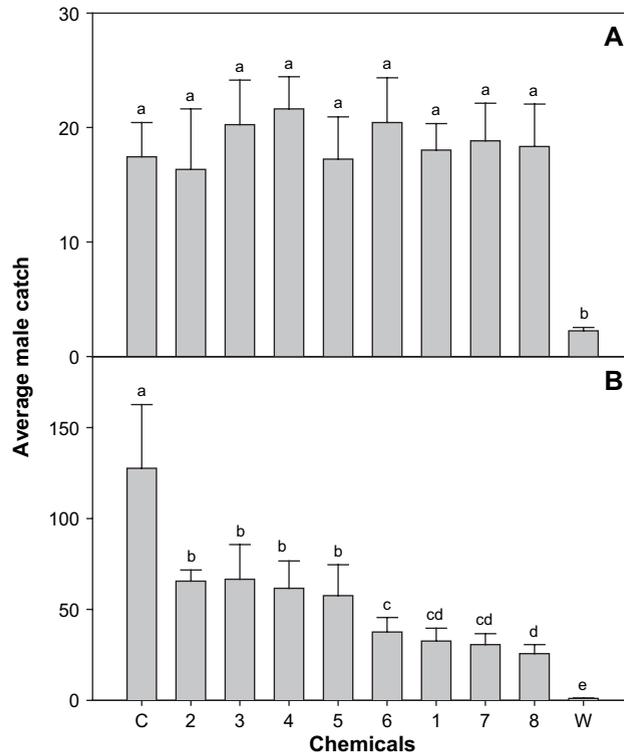


Fig. 4. Average sterile male catch at traps in an outdoor olfactometer (A) and in a macadamia nut orchard (B). The treatments in (A) were 1.0 ml of  $\alpha$ -ionol and 0.2 ml cade oil, 1.0 ml  $\alpha$ -ionol alone, 1.2 ml water alone or 1.0 ml of  $\alpha$ -ionol and 0.2 ml of a 5000 ppm solution of each of the seven potential synergists. The treatments in (B) were 0.4 ml of  $\alpha$ -ionol and 0.1 ml cade oil, 0.4 ml  $\alpha$ -ionol alone, 0.4 ml water alone or 0.4 ml  $\alpha$ -ionol and 0.1 ml of a 5000 ppm solution of each of the seven potential synergists. Trap catches marked by the same letter are not significantly different at the  $\alpha = 0.05$  level. Cade oil (C) and water (W).

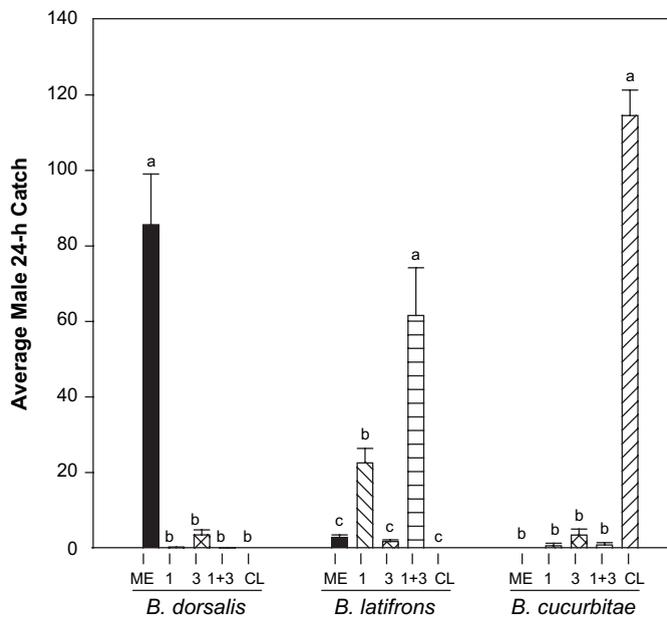


Fig. 5. Average 24 h male catch of *B. dorsalis*, *B. latifrons*, and *B. cucurbitae* in a macadamia nut orchard in traps baited with methyl eugenol (ME),  $\alpha$ -ionol (1), eugenol (3),  $\alpha$ -ionol + eugenol, or cuelure (CL). For a given fruit fly species, means with the same letter are not significantly different (at the  $\alpha = 0.05$  level) based on ANOVA of square-root transformed trap catch data.

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