# Assessing Methyl Eugenol and Dietary Protein on Peach Fruit Fly, *Bactrocera zonata* Saunders (Diptera: Tephritidae) Males for Enhancing the Effectiveness of SIT Application

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# Assessing Methyl Eugenol and Dietary Protein on Peach Fruit Fly, *Bactrocera zonata* Saunders (Diptera: Tephritidae) Males for Enhancing the Effectiveness of SIT Application

**A PhD dissertation submitted in the partial fulfillment for the degree of Doctor of Philosophy (PhD) in Plant Sciences**


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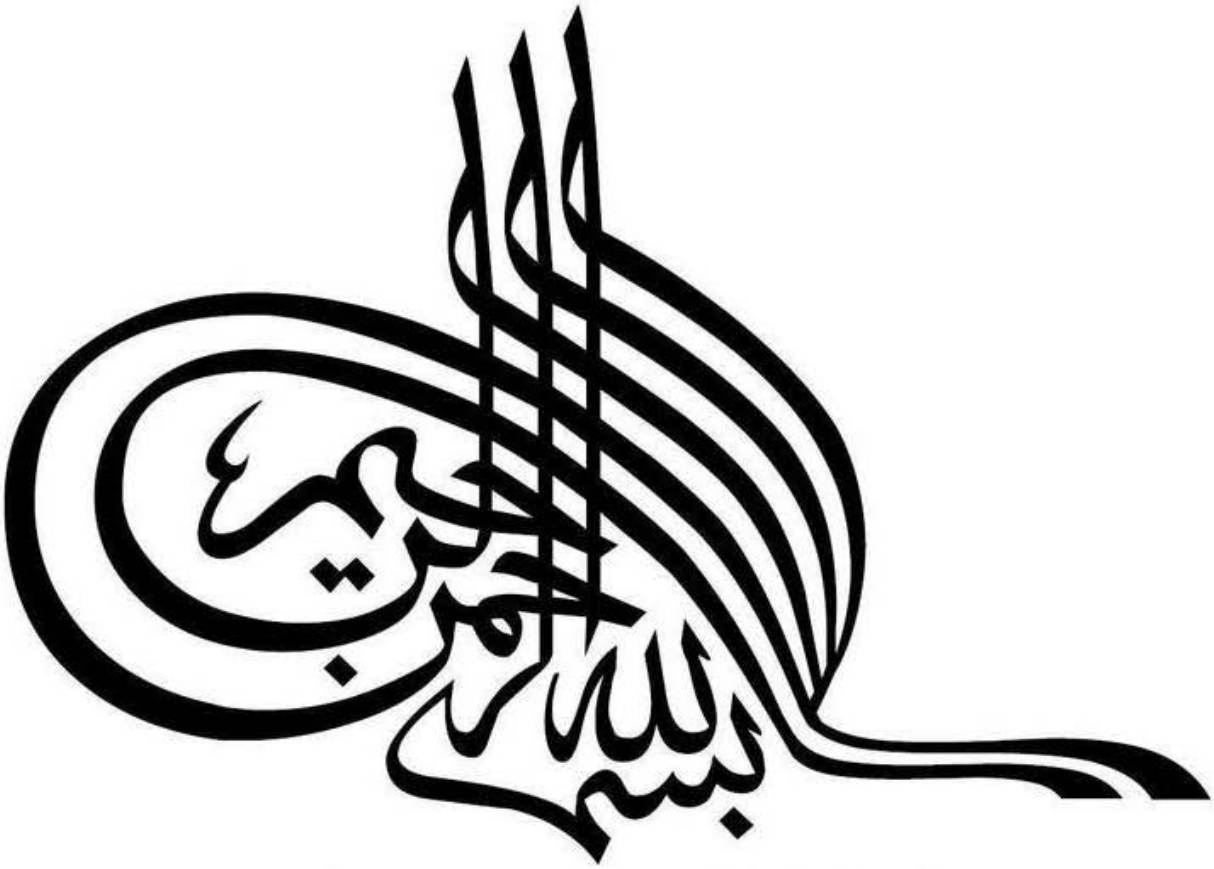


*In the name of Allah,*
*the Most Beneficent,*
*the Most Merciful*

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This is to certify that the research work in this thesis, entitled "**Assessing Methyl Eugenol and Dietary Protein on Peach Fruit Fly, *Bactrocera zonata* Saunders (Diptera: Tephritidae) Males for Enhancing the Effectiveness of SIT Application** " was conducted by **Mr. Awais Rasool** under the supervision of **Dr. Muhammad Farooq Hussain Munis**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to **Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad** in partial fulfilment of requirements for the degree of **Doctor of Philosophy** in the field of **Plant Sciences.**

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**"To my parents,**

**who never stopped believing in me."**

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AWAIS RASOOL

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| | |
|---|---|
| ANOVA | Analysis of Variance |
| AW-IPM | Area Wide-Integrated Pest Management |
| *B. zonata* | *Bactrocera zonata* |
| cm | Centimeter |
| D | Dark |
| d.f | Degrees of Freedom |
| DMP | Dimethoxy Phenol |
| DPT | Days Post Treatment |
| EPPO | European and Mediterranean Plant Protection Organization |
| g | Gram |
| h | Hours |
| L | Light |
| m | Meter |
| MAT | Male Annihilation Technique |
| ME | Methyl Eugenol |
| min | Minutes |
| mL | Milliliter |
| n | Number |
| P- | Sugar-only |
| P+ | Dietary Protein |

| | |
|---|---|
| *P* | Probability |
| R.H. | Relative Humidity |
| SE | Standard Error |
| SIT | Sterile Insect Technique |
| spp. | Species |
| Z-CF | (Z)-coniferyl Alcohol |

# ABSTRACT

Methyl eugenol (ME) naturally occurs in several plant species. It strongly attracts males of many *Bactrocera* species. The peach fruit fly, *Bactrocera zonata* Saunders (Diptera: Tephritidae) is endemic to South and Southeast Asia. The species is a quarantine pest and interferes with the international trade of horticultural produce. The male annihilation technique (MAT) incorporating the ME is an environmentally benign technique that can be used to suppress *B. zonata* population. Due to certain limitations of MAT, the sterile insect technique (SIT) has been used to eliminate several pests successfully and has the potential to be applied against *B. zonata*. The SIT inherently relies upon mass rearing of males of the target insects, sterilizing them with ionizing radiations and releasing them in the target area where mating of the sterile males with wild females will not produce offspring. The sustained releases of sterile males can lead to eradication/suppression of the target population. For the success of SIT, sterile males must be competitive, and the increased mating success of sterile males can enhance the efficiency of SIT. ME can increase the mating success of several *Bactrocera* species males but the necessary knowledge on how ME can be utilized to increase *B. zonata* male mating success for enhancing the efficiency of SIT was not available. The simulation model suggested that the simultaneous application of MAT and SIT can enhance the cost-effectiveness of fruit fly control programmes manifold. The objectives of the current studies were to assess, i) Effect of methyl eugenol on *B. zonata* male age-dependent response and mating success, ii) ME delivery system, adoptable in SIT operational programs, and assess the ME delivery system for suppressing the subsequent response of ME treated males to ME-baited traps for simultaneous application of MAT and SIT, iii) Effect of methyl eugenol treatment and dietary protein on *B. zonata* male mating success, and iv) Effect of methyl eugenol treatment and dietary protein on *B. zonata* male survival. The results showed that males initiated their mating at 8 days of age and attained the highest mating success at 16 days of age. A proportion of immature males responded to ME, the percentage of responders increased with age and the maximum response was observed during the sexual maturity age. ME treatment at sexual maturity age significantly enhanced the male mating success over untreated males. The males treated with ME at a sexually immature age (5 days; a standard protocol of holding males in sterile males holding and release facility), achieved significantly higher mating success after reaching sexual maturity age. These findings suggested that *B. zonata* males can be treated with ME at 5 days of

age in sterile male holding and release facilities and released in the field to achieve higher mating success after attaining sexual maturity.

Owing to ME-feeding impracticable, ME-aromatherapy which is a practicable delivery system in sterile male holding and release facility was developed. The ME-aromatherapy given at 5 days of age yielded higher mating success of males, after attaining sexual maturity. The ME-aromatized males also exhibited reduced response to ME-baited traps. ME-aromatherapy will allow the simultaneous application of MAT and SIT.

For assessing the effect of dietary protein and ME, the males were provided with a) dietary protein and ME (ME+P+) b) dietary protein (ME-P+) only, c) sugar-only diet and ME (ME+P-), and d) untreated (ME-P-) and their mating success was evaluated in the field cages. The protein-fed males treated with ME at sexual maturity age achieved higher mating success over protein-fed or protein-deprived males and ME did not enhance mating success of protein-deprived males. The ME+P+ males that were fed on dietary protein for 5 days of age, treated with ME and then switched to a sugar-only diet, achieved higher mating success over ME-P+, ME+P- and ME-P- males after attaining sexual maturity age. The study showed that prerelease dietary protein and exposure of ME at 5 days of age is sufficient for males to achieve higher mating success and this protocol is expected to enhance the efficiency of SIT application in the field.

In the last section of the current study, the effect of ME and dietary protein on the survival of *B. zonata* males was investigated. The males were fed for 5 days on their respective diets and were assessed under extreme scenarios of the absence of food and water and then with access to water. The results showed that sugar-fed males (ME-P-) had significantly more survival than other males. The protein-fed males (ME-P+) had the lowest survival. The present study also indicated that ME+P+ males had less survival than sugar-fed males, however, they performed better than the males of other treatments. The current study indicated that ME-aromatized males had better survival than ME-fed males. ME-aromatherapy can be adopted as a ME delivery system in sterile male holding and release facilities and such treatment enhances male mating success, with a positive effect on male survival. The current study developed the protocols for treating *B. zonata* males with ME that will enable the simultaneous application of MAT and SIT and enhance the efficiency of the control programme incorporating the SIT.

# Chapter 1: General Introduction

Pests can deteriorate more than 40% of the total food production of the world (Oerke, 1994). Among all pests, the insect pests are responsible for 14% yield losses (Pimentel, 2007). Insect pests cause direct and indirect losses to agriculture production. The dipteran family Tephritidae is a large family of true fruit flies, that has more than 4000 species (White & Elson-Harris, 1992). The females of Tephritids typically have a posteriorly extended ovipositor that is used to penetrate the skin of fruits or in some cases flowers to deposit eggs. These eggs hatch to produce larvae which feed inside the fruit or flower and undergo three larval instars. After 3$^{rd}$ instar, the larvae pop out of the host and drop on the soil for pupation. The adults emerge from soil within weeks or months, depending upon environmental conditions and start their reproduction again (Syed, 1971; White & Elson-Harris, 1992).

Among Tephritidae, the genus *Bactrocera* has more than 45 species that are endemic to Southeast Asia, the South Pacific and Australia (Doorenweerd et al., 2018). Some of these species also have invaded Africa and South America (Clarke et al., 2005). More than 50 species of *Bactrocera* are serious pests of fruits as well as vegetables (Vargas et al., 2015). Due to their polyphagous nature, these fruit flies cause severe losses in fruits and vegetables. Economic damage is not only caused by direct losses to crop yield but also indirectly due to deprivation of export markets. According to a study, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) is reported to cause annual losses of USD 1.5 billion in California (Siebert, 1999). In Pakistan, *Bactrocera* species are estimated to cause losses of USD 200 million (Stonehouse et al., 1998).

## 1.1 THE PEACH FRUIT FLY, *Bactrocera zonata* Saunders (Diptera: Tephritidae)

*Bactrocera zonata* can attack economically important fruits (Qureshi et al., 1991; Mosleh et al., 2011; Sarwar et al., 2013, 2014; Ashfaq et al., 2020; Mahmoud et al., 2020). It is highly invasive in nature and due to its quarantine pest status, it interferes with horticultural trade (Qureshi et al., 1975; White & Elson-Harris, 1992; Kapoor, 1993; Stonehouse et al., 1998; Zingore et al., 2020). It is usually managed by synthetic insecticides, MAT application, and protein bait sprays. However, these management strategies have generally remained unsuccessful in suppressing the

population of *B. zonata* to an acceptable level (Nadeem et al., 2014; Al-Eryan et al., 2018; Khan & Akram, 2018).

## 1.2 MALE ANNIHILATION TECHNIQUE

Methyl eugenol (ME) is naturally present in more than 450 species of plants (Metcalf & Metcalf, 1992; Tan & Nishida, 2012). It is a phenylpropanoid compound (1, 2--4-(2-propenyl) benzene), and attracts males of several species of *Bactrocera* and *Dacus* genera (Howlett, 1912, 1915; Drew, 1974, 1989; Cunningham & Suda, 1985; White & Elson-Harris, 1992; Shelly et al., 2010). ME mixed with insecticide has been extensively used for managing *Bactrocera* fruit fly populations by luring and killing the males which is termed as male annihilation technique (MAT) (Steiner & Lee, 1955; Vargas et al., 2010). MAT is an environmentally benign technique that has been used against *Bactrocera dorsalis* (Hendel) of Micronesia and Japan (Steiner et al., 1965; Koyama et al., 1984). Various *Bactrocera* species were eradicated from California (Chambers, 1977). However, MAT was not successful in eradicating *B. dorsalis* population in Japan, as males did not respond to ME (Christenson, 1963).

The efficiency of MAT may also be reduced due to the presence of abundant source of ME in the form of natural flora. MAT is also to be applied on areawide basis in a very planned manner as a few surviving males can still inseminate many females, causing significant economic losses by ovipositing females (Steiner et al., 1970; Cunningham, 1989). MAT was applied to reduce fruit fly populations and then SIT was used for suppression (Steiner et al., 1970; Itô & Iwahashi, 1974; Koyama et al., 1984, 2004; Pérez-Staples et al., 2021). Although MAT has been applied to manage *B. zonata* (Qureshi et al., 1981), no eradication of its population on an Area-Wide (AW) scale has been reported so far. Therefore, the SIT that has been proven very successful in suppressing/eradicating many species of fruit flies, will have the potential for suppressing the *B. zonata* population.

## 1.3 STERILE INSECT TECHNIQUE

The SIT is a cost-effective, efficient and environment friendly technique that involves the rearing of the target insect on a large scale (Knipling, 1955). It is a part of regional integrated pest management programmes to decrease fruit fly populations and other pest insects (Hendrichs &

Robinson, 2009). Its success hinges upon the insemination of wild females by sterile males, and it is ideal to release sexually mature sterile males in the target area. Conversely, releasing sexually immature sterile males will expose them to mortality factors such as predation and abiotic stresses, before inseminating wild females, which may adversely affect the SIT efficacy (Hendrichs & Hendrichs, 1998; Rao et al., 2014; González-López et al., 2016).

Sustained and planned releases of sterile males can eradicate the target population. The SIT has successfully eradicated many insect species in different regions of the world. The cost-benefit analysis showed that nearly half of the cost of chemical control was spent on SIT application for eradicating melon fruit fly, *Zeogudacus cucurbitae* (Coquillett) in Japan. Continuous efforts are being made to enhance the efficiency of SIT application. These efforts involve improving the rearing protocols, producing the 'super males' by treating them with dietary supplements and certain chemicals. Detailed knowledge on ME enhancing the mating performance of *B. dorsalis* is available but little information is available on whether and how ME affects the mating performance of the males of *B. zonata*.

## 1.4 MATING SUCCESS

For SIT application, sterile males must be highly competitive, after their introduction in the field. Semiochemicals have been reported for this purpose (Shelly & McInnis, 2001; Shelly, McInnis, et al., 2005; Segura et al., 2018; Khan et al., 2019). ME has also been described to enhance the male mating competitiveness of several *Bactrocera* species (Tan & Nishida, 1996; Wee et al., 2007, 2018; Shelly et al., 2010; Ji et al., 2013). In all these studies, males were treated with ME at their sexual maturity age. However, the effect of ME applied at sexual immaturity age and mating success at sexual maturity age of *B. dorsalis* males was also assessed. These studies showed that sexually immature males exposed to ME were not able to gain mating advantage after reaching their sexual maturity age (Shelly et al., 2008; Shelly, 2020). Thus, holding the sterile males in sterile male holding and release facility until they are sexually mature for ME treatment or releasing them as sexually immature, has been paradoxical.

The studies on *B. dorsalis* males showed that exposure of males to ME at sexual maturity age only can enhance their mating success. However, sexual maturity age and the age-related response of *B. zonata* males to ME is still not understood well. The *Bactrocera* males generally

require more than one week to attain sexual maturity. Holding the sterile males for more than one week adds cost to the control programme and alternatively releasing the sexually immature males may increase their exposure duration to mortality factors i.e., abiotic factors and predation risk. The knowledge on whether *B. zonata* males treated with ME at the age of 4-5 days which is a protocol being practiced in programmes involving the SIT application, can enhance their mating success immediately after ME treatment or at their sexual maturity age, is critical but not known so far.

## 1.5 ME-AROMATHERAPY

Pre-release feeding on ME has limited practicality at present because feeding ME to hundreds of thousands or even millions of sterile males daily is not logistically feasible. The difficulty arises because no ME delivery system has yet been developed that allows access to an ME source for large numbers of flies (any source offered would quickly become covered with flies, rendering the ME available only to those flies in contact with the source) and limits the amount of time spent feeding by individual males as overfeeding on ME may be lethal (Steiner, 1952). In one study, scientists have designed a machine to feed males through a conveyor belt impregnated with ME, allowing the male to feed on ME briefly before being brushed off (Tan & Tan 2013). But this system of ME-feeding also seems inadequate for treating millions of flies in a SIT facility daily. Pre-release exposure to ME could be more easily incorporated into SIT programs if exposure to the odour of ME affected the same male responses as ingestion of the chemical.

This procedure termed aromatherapy has been developed (Shelly & McInnis, 2001; Shelly, McInnis, et al., 2005; Shelly et al., 2007) and is currently in use in SIT programs against *C. capitata* (Shelly, 2008; Paranhos et al., 2013; Silva et al., 2013; Steiner et al., 2013). ME-aromatherapy has been described earlier to enhance the male sexual performance of *Bactrocera carambolae* (Drew and Hancock) and *B. dorsalis* by Haq et al. (2014, 2015, 2018).

## 6 SIMULTANEOUS APPLICATION OF MAT AND SIT

In integrated pest management programmes for fruit flies, the SIT was applied after the population was suppressed by MAT application. However, the population modelling studies

predicted that the combined application of MAT with SIT can enhance the cost-effectiveness of fruit fly control programmes (Barclay et al., 2014).

Until recently, actions of MAT and SIT were considered incompatible, since the presence of ME-baited traps would kill large numbers of sterile males, thus greatly reducing the efficacy of an SIT program. SIT is more effective when employed after the reduction of fruit fly populations through a MAT programme (Dyck et al., 2005). The efficacy of SIT increases under reduced fruit fly populations. The strategy has successfully eradicated *B. dorsalis* populations (Steiner et al., 1970). MAT and SIT can potentially be used simultaneously. However, the main limitation associated with combined application of MAT and SIT, is that the MAT traps may lure a large number of released sterile males and get killed along with wild males. Shelly (1994) demonstrated that ME-fed mature *B. dorsalis* males are less enticed to ME-baited traps. Significant reduction in response to lures after initial feeding has also been reported in other ketone/cue lure responding and ME-sensitive species (Chambers et al., 1972; Shelly & Villalobos, 1995; Akter et al., 2017).

The basic consideration for the simultaneous application of SIT with MAT is that males treated with ME in the release facility should not be trapped in ME-baited devices/traps in the field. Therefore, prelease exposure to lure may allow concurrent application of MAT and SIT. Simultaneous application of MAT and SIT may increase efficiency and cost effectiveness of the control programme. Furthermore, ME delivery by aromatherapy which has been foreseen as a practicable system is needed to be investigated whether it is effective for suppressing repeat feeding response in ME to *B. zonata* males, the information on ME repeat feeding response of *B. zonata* males is also lacking.

The ME treatment which enhances the male mating success by triggering the metabolism may require more energy fuel which can be supplied by dietary protein. Thus, the role of dietary protein in interaction with ME is needed to be investigated. Furthermore, knowledge of dietary protein supplements either alone or in combination with ME for enhancing the mating success of *B. zonata* males is scarce.

## 1.7 DIET SUPPLEMENTATION

Most of the tephritid flies are anautogenous and need nutrition for their sexual development (Drew & Yuval, 2000). The fruit flies forage for carbohydrates and protein to sustain their

metabolic activities and sexual development (Hendrichs & Prokopy, 1994). In nature, the nutrition comes from a variety of sources such as plant exudates, bird feces, bacteria, yeast, honeydews and floral products (Hagen, 1958; Neilson & Wood, 1966; Drew et al., 1983; Drew & Lloyd, 1987; Hendrichs & Hendrichs, 1990; Hendrichs et al., 1991, 1993). A yeast hydrolysate and sugar mixture is consumed as an adult diet in fruit fly rearing colonies, for optimum sexual development and production (Perez-Staples et al., 2007; Fanson & Taylor, 2012).

Mating performance has been reported to be enhanced by dietary protein in Mediterranean fruit fly, *C. capitata* (Blay & Yuval, 1997; Kaspi & Yuval, 2000; Shelly & Kennelly, 2002; Shelly et al., 2003; Yuval et al., 2003). Similarly, other researchers have also described the positive effect of dietary protein on the mating performance of Queensland fruit fly, *Bactrocera tryoni* (Froggatt), Oriental fruit fly, *B. dorsalis*, melon fruit fly, *Z. cucurbitae* (Haq et al., 2010) and several *Anastrepha* species (Aluja et al., 2001; Liedo et al., 2013). While a prelease diet may provide sterile males with nutritional resources to increase mating success, additional treatments with different semiochemicals such as ME can further enhance their mating competitiveness.

The accelerated metabolism and sexual activities due to ME treatment and dietary protein to sterile males and their cut-off supply at the time of their release in the filed may cost their survival in the wild (Johansson et al., 2005; Papadopoulos et al., 2010).

## 1.8 SURVIVAL

Post-feeding impact of a protein diet on survival is significantly variable in different tephritid species. Kaspi & Yuval (2000) reported high mortality of protein-fed males of *C. capitata*. Similarly, protein feeding had a negative impact on the survival of *C. capitata* male after starvation (Maor et al. 2004). While other studies did not find any negative role of dietary protein on survival of *C. capitata* males (Shelly & Kennelly, 2002; Shelly & McInnis, 2003). In *B. tryoni*, protein diet increases longevity of males (Perez-Staples et al., 2008; Taylor et al., 2013). Similarly, dietary protein also positively influences the survival of *Z. cucurbitae* males (Haq & Hendrichs, 2013). ME treatment enhances wing fanning/signaling (Shelly, 1994; Shelly et al., 1996) and switching off the males from dietary protein before release in the field may have an adverse effect on male survival. Therefore, the effect ME treatment and dietary protein on survival of *B. zonata* males needed to be investigated.

## 1.9 OBJECTIVES

The specific objectives of this study were to evaluate:

- Effect of methyl eugenol (ME) on *B. zonata* male age-dependent response and mating success.
- Effect of ME-aromatherapy on mating success of *B. zonata* males and suppressing response to methyl eugenol for simultaneous usage of male annihilation and sterile insect techniques
- Effect of ME treatment and dietary protein on *B. zonata* male mating success.
- Effect of ME treatment and dietary protein on *B. zonata* male survival

# Chapter 2: Age-dependent Effect of Methyl Eugenol on the Peach Fruit Fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae), Male Mating Success


## ABSTRACT

Feeding on ME enhances male mating success in several species of *Bactrocera*. Such an effect can increase the efficacy of the sterile insect technique (SIT). Present study was designed to identify the *B. zonata* male sexual maturity age, the relation between the age of the male and its attraction to ME, and the ultimate effect of ME on successful mating of the males. Results of this study showed that males initiated their mating at 8 days of age and attained the highest mating success at 16 days of age. A proportion of immature males responded to ME, the percentage of responders increased with age and the maximum response was observed during the sexual maturity age. ME treatment at sexual maturity age significantly enhanced the male mating success over untreated males. The males treated with ME at sexually immature age (5 days old), achieved significantly higher mating success after reaching sexual maturity age. These findings suggested that *B. zonata* males can be treated with ME at the age of 5 days in sterile male holding and release facilities. After releasing them in the field, these insects are expected to achieve higher mating success after attaining sexual maturity. The results are discussed as a valid approach for increasing the usefulness of SIT application against *B. zonata*.


## 2.1 INTRODUCTION

Methyl eugenol (ME) has the ability to attract males of several tropical tephritid species (Howlett, 1912; Drew, 1974, 1989; Cunningham & Suda, 1985; White & Elson-Harris, 1992; Shelly et al., 2010). ME mixed with an insecticide has been extensively used for managing *Bactrocera* fruit fly populations by luring and killing the males which is termed as male annihilation technique (MAT) (Vargas et al., 2010). In certain cases, where the MAT failed to eliminate the wild population, sterile insect technique (SIT) was applied (Steiner et al., 1970; Koyama et al., 1984, 2004; Pérez-Staples et al., 2021).

The application of SIT has successfully suppressed the population of fruit flies and different insect pests (Hendrichs & Robinson, 2009). The outcome of SIT application hinges upon the insemination of wild females by sterile males. Ideally, the sterile males must be released in the target area after their sexual maturation. Conversely, releasing sexually immature males expose them to different mortality factors such as predation and abiotic stresses, before inseminating wild females, which may adversely affect the SIT efficacy (Hendrichs & Hendrichs, 1998; Rao et al., 2014; González-López et al., 2016).

In most of AW-IPM programmes involving the SIT application, the sterile males are withheld for 4-5 days in sterile male holding and release facilities due to space and cost constraints. Such duration suits to Mediterranean fruit fly, *Ceratitis capitata*, which is mature at the time of release (McInnis et al., 2013). However, in melon fly, *Zeugodacus cucurbitae* (Coquillett), eradication programme in Okinawa Islands, Japan, though the males required more than one week to attain sexual maturity, they were also released at the age of 3-4 days (Nakamori & Kuba, 1990).

In SIT, the sterile males must be competitive. Hence, their competitiveness is one of the key factors. The mating competitiveness of different tephritid males has been reported to be enhanced by different semiochemicals (Shelly & McInnis, 2001; Shelly, McInnis, et al., 2005; Segura et al., 2018; Khan et al., 2019). ME has been evaluated and described to enhance significantly the male mating competitiveness of several species of *Bactrocera* (Tan & Nishida, 1996; Wee et al., 2007, 2018; Shelly et al., 2010; Ji et al., 2013). In all these studies, males were treated with ME at their sexual maturity age. However, in a few studies on *Bactrocera dorsalis* (Hendel) males, the effect of ME applied at sexual immaturity age and mating success at sexual maturity age was also assessed. These studies showed that after exposing sexually immature males to ME, they were not able to gain a mating advantage after reaching their sexual maturity age (Shelly et al., 2008; Shelly, 2020). Thus, holding the sterile males in sterile male holding and release facility until they are sexually mature for ME treatment or releasing them as sexually immature, has been paradoxical.

*Bactrocera zonata* is highly invasive and due to its quarantine pest status, it interferes with horticultural trade (Qureshi et al., 1975; White & Elson-Harris, 1992; Kapoor, 1993; Stonehouse et al., 1998; Zingore et al., 2020). Its management so far has been relied on the application of

synthetic insecticides, MAT application, and protein bait sprays. However, these management strategies have generally remained unsuccessful in suppressing the *B. zonata* population (Nadeem et al., 2014; Al-Eryan et al., 2018; Khan & Akram, 2018). Therefore, SIT application is advisable for the control and successful management of *B. zonata.* Studies have reported that ME can enhance *B. zonata* male mating success (Quilici et al., 2004; Sookar et al., 2009; Ndzana et al., 2016), but the information was of preliminary nature. Detailed studies on the interaction of ME with *B. zonata* males can provide the basis for enhancing the effectiveness of SIT application.

The brief objectives of this study were to identify, i) the sexual maturity age of *B. zonata* males, ii) the relation between male age and their attraction to ME, and iii) the effect of ME treatment on mating success of males.

## 2.2 MATERIALS AND METHODS

### *2.2.1 Study insects*

*Bactrocera zonata* flies utilized in experiments originated from a colony that was started from infested guava fruits (*Psidium guajava* L.) (Myrtaceae) at the Insect Pest Management Program, National Agricultural Research Centre, Islamabad, Pakistan. At experimental time, the flies were ~34 generations under laboratory rearing. The colony was maintained on a standard wheat bran derived diet (Hooper, 1987). After emergence, these adults were fed on protein diet, consisting of a mixture of hydrolyzed yeast (MP, Biomedicals, LLC, USA) and sugar (1:3) and water *ad libitum*. The eggs were collected in perforated plastic bottles containing guava juice. The eggs were poured out on a piece of tissue paper that was placed on a wet sponge set in a petri dish. After 24 h of incubation at 25 ± 2 ℃, 60 ± 5% R.H., the eggs were seeded on a larval diet. The flies were reared at a low density of 4-5 larvae/g of diet. The pupae were collected in sawdust and placed in screened cages for the emergence of flies. The males and females were parted within 24 h of emergence, much earlier than attaining sexual maturity. The experimental flies were kept in a separate room at 25 ± 2 ℃, 60 ± 5% R.H. and photoperiod of L14: D10. The photoperiod (initiation of darkness) was adjusted according to the natural photoperiod.

### *2.2.2 Sexual maturity age of B. zonata males under laboratory conditions*

The experiment was performed in a room under controlled conditions at 25 ± 2 ºC and 60 ± 5% R.H. The sexual maturity age of males was identified by evaluating them from age of 2 to 30 days. Five virgin males of each age were introduced 90 min before sunset in Plexiglas transparent screened cages (20 × 15 × 15 cm), placed adjacent to windows that allowed natural light to pass through. Five same-age virgin females as the males were introduced into the cage 15 min later. No food and water were provided to the flies during the experiment. The lights were switched off and mating was noticed under semi-dark conditions. The mated couples were collected in vials after mating. Five replicates for each age were conducted simultaneously. The experiment was proceeded till 1 h after sunset, when males ceased calling in darkness.

### *2.2.3 Age-dependent response of B. zonata males to ME under laboratory and semi-natural conditions*

Age-dependent response of males to ME was evaluated in an isolated room under 25 ± 2 ºC and 60 ± 5% R.H. Ten males were transferred to Plexiglas transparent screened cages (20 × 15 × 15 cm) without food and water at 09:00 hours. 0.1 mL of ME (99% purity; Merck, Darmstadt, Germany) was applied on a filter paper disc (Watman® No. 1) of 9 cm in diameter and placed on an aluminum foil-lined Petri dish. The Petri dish containing filter paper impregnated with ME was transferred into each cage. The response of males to ME from age 2 to 20 days was monitored continuously for 2 h duration each day. The ME-fed males were removed from the cage with forceps and counted as feeding response. For each age group of males, 5 replicates were evaluated.

In the second experiment, the age-dependent response of males to ME was evaluated in a screen house (15.2 × 9.1 m). The ceiling of the screen house was 3 m high at the center and sloped down to a height of 1.8 m on either side. The screen house had side walls of stainless-steel mesh. The ceiling was opaque and made of two metal sheets with one Styrofoam (8 cm thick) in the middle. Inside the screen house, six large cages (each 7.9 × 2.3 × 1.8 m), made of iron frame were fixed and covered with screen cloth. The cages were separated from each other by screened curtains. The curtains at the entrance were overlapped, permitting the observers to pass through by flipping over but preventing the flies from crossing one cage to another cage. There was a 60 cm

wide pathway between the screened cages and the surrounding stainless-steel mesh which served as a buffer zone from the outer environment.

Methyl eugenol (0.5 mL) was applied on a cotton wick, and placed on a piece of aluminum foil, inside the Steiner type traps. These traps were installed at one side of each screened cage. The males (n= 20) of the given age were released in each cage at 08:30 hours. The attraction of males to ME from age 2 to 30 days was assessed. The number of trapped males was scored after 3 h. The temperature remained 24-32 ℃ during the experiment. Six replicates for a given age were carried out simultaneously.

### *2.2.4 Effect of ME on mating success of B. zonata males in field cages*

*2.2.4.1 ME treatment*

ME treatment was carried out in a separate room. The males were marked on the thorax, one day before treatment. The males (n= 120) were shifted to a Plexiglas transparent screened cage (20 × 15 × 15 cm). On a filter paper disc (Watman® No. 1) of 9 cm diameter, ME (0.5 mL) was applied and placed in a Petri dish containing an aluminum foil that was introduced in the cage at 09:00 hours. The males (hereafter called ME-treated males) were then exposed to ME for 1 h. Food and water were not provided to the males during exposure to ME. After ME treatment, the males were maintained at 25±2 ℃, 60±5% R.H. and L14: D10 photoperiod. The males were provided with a protein diet consisting of a mixture of hydrolyzed yeast and sugar (1:3) and water *ad libitum*.

*2.2.4.2 No-ME treatment*

The males that were not exposed to ME (hereafter called untreated males) were maintained in an isolated room at 25 ± 2 ℃, 60 ±5 % R.H. and L14: D10 photoperiod. The untreated males were marked and provided with a protein diet and water *ad libitum*.

*2.2.4.3 Field cages*

Semi-circular walk-in screened clothing field cages (2 m high, 1.5 m diameter) were used for the experiments. Eight field cages (90 cm apart) were placed inside two adjacent glass houses of the same size (4 × 4 × 3.8 m). Each glasshouse had an exhaust fan for ventilation and a temperature of 26 ± 2 ℃ with 60 ± 5% R.H was maintained during the experiment. A potted artificial tree mimic of a citrus tree (1.8 m high, canopy 1 m in diameter) was placed in each field

cage. The light passing through the roof and side walls of the glasshouse provided illumination, making the environment semi-natural. The experimental setup allowed running eight replicates each day simultaneously.

*2.2.4.4 Mating experiments*

Four sets of experiments were carried out to ascertain the impact of ME on male mating success. In the first set of experiments, sexually mature males were evaluated for the effect of ME on mating success in field cages. The males were treated with ME at age of 15 days. ME-treated males were tested against untreated males for mating success in field cages after 1-, 2- and 3-days post-treatment (DPT). Thus, the age of both ME-treated and untreated males was 16-18 days at the time of the experiment. ME-treated and untreated males (n= 20) were released at the same time in each field cage ~90 minutes before sunset and were allowed to settle down before starting their mating activity. Virgin females (n= 20) were introduced in the field cages 15 minutes after releasing the males. The age of females was the same as that of males. The couples were collected in separate vials with screened lids as soon as mating occurred and were allowed to complete mating. Mating was observed up to 1 hour after sunset when males ceased calling in complete darkness. The couples were then brought to the laboratory the next day for their identification. Eight replicates were performed on each day post-treatment.

In the second set of experiments, the males were treated at the age of 12 days (when more than 60% of males were sexually mature) and tested for their mating success at age of 13 and 15 days. Mature virgin females (15 days old) were used in each experiment. The flies were used only once in each experiment. Each experiment was replicated eight times.

In the third set of experiments, the males were treated with ME at 8 days of age (onset of sexual maturity) and were evaluated for their mating success at 9 and 15 days of age. Mature virgin females (15 days of age) were used in each experiment and eight replicates were evaluated.

In the fourth set of experiments, the males were treated with ME at 5 days of age and were tested for mating success against untreated males at age of 6, 8,12, and 15 days. The reason for selecting such an age was that holding males for 5 days suits the protocols of existing SIT operational programmes. The ME-treated and untreated males were supposed to be competing for mating with mature females (16 days of age). The experiment was replicated eight times for each

age except for the age of 6 days where due to no mating, only four replicates were carried out. The flies were used only once in each experiment.

### *2.2.5 Statistical analyses*

Data on male maturity age and male response to ME in the laboratory were normally distributed, whereas data on male response to ME in field cages were normalized by using the inverse distribution function. During the age of 2-7 days, there was no mating. For peak maturity age, data from age of 8 days and onward were analyzed. Mating success and the number of males who responded to ME were response variables in peak maturity age and age-dependent response to ME experiments. Data from both experiments were analyzed by one-way ANOVA. Tukey's test performed all pairwise comparison of means ($P$= 0.05). Data for all experiments on the effect of ME on male mating success (expressed as a percentage of all possible mating) were normally distributed and the data were analyzed by using an unpaired Student's t-test ($P$=0.05). Data regarding mating success achieved by the males at different days post-treatment (DPT) were analyzed by one-way ANOVA. Data analyses were performed by using SPSS version 26.

## 2.3 RESULTS

### *2.3.1 Sexual maturity age of B. zonata males under laboratory conditions*

The male mating success depended on male age ($F_{14,60} = 18.13$, $P < 0.001$; Figure 2.1). No mating was observed from the age of 2 to 7 days. The first mating activity was observed at the age of 8 days and the mating success was 16% only. The mating success increased consistently afterwards and reached the highest (92%) at 16 days of age. A sharp decline in mating success was observed after 20 days of age.

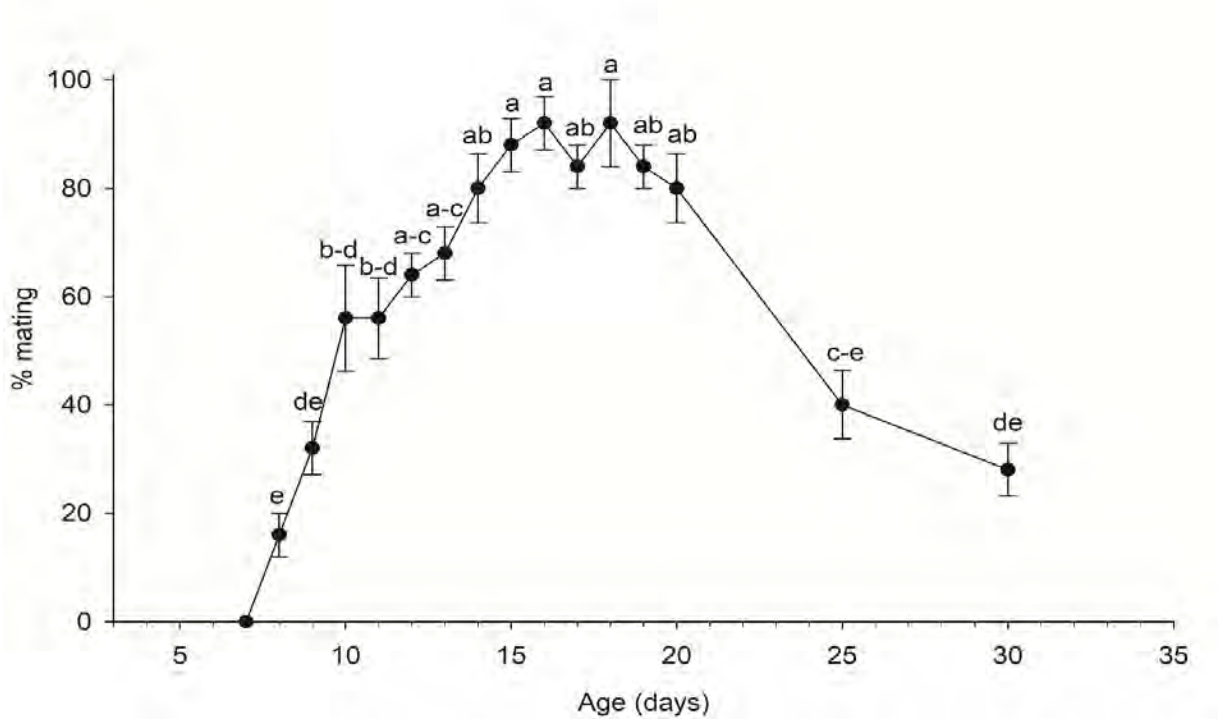


Figure 2.1 Percent mating success (Mean ± SE) achieved by *B. zonata* males during the age of 2-30 days under laboratory conditions. Mean values followed by different letters are significantly different from each other (one-way ANOVA, $P < 0.05$).

### *2.3.2 Age-dependent response of B. zonata males to ME under laboratory and seminatural conditions*

There was a significant difference in response to ME among males aged 2 to 20 days under laboratory experiments ($F_{14,60}$=19.7, $P < 0.001$; Figure 2.2). The males showed a response (48%) to ME at the earliest observed age of 2 days. The response increased steadily and peaked at the age of 8 days and afterwards, it became uniform up to the age of 20 days.

Under seminatural conditions, response of *B. zonata* males to ME varied significantly at different ages ($F_{16,85}$= 19.25, $P < 0.001$; Figure 2.3). The males (7.5%) started responding to ME as early as the age of 2 days, which increased markedly with age and peaked (94%) at the age of 15 days. There was a decline in the number of males trapped from age of 20 to 30 days.

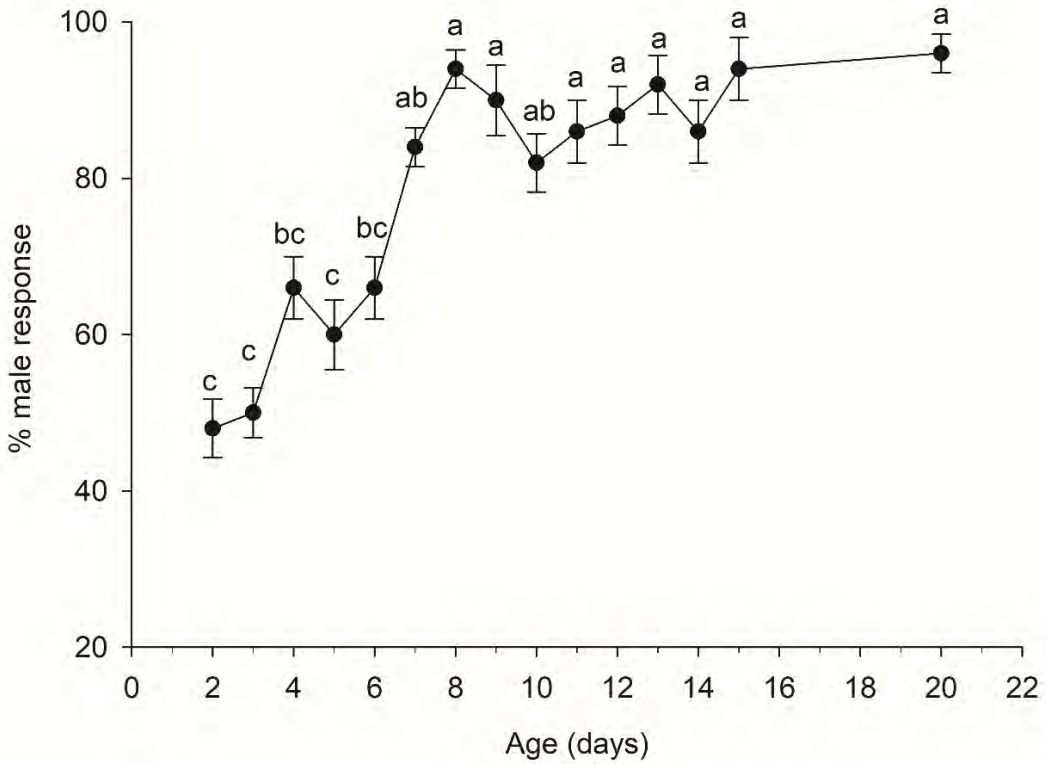


Figure 2.2 Percent response of *B. zonata* males (Mean ± SE) to methyl eugenol during the age of 2-20 days under laboratory conditions. Mean values followed by different letters are significantly different from each other (one-way ANOVA, $P < 0.05$).

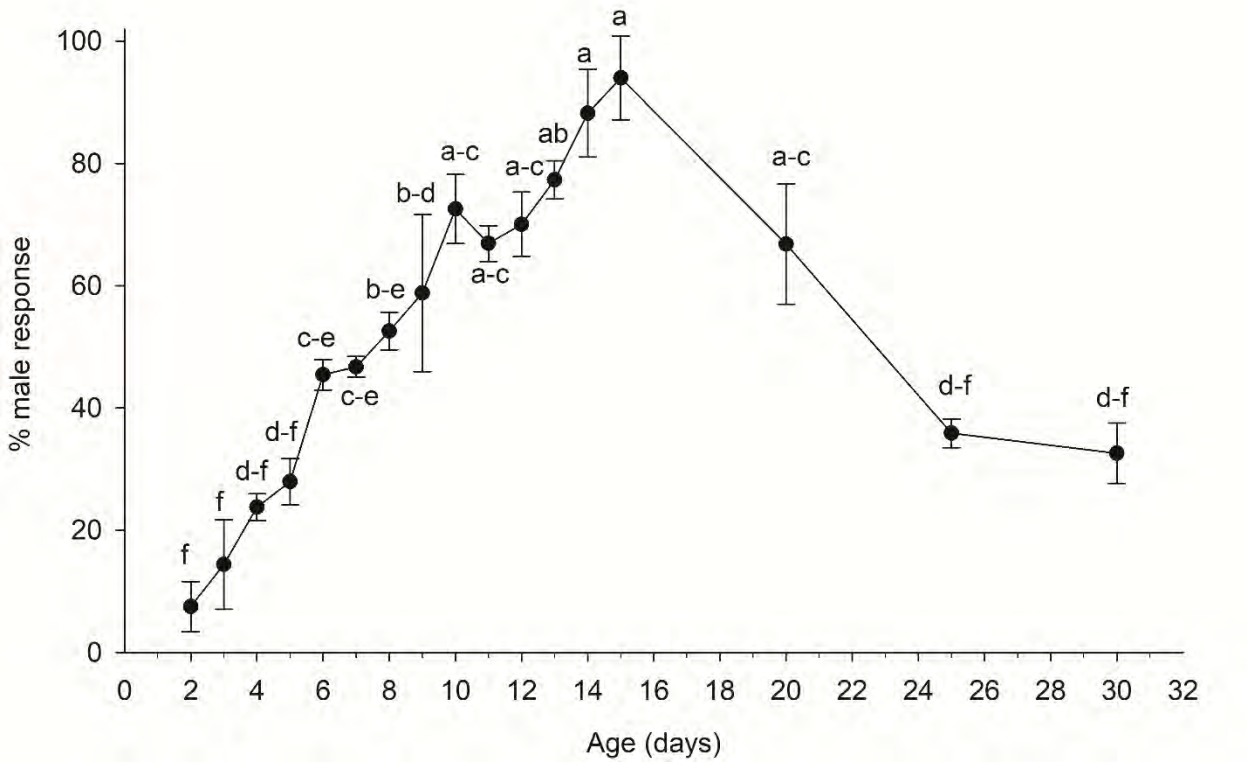


Figure 2.3 Percent response of *B. zonata* males (Mean ± SE) to methyl eugenol during the age of 2-30 days under semi-natural conditions. Mean values followed by different letters are significantly different from each other (one-way ANOVA, $P < 0.05$).

### *2.3.3 Effect of ME on mating success of B. zonata males in field cages*

*B. zonata* males treated with ME at peak sexual maturity age (15 days) achieved higher mating success than untreated males at 1 DPT (t = 8.02, d.f= 14, $P < 0.001$), 2 DPT (t= 5.60, d.f= 14, $P < 0.001$) and 3 DPT (t= 8.23, d.f= 14, $P < 0.001$) (Table 1), respectively. There was no significant difference in male mating success on different days post-treatment ($F_{2,47} = 0.21$, $P$= 0.81).

The males treated with ME at the age of 12 days achieved higher mating success at the age of 13 days (t= 5.16, d.f= 14, $P < 0.001$; Table 1) and 15 days (t= 7.89, d.f= 14, $P < 0.001$; Table 1) than untreated males of the same age. Similarly, the males that were treated with ME at the age of 8 days, achieved higher mating success at the age of 9 days (t= 6.30, d.f= 14, $P < 0.001$; Table 1) and 15 days (t= 5.14, d.f= 14, $P < 0.001$; Table 1) than untreated males of the same age.

When the males were treated with ME at the age of 5 days, no mating was observed at all at the age of 6 days. Only 3.10 % of mating (5 out of 160) occurred at the age of 8 days and all mated males were ME-treated. At the age of 12 days (t= 3.71, d.f= 14, *P*= 0.002; Table 2.1) and 15 days (t= 5.05, d.f= 14, *P* < 0.001; Table 2.1), the effect ME remained visible and the treated males achieved higher mating success than untreated males of similar age.

Table 2.1 Percent mating success (Mean ± SE) of ME-treated vs. untreated *Bactrocera zonata* males. The males were treated with ME at the age of 15, 12, 8, and 5 days. The treated males were competing with untreated males at various phases of sexual maturity for mating with mature virgin females (15-18 days old).

| Male age (days) at ME treatment | Male age (days) at the mating experiment | Mating success (%) | | t-value | P-value |
|---|---|---|---|---|---|
| | | ME-treated | Untreated | | |
| 15 | 16 | 59.38 (± 2.00) A | 34.38 (±2.40) B | 8.02 | <0.001 |
| | 17 | 60.63 (± 2.90) A | 38.75 (±2.63) B | 5.60 | <0.001 |
| | 18 | 60.63 (±2.20) A | 38.13 (±1.62) B | 8.23 | <0.001 |
| 12 | 13 days | 51.88(±1.32) A | 40.00 (±1.90) B | 5.16 | <0.001 |
| | 15 days | 64.38(±2.58) A | 35.63(±2.58) B | 7.89 | <0.001 |
| 8 | 9 days | 27.50 (±2.32) A | 6.25 (±2.46) B | 6.30 | <0.001 |
| | 15 days | 59.38(±2.90) A | 35.63(±3.60) B | 5.14 | <0.001 |
| 5 | 6 days | 0 | 0 | - | - |
| | 8 days | 3.1 | 0 | - | - |
| | 12 days | 41.88(±3.65) A | 23.75(±3.24) B | 3.71 | 0.002 |
| | 15 days | 57.50(±2.10) A | 39.38(±2.91) B | 5.05 | <0.001 |

Mean mating success in each row followed by different letters is significantly different from each other (Student's t-test, *P*<0.05).

## 2.4 DISCUSSION

Tephritid fruit flies are anautogenous, where the adults gather resources after emergence (Drew & Yuval, 2000), take several days to reach sexual maturity and the precocious period varies in different species. Sexual maturity age in some *Bactrocera* wild flies has been reported as 2-3 weeks (Wee & Tan, 2000; Wee et al., 2018). Laboratory colonization is reported to shorten the precocious duration (Wong et al., 1989; Shelly & Manoukis, 2022; Haq et al., 2010; Shelly, 2020). In the present study, 8 days of age was recorded as the male mating initiation age in both laboratory

and field cage experiments. In the laboratory experiment, males and females of the same age were evaluated. Males may attain sexual maturity slightly earlier than females; thus, a question arises that males may have initiated their mating earlier if they would have been exposed to mature females. However, in field cage experiments, evaluating young males against sexually mature females, no mating occurred at 6 days of age and the first incidence of mating occurred at 8 days of age. Qureshi et al. (1974) also reported 8 days as the mating initiation age of the *B. zonata* laboratory strain in Pakistan. *B. zonata* strain under rearing for ~233 generations in Mauritius, attain sexual maturity at 6 days of age (Sookar, personal communication) which is evidence of a reduction in precocious duration due to a greater number of generations under laboratory rearing.

In the experiments on the age-dependent response of males to ME, both under laboratory and seminatural conditions, a proportion of immature males responded to ME, the percentage of responders increased with age and the maximum response was observed during sexual maturity age. These results correspond with previous studies on both laboratory and wild strains of *B. dorsalis* (Wong et al., 1989; Shelly et al., 2008; Shelly, 2020). However, *Bactrocera correcta* (Bezzi) males respond to ME only at their sexual maturity age (Kamiji et al., 2018). The male response to ME at pre-maturation age has implications for the success of MAT application. The attraction of males to ME at immature age has been suggested as of key importance in MAT application (Wong et al., 1989). Our findings also suggested that MAT could have the potential for success against *B. zonata.* In addition to ME incorporation in MAT application, the ME has been reported to improve the mating performance of males of many *Bactrocera* species (Tan & Nishida, 1996; Wee et al., 2007, 2018; Shelly et al., 2010; Ji et al., 2013). The duration required to improve the male mating achievement after ME treatment, is variable in various species. For example, *B. dorsalis* males required 1 day (Tan & Nishida, 1996; Haq et al., 2018), while *B. carambolae* males required 3 days post-ME treatment to achieve higher mating success (Wee et al., 2007). In the case of *B. zonata*, ME is reported to enhance male mating success (Quilici et al., 2004; Sookar et al., 2009; Ndzana et al., 2016). To determine the duration, Tan et al. (2011) performed biochemical studies and reported that 1 day was sufficient to metabolize the ME to sex pheromones in *B. zonata* males. Findings of such biochemical studies were the indication of enhanced mating success 1 day after ME-feeding but the evidence from mating behavioral studies

was lacking. The current study demonstrated that 1-day post-ME treatment was sufficient to achieve mating advantage.

In the context of ME application for enhancing the effectiveness of SIT application, studies on *B. dorsalis* males revealed that the ME effect persisted for 35 days after feeding (Shelly & Dewire, 1994). But the males could get mating advantage only when they were fed on ME at sexual maturity age (Shelly et al., 2008). It warrants confining the sterile males for ME treatment until they attain their sexual maturity, which limits the application of ME in SIT operational programmes. In the current study, *B. zonata* males treated with ME at sexual maturity age enhanced their mating success. ME treatment at sexually immature (5 days) age, also enhanced the male mating success but after reaching sexual maturity age. The male age of 5 days for ME treatment was selected based on their response to ME and it was closest to the sterile males holding age, practiced in the existing SIT operational programs (Koyama et al., 1984; McInnis et al., 2013). However, the minimum number of days required to hold *B. zonata* males for ME treatment in an SIT facility needs further investigation.

Despite ME effects on enhancing male mating success, ME application to sterile males is limited due to the absence of a viable delivery system. Furthermore, the combined application of MAT and the SIT has been suggested for enhancing the effectiveness of *Bactrocera* fruit flies management programmes (Barclay et al., 2014). The prerequisite for combined application is that the sterile males treated with ME should not exhibit repeat feeding in ME-baited devices/traps. The repeat feeding behavior of *B. zonata* ME-treated males is not known and should be investigated.

### *2.4.1 Conclusion*

A proportion of the males responded to ME traps before mating initiation age that has implications for MAT application for control of *B. zonata*. ME treatment enhanced mating success of mature *B. zonata* males and the males could achieve mating advantage after 1-day post-treatment. ME treatment of the males at immature age of 5 days indicated that the effect of ME persisted and males gained mating advantage after reaching sexual maturity. Treating males with ME at 5 days of age is compatible with current sterile males holding and release system and can enhance effectiveness of SIT.

# Chapter 3: Effect of Methyl Eugenol Aromatherapy on *Bactrocera zonata* Male Mating Success and Suppressing Response to Methyl Eugenol for Simultaneous Application of Male Annihilation and Sterile Insect Techniques


## ABSTRACT

The male annihilation technique (MAT) and the sterile insect technique (SIT) are environmentally friendly techniques used to decrease fruit fly populations. The MAT and SIT are typically used sequentially; however, the MAT and SIT can potentially be used simultaneously and thereby increase the overall efficiency of control programmes. Methyl eugenol (ME) by feeding has been reported to enhance the male mating performance of many *Bactrocera* spp., including *B. zonata,* but its use in SIT holding and release facilities is limited due to the absence of a viable delivery system for ME. In the present study, we demonstrated that ME-aromatherapy, a practical method for delivering ME, enhances the mating success of *B. zonata* males. When given to 5 days old immature males, ME-aromatherapy resulted in elevated mating success of males tested when sexually mature. The ME-aromatized males also exhibited reduced responses to ME-baited traps. Treating males at 5 days of age is in accordance with the males holding duration protocol adopted in sterile males holding and release facilities. Thus, ME-aromatherapy appears to be a practical means to simultaneously combine MAT and SIT.


## 3.1 INTRODUCTION

Tephritid fruit flies inflict damage to fruits and interfere with horticultural trade due to their quarantine pest status (Qureshi et al., 1975; White & Elson-Harris, 1992; Kapoor, 1993; Stonehouse et al., 1998; Zingore et al., 2020). Synthetic insecticides have largely failed to suppress fruit fly populations. Furthermore, due to health hazards, synthetic insecticides are being replaced with alternative methods that are effective as well as environmentally friendly.

The male annihilation technique (MAT) is an environmentally benign technique used against *Bactrocera* spp. that involves luring males to certain chemicals, i.e., methyl eugenol (ME) mixed with a toxicant. Subsequent ingestion of this mixture is lethal to the males. ME is highly

attractive to males of certain *Bactrocera* spp. (Metcalf & Metcalf, 1992; Tan & Nishida, 2012). Owing to this powerful attraction, ME has been used for population monitoring and population suppression (Steiner et al., 1970; Vargas et al., 2010). For example, ME-based MAT was applied to eliminate *Bactrocera dorsalis* (Hendel) from the Marianas Islands, Micronesia, and Okinawan Islands, Japan (Steiner et al., 1965; Koyama et al., 1984).

MAT application could not eradicate *B. dorsalis* in Japan, and therefore, the sterile insect technique (SIT) was subsequently applied for this purpose. The application of SIT has proven successful in suppressing populations of fruit flies (Hendrichs & Robinson, 2009). The SIT becomes more efficacious at reduced wild population (Knipling, 1979; Dyck et al., 2005). Thus, the application of SIT after suppressing *B. dorsalis* population by MAT application was successful in eradicating the population in the Marianas Islands (Steiner et al., 1970).

For the eradication of fruit flies generally, SIT has been applied sequentially, i.e., SIT after MAT application. Theoretical models, however, indicate that concurrent application of MAT and SIT would achieve more effective suppression of pest populations (Barclay et al., 2014). Until recently, the actions of MAT and SIT were considered incompatible, since the presence of ME-baited (or MAT) traps would kill large numbers of sterile males, thus greatly reducing the efficacy of an SIT program (Barclay & Hendrichs, 2014). However, studies of *B. dorsalis* demonstrated that, after an initial feeding on ME, males showed less attraction to ME-baited devices/traps (Shelly, 1994).

The reduction in response to lures after initial feeding has also been reported in other ME and raspberry ketone/cue lure responding species (Chambers et al., 1972; Shelly & Villalobos, 1995; Akter et al., 2017). Therefore, SIT and MAT can be implemented, simultaneously (Chambers et al., 1972; Barclay et al., 2014). Furthermore, ME-feeding also enhances mating competitiveness of *Bactrocera* males (Quilici et al. 2004; Wee et al. 2007, 2018; Shelly et al. 2008, 2010; Sookar et al. 2009; Ndzana et al. 2016; Shelly 2020). In *B. dorsalis*, the age at which males feed on ME is critical, as ME-feeding enhances male mating success only when provided to sexually mature males. Exposing sexually immature males to ME had no effect on their mating success when tested as sexually mature adults (Shelly et al., 2008). This fact complicates the integration of pre-release ME exposure into SIT programmes, as such programmes release sterile

*Bactrocera* males as immatures (4-5 days old) owing to a lack of space and resources to hold the sterile males for longer time intervals.

*Bactrocera zonata* poses a severe threat to commercial crops (White & Elson-Harris, 1992; Kapoor, 1993). It is considered an A1 quarantine pest in Europe (EPPO, 2005). Although *B. zonata* has received less attention than *B. dorsalis*, available data suggest that pre-release ME exposure might be more easily incorporated into SIT efforts against this species. Rasool et al., (2023) demonstrated that *B. zonata* males fed ME at 5 days of age (when sexually immature) had a mating advantage over untreated males when tested after attaining sexual maturity. Holding adult *Bactrocera* males for 4-5 days post-emergence is standard protocol in SIT operational programmes (Nakamori & Kuba, 1990; McInnis et al., 2013), and the enhanced mating success of *B. zonata* males fed on ME when 5 days old opens the opportunity of releasing sterile males treated with ME.

Despite this promising finding, pre-release feeding on ME has limited practicality at present, because feeding ME to hundreds of thousands or even millions of sterile males daily is not logistically feasible. The difficulty arises, because no ME delivery system has yet been developed that (i) allows access to a ME source for large numbers of flies (any source offered would quickly become covered with flies, rendering the ME available only to those flies in contact with the source) and (ii) limits the amount of time spent feeding by individual males (as overfeeding on ME may be lethal, (Steiner, 1952; Haq et al., 2018).

Pre-release exposure to ME could be more easily incorporated into SIT programmes if exposure to the odour of ME affected the same male responses as ingestion of the chemical. This procedure, termed aromatherapy, has been developed and is currently in use in SIT programmes against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), where the semiochemical used is, not ME, but ginger root oil (Shelly, 2008; Paranhos et al., 2013; Silva et al., 2013; Steiner et al., 2013). Haq et al. (2018) devised a method to deliver ME volatiles to *B. dorsalis* males and demonstrated that the method enhanced male mating success like ME-feeding.

In the present study, we expanded our research on *B. zonata* and examined whether (i) ME-aromatherapy enhanced the mating success of *B. zonata* males and (ii) ME-aromatherapy reduced male response to ME-baited devices/traps. Answers to these questions will allow assessment of

the potential for simultaneous application of MAT and SIT for controlling *B. zonata* in the field.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Study insects*

At the time of the experiments, the flies were ~ 37 generations, under laboratory rearing. Eggs were collected and flies were reared according to previously described conditions under the heading "2.3.1 Study insects".

### *3.2.2 ME-feeding*

The ME (https://www.sigmaaldrich.com, 99% purity) feeding was performed in a room separated from all other flies. The males (n= 120) were marked with the paint on the thorax, one day before treatment and shifted to a transparent Plexiglass cage (20 × 15 ×15 cm). The following day 0.5 mL ME was dispensed on a filter paper disc (Watman® No. 1, 9 cm diameter), and placed in a petri dish lined with aluminum foil. The filter paper impregnated with ME was introduced in the cage at 09:00 hours. The males (hereafter termed ME-fed males) were fed on ME continuously for one hour; the feeding activity of individual males was not monitored. No food and water were provided during the ME-feeding period. After ME-feeding the petri dish was taken out of the cage, and the filter paper was removed and sealed inside a polyethylene bag for disposal. The males were provided with food and water *ad libitum*. The males were transferred to another room separate from all other flies with a temperature of 25±2 ℃, 60±5% R.H. and L14: D10 photoperiod.

### *3.2.3 ME-aromatherapy*

ME-aromatherapy of the males was executed in an isolated room (different from the ME-feeding room) following the procedure developed by Haq et al. (2018). As above, 0.5 ml ME was dispensed on a filter paper disc of 9 cm diameter, which was placed in a petri dish lined with aluminum foil. The petri dish containing ME laden filter paper disc was placed inside a transparent Plexiglas rectangular cage (30 × 23 × 23 cm). The cage was open at each end, with one end covered with nylon mesh and the other end uncovered. A cage of similar size with both openings covered with nylon mesh, holding the marked males, was placed immediately next to the cage containing the ME source, such that the screened opening of the male-containing cage was positioned directly next to the uncovered opening of the ME-containing cage. A small table fan placed at the covered

end of the ME-containing cage was used to blow air through that cage into the male-containing cage over a 5 h period (09:00-14:00 hours). The males congregated on the mesh and were observed pumping their proboscis over a 5 h period. After 5 h they stopped their pumping activities and moved away from the mesh. Therefore, ME treatment was stopped after 5 h. After treatment, the males (hereafter termed ME-aromatized) were maintained in a separate room with the temperature at 25±2 ℃, 60±5% R.H. and L14: D10 photoperiod. The males were provided with a standard adult protein diet and water *ad libitum*.

### *3.2.4 No-ME treatment*

Untreated males, i.e., those who did not provide any access to ME, were maintained in a separate room at 25±2 ℃, 60±5% R.H. and L14: D10 photoperiod. The ME-deprived males were marked in the same manner as ME-fed and ME-aromatized males. The males were provided with a protein diet and water *ad libitum*. Thus, untreated, ME-aromatized, ME-fed males and colony flies were maintained in separate rooms under similar abiotic conditions.

### *3.2.5 Field cages*

Circular, walk-in, nylon-screened field cages (2 m high and 1.5 m in diameter) were used for the experiments. Eight field cages were placed inside two adjacent glass houses (each 3.96 × 3.96 × 3.81 m). A potted, artificial mimic of a citrus tree (height 1.8 m; canopy 1 m in diameter) was placed in each field cage. The glasshouses were fitted with exhaust fans for ventilation and maintained at 26±2℃ and 60±5% R.H. during the experiments. The roof of glasshouses and glass side walls provided natural illumination, making the environment semi-natural. The experimental setup allowed eight simultaneous replications per day.

### *3.2.6 Experiments*

A total of 8 experiments were conducted, which addressed 3 main topics. Experiments 1-3 examined ME-aromatherapy given to sexually mature males and its effect on mating success, and Experiments 4-6 examined ME-aromatherapy given to immature males and its effect on male mating success at sexual maturity. The impact of ME-aromatherapy on subsequent ME attraction was addressed in Experiments 7-8.

### *3.2.7 Effects of ME-aromatherapy on mature males*

*3.2.7.1 Experiment 1: Competition between ME-aromatized and untreated males*

Males were treated with ME-aromatherapy at 15 days of age and evaluated the next day for their mating success. ME-aromatized and untreated males (n= 20 each) were released in each field cage 90 min before sunset, and virgin females (n= 20 per cage) were introduced 15 min later. The age of all flies was the same (16 days) at the time of the experiment. Mated couples were collected in plastic vials with screened lids. The experiment was continued till 1 h after sunset. The couples were brought to the laboratory for male identification (females were unmarked in all experiments). Eight replications were evaluated. Four observers ran the experiment, with each observer collecting couples from two cages by moving from one cage to the other every 5-10 min.

*3.2.7.2 Experiment 2: Competition between ME-aromatized and ME-fed males*

Similar experimental protocols were adopted as in Experiment 1 except that ME-aromatized males competed with ME-fed males for mating. The experiment was replicated eight times.

*3.2.7.3 Experiment 3: Competition between ME-aromatized, ME-fed, and untreated males*

Similar experimental protocols were adopted as in Experiment 1 except that ME-aromatized males, ME-fed males, and untreated males of the same age competed for copulations. Although fly density was higher than in the preceding experiments (80 total flies per cage versus 60 total flies), the higher density did not appear to affect the experiment as the average number of matings observed was similar for experiments conducted at the lower fly density.

### *3.2.8 Effects of ME-aromatherapy on immature males*

*3.2.8.1 Experiment 4: Competition between ME-aromatized (when immature) and untreated males*

The experiment followed the same procedure as adopted in Experiment 1 except that males were treated with ME-aromatherapy at 5 days of age (i.e., when sexually immature). At 15 days of age, the ME-aromatized males were tested for mating success against 15 days old untreated males in field cages. Virgin, 15 days old females were used, and eight replications were evaluated.

*3.2.8.2 Experiment 5: Competition between ME-aromatized (when immature) and ME-fed (when immature) males*

The same experimental protocol was adopted as in Experiment 4 except that ME-aromatized males competed with ME-fed males. Males were treated with ME by aromatherapy or feeding at 5 days of age and evaluated for mating at 15 days of age with 15 days old females. Eight replications were evaluated.

*3.2.8.3 Experiment 6: Competition between ME-aromatized (when immature), ME-fed (when immature), and untreated males*

The same experimental protocol was followed as in experiment 4 except that ME-aromatized males, ME-fed males and untreated males competed for matings. As in experiment 3, the density was increased from 60 to 80 total number of flies per cage. Eight replications were evaluated.

### *3.2.9 Effects of ME treatment on subsequent attraction to ME*

*3.2.9.1 Experiment 7: ME treatment on mature males*

*Bactrocera zonata* males treated with ME by aromatherapy or feeding at 15 days of age were tested for subsequent attraction to ME at 1 DPT, 5 DPT, 10 DPT and 15 DPT. Untreated males of the same age were tested as well. The experiment was conducted in a screen house (15.24 × 9.14 m). The ceiling of the screen house was 3 m high at the center and sloped down to a height of 1.82 m on either side. The screen house had side walls of stainless-steel mesh. The ceiling was made of two metal sheets with a layer of styrofoam (8 cm thick) between them. The ceiling was opaque, and the sunlight passing through the mesh walls made the environment semi-natural. Within the screen house, four cages (each cage 7.9 × 2.28 × 1.82 m) with a metal frame were aligned side-by-side and covered by nylon mesh. The cages were separated from each other by a curtain of nylon mesh. The curtain at the passage was overlapped, such that observers could move easily between cages while preventing fly movement between cages. There was a pathway 0.6 m wide between the cages and the surrounding stainless-steel mesh walls.

ME (0.5 mL) without insecticide was applied to a cotton wick, which was placed on aluminum foil inside a Steiner type trap (locally made). One trap was installed at the shorter length side (2.28 m) of each cage, and the treated males (by aromatherapy or feeding) (n= 20 each) and

the untreated males (n= 20) were released on the opposite end, 7.9 m (long side of the cage) from the ME source at 08:30 hours. ME-baited traps were suspended with a metal frame 1.8 m above ground. The width of each cage was 2.28 m, and the traps were suspended in the middle of each cage, thus traps were 2.28 m distant from each other. The number of trapped males was scored after 3 h. The temperature was 28-32℃ during experiments. A total of eight replications (4 replications per day) were evaluated.

*3.2.9.2 Experiment 8: ME treatment on immature males*

The same experimental procedures were followed as in experiment 7 except that ME-aromatized and ME-fed males were treated at 5 days of age (i.e., when immature). The response of ME-aromatized and ME-fed males was assessed at 1 DPT, 3 DPT, 7 DPT, 10 DPT, 15 DPT, 20 DPT, and 25 DPT. Untreated males of the same age as that of treated males were also included as a control treatment.

### *3.2.10 Statistical analyses*

The data in all experiments fulfilled parametric assumptions (data were homoscedastic and normally distributed). Data were analyzed by paired Student's t-test and one-way ANOVA. Pairwise comparisons of means were performed by Tukey's test. The significance level used for analysis was 95%, $P \leq 0.05$. Analyses were performed by using GraphPad Prism version 8.0.2.

## 3.3 RESULTS

### *3.3.1 Experiment 1: Competition between ME-aromatized and untreated males*

ME-aromatized males achieved significantly higher mating success ($t = 4.07$, $df = 7$, $P = 0.005$) than untreated males (Figure 3.1).

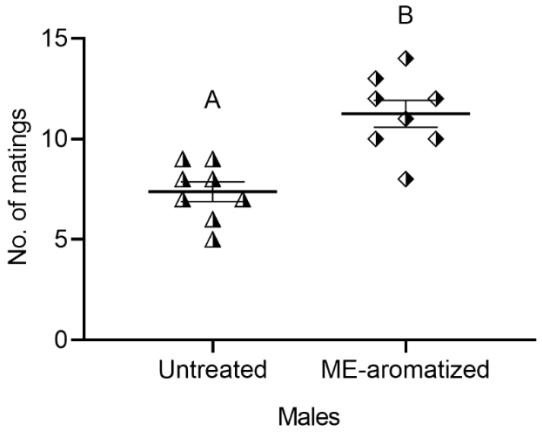


Figure 3.1 Mating success of ME-aromatized and untreated sexually mature *B. zonata* males. Males were treated with ME at 15 days of age and tested 1-day post-treatment. Mean relative mating frequencies ± SE are represented by horizontal lines; symbols represent results from the 8 individual replicates. Mean number of mating by each treatment shared out of 20 mating in each replication. Mean frequencies denoted by different letters were significantly different from one another (Student's t-test, $P < 0.05$).

### *3.3.2 Experiment 2: Competition Between ME-aromatized and ME-fed males*

ME aromatized males achieved significantly higher mating success (t= 3.95, df = 7, $P$ = 0.005) than ME-fed males (Figure 3.2).

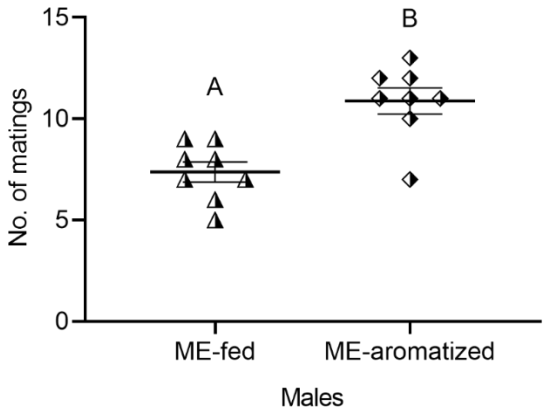


Figure 3.2. Mating success of ME-aromatized and ME-fed sexually mature *B. zonata* males. Males were treated with ME at 15 days of age and tested 1-day post-treatment. Mean relative mating frequencies ± SE are represented by horizontal lines; symbols represent results from the 8 individual replicates. Mean number of mating by each treatment shared out of 20 mating in each replication. Mean frequencies denoted by different letters were significantly different from one another (Student's t-test, $P < 0.05$).

### *3.3.3 Experiment 3: Competition between ME-aromatized, ME-fed, and untreated males*

There was significant variation among the treatments ($F_{2,21} = 24.35$, $P < 0.001$). ME-aromatized males achieved significantly higher mating success than ME-fed males and untreated males, however, no significant difference was observed in the mating success of ME-fed and untreated males (Figure 3.3).

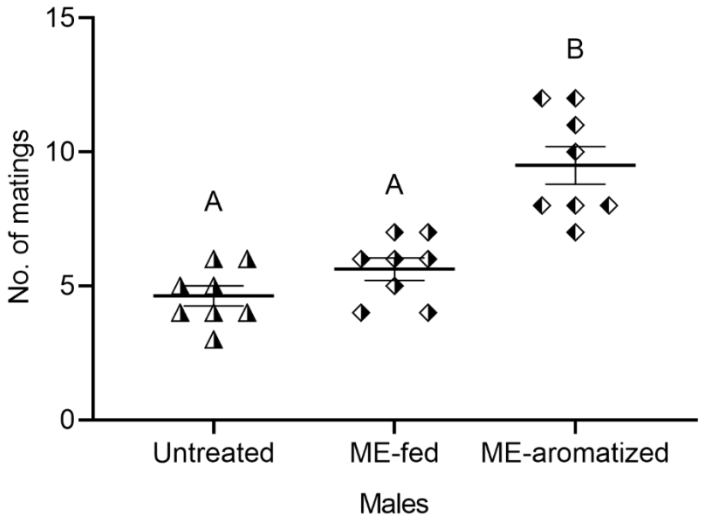


Figure 3.3 Mating success of ME-aromatized, ME-fed and untreated sexually mature *B. zonata* males. Males were treated with ME at 15 days of age and tested 1-day post-treatment. Mean relative mating frequencies ± SE are represented by horizontal lines; symbols represent results from the 8 individual replicates. Mean number of mating by each treatment shared out of 20 mating in each replication. Mean frequencies denoted by different letters were significantly different from one another (one-way ANOVA, $P < 0.05$).

### *3.3.4 Experiment 4: Competition between ME-aromatized (when immature) and untreated males*

ME-aromatized males achieved significantly higher matting success ($t = 4.67$, $df = 7$, $P = 0.002$) than untreated males (Figure 3.4).

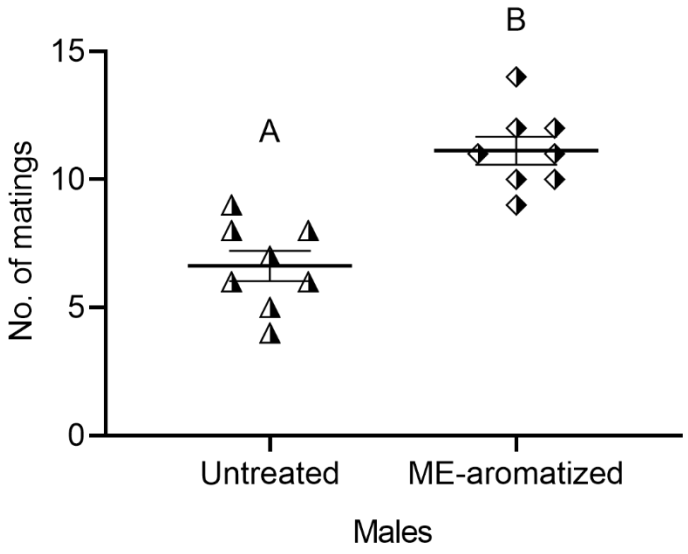


Figure 3.4 Mating success of ME-aromatized and untreated *B. zonata* males. Males were treated with ME at immature age of 5 days and tested for mating success at sexual maturity (15 days). Mean relative mating frequencies ± SE are represented by horizontal lines; symbols represent results from the 8 individual replicates. Mean number of mating by each treatment shared out of 20 mating in each replication. Mean frequencies denoted by different letters were significantly different from one another (Student's t-test, $P < 0.05$).

### *3.3.5 Experiment 5: Competition between ME-aromatized (when immature) and ME-fed males*

ME-aromatized males achieved significantly higher mating success (t = 4.13, df = 7, *P*= 0.004) than ME-fed males (Figure 3.5).

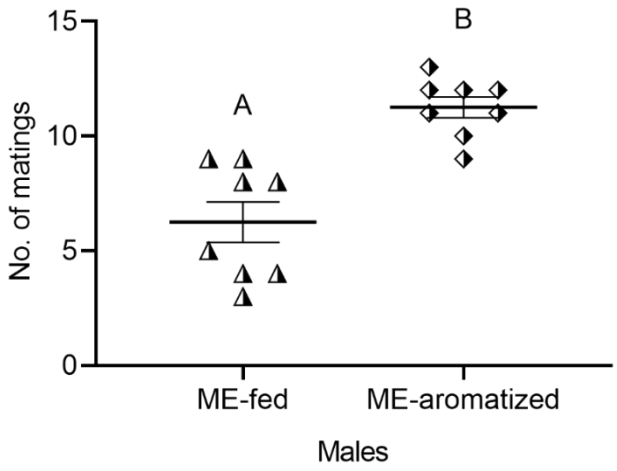


Figure 3.5 Mating success of ME-aromatized and ME-fed *B. zonata* males. Males were treated with ME at immature age of 5 days and tested for mating success at sexual maturity (15 days). Mean relative mating frequencies ± SE are represented by horizontal lines; symbols represent results from the 8 individual replicates. Mean number of mating by each treatment shared out of 20 mating in each replication. Mean frequencies denoted by different letters were significantly different from one another (Student's t-test, $P < 0.05$).

### *3.3.6 Experiment 6: Competition between ME-aromatized (when immature), ME-fed, and untreated males*

There was significant variation among the treatments ($F_{2,21} = 42.61$, $P < 0.001$). ME-aromatized males achieved significantly higher mating success than ME-fed males, which, in turn, had significantly higher mating success than untreated males (Figure 3.6).

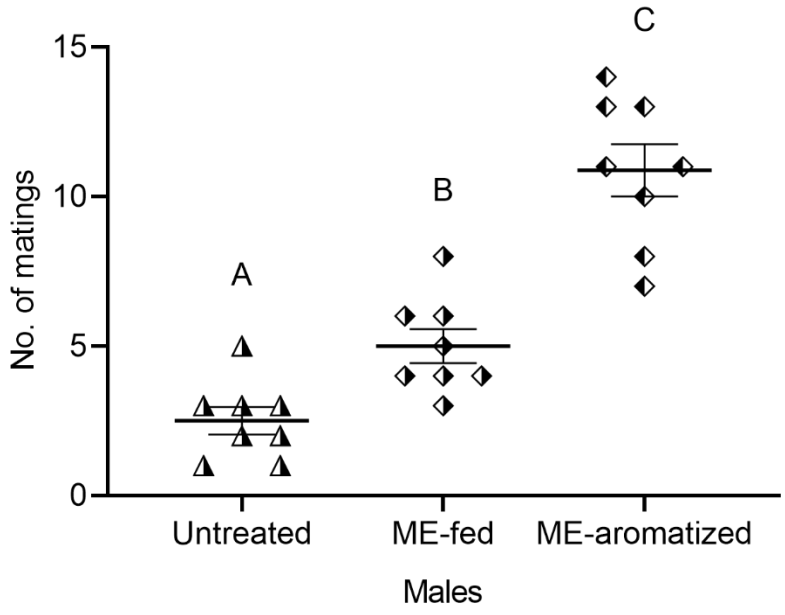


Figure 3.6 Mating success of ME-aromatized, ME-fed and untreated *B. zonata* males. Males were treated with ME at immature age of 5 days and tested for mating success at sexual maturity (15 days). Mean relative mating frequencies ± SE are represented by horizontal lines; symbols represent results from the 8 individual replicates. Mean number of mating by each treatment shared out of 20 mating in each replication. Mean frequencies denoted by different letters were significantly different from one another (one-way ANOVA, $P < 0.05$).

### *3.3.7 Experiment 7: ME attraction following ME treatment on mature males*

The numbers of trapped males varied significantly among the male groups at 1 DPT, 5 DPT, 10 DPT, and 15 DPT ($F_{2,21} = 38.32$, $P < 0.001$; $F_{2,21} = 25.48$, $P < 0.001$; $F_{2,21} = 33.80$, $P < 0.001$; and $F_{2,21} = 29.58$, $P < 0.001$, respectively; Table 3.1). Similar numbers of ME-aromatized and ME-fed males were trapped at all days post treatment (DPT), and these numbers were significantly lower than those observed for untreated males. Thus, ME-treated males showed a reduced tendency to revisit ME for as long as 15 days after initial exposure.

Table 3.1 Number (mean ± SE) of ME-aromatized, ME-fed and untreated *B. zonata* males captured in ME-baited traps. The males were treated with ME at 15 days (mature) of age and were evaluated for their subsequent attraction to ME at different days post-treatment (DPT). Untreated males of the same age as that of treated males were included as a control treatment.

| Male age at 1st exposure to ME | Male age when tested (DPT) | Mean number ± SE of males captured in ME-traps | | | F-value | P-value |
|---|---|---|---|---|---|---|
| | | ME-aromatized | ME-fed | Untreated | | |
| 15 | 16 (1) | 7.38 ±0.89 A | 6.13±1.03 A | 16.00±0.65 B | 38.32 | <0.001 |
| | 20 (5) | 6.00±0.82 A | 4.13±0.69 A | 11.50±0.76 B | 25.48 | <0.001 |
| | 25 (10) | 5.25±0.53 A | 3.75±0.31 A | 10.38±0.82 B | 33.44 | <0.001 |
| | 30 (15) | 4.88±0.67A | 4.38±0.46 A | 11.38±0.94 B | 29.58 | <0.001 |

Mean response values are out of 20 males in each replication, and 8 replications were evaluated. Mean values followed by different letters are significantly different from each other (one-way ANOVA, $P$ <0.05).

### *3.3.8 Experiment 8: ME attraction following ME treatment on immature males*

The numbers of trapped males varied significantly among the male groups at 1 DPT, 3 DPT, 7 DPT, 10 DPT, 15 DPT, 20 DPT, and 25 DPT ($F_{2,21} = 8.76$, $P < 0.001$; $F_{2,21} = 44.14$, $P < 0.001$; $F_{2,21} = 88.44$, $P < 0.001$; $F_{2,21} = 44.00$, $P < 0.001$; $F_{2,21} = 68.32$, $P < 0.001$; $F_{2,21} = 60.64$, $P < 0.00$ and $F_{2,21} = 79.41$, $P < 0.001$, respectively; Table 3.2). Similar numbers of ME-aromatized and ME-fed males were trapped at all days post treatment (with the exception of 7 DPT), and these numbers were significantly lower than those recorded for untreated males (again, with the exception of 7 DPT). Thus, ME-treated males exhibited a reduced tendency to revisit ME for as long as 25 days after initial exposure.

Table 3.2 Number (mean ± SE) of ME-aromatized, ME-fed and untreated *B. zonata* males captured in ME-baited traps. The males were treated with ME at 5 days (immature) of age and were evaluated for their subsequent attraction to ME at different days post-treatment (DPT). Untreated males of the same age as that of treated males were included as a control treatment.

| Male age at 1st exposure to ME | Male age when tested (DPT) | Mean number ± SE of males captured in ME-traps | | | F-value | P-value |
|---|---|---|---|---|---|---|
| | | ME-aromatized | ME-fed | Untreated | | |
| 5 | 6 (1) | 4.50±0.60 A | 4.13±0.40 A | 7.25±0.75 B | 8.76 | 0.002 |
| | 8 (3) | 4.13±0.35 A | 3.00±0.38 A | 7.50±0.33 B | 44.14 | <0.001 |
| | 12 (7) | 7.13±0.30 A | 5.38±0.38 B | 12.25±0.45 C | 88.44 | <0.001 |
| | 15 (10) | 6.88±0.71 A | 4.75±0.88 A | 14.50±0.71 B | 44.00 | <0.001 |
| | 20 (15) | 6.75±0.45 A | 4.63±0.63 A | 14.25±0.73 B | 68.32 | <0.001 |
| | 25 (20) | 6.13±0.61 A | 3.63±0.71 A | 14.13±0.79 B | 60.44 | <0.001 |
| | 30 (25) | 4.50±0.38 A | 3.25±0.31 A | 10.13±0.52 B | 79.41 | <0.001 |

Mean response values are out of 20 males in each replication, and 8 replications were evaluated. Mean values followed by different letters are significantly different from each other (one-way ANOVA, $P < 0.05$).

## 3.4 DISCUSSION

The present study demonstrated that ME exposure significantly increased male mating success of *B. zonata* as noted for other *Bactrocera* spp. (Quilici et al., 2004; Sookar et al., 2009; Ndzana et al., 2016). If ingested by *B. zonata* males, ME is transformed into two derivatives, dimethoxy phenol (DMP) and (Z)-coniferyl alcohol (Z-CF) in a 1:1 ratio (Tan et al., 2011). When sexually signaling, these metabolites are released as components of the male pheromone and make the pheromone more attractive to females (Nishida et al., 1988; Tan & Nishida, 1996; Wee et al., 2007). Due to the increased attraction of females to male pheromones containing ME metabolites, it was assumed that male feeding on ME is required to confer a mating advantage but Haq et al. (2014), demonstrated that males treated with ME by aromatherapy without direct access to ME achieved higher mating success. Conversely, Shelly and Dewire (1994) reported that, when denied direct contact with ME, *B. dorsalis* males gained no mating advantage. However, the current study demonstrated that sexually mature males when exposed to ME volatiles through aromatherapy gained higher mating success. These findings are consistent with Haq et al. (2014, 2015, 2018), who reported that ME-aromatherapy enhanced the mating success of *Bactrocera carambolae*

(Drew & Hancock) and *B. dorsalis* males over untreated males. In the present study, ME-aromatherapy increased male mating success more dramatically than did ME-feeding, and this trend was consistent in all experiments. One potential explanation of such a dramatic effect might be that the ME-aromatized males were able to receive the optimal amount of ME as the males were able to move away from the air stream containing ME after attaining 'satiation'. In the feeding setup, however, males could not stop and over-consumption of ME appeared to temporarily reduce male agility (Haq et al., 2018). The physiological mechanism by which males acquire ME volatiles and process these internally is still unknown. Males may have taken in ME volatiles through the pumping of the proboscis and/or through direct absorption through the cuticle (Haq et al., 2018). Interestingly, Wee (personal communication) detected ME in the rectal glands of *B. dorsalis* males exposed to ME through membrane/screen mesh. Biochemical analysis of rectal glands and sex pheromones may shed light on the absorption and metabolism of ME volatiles in ME-aromatized males.

Treating males at maturity (either by feeding or aromatherapy) is not possible with the current practices of sterile fly emergence and release system, as most SIT programmes release sterile males at 4-5 days of age. Holding males beyond 4-5 days until their maturity would add costs to the programme and may decrease the quality of males due to overcrowded conditions (Koyama et al., 2004). Rasool et al. (2023) demonstrated that 5 days old *B. zonata* males treated with ME by feeding had a mating advantage over untreated males in trials conducted at the onset of sexual maturity (8 days) age and onward. Holding males for 8-9 days for ME treatment is not practicable due to financial costs, therefore, treating males at 5 days of age and releasing them at 6 days of age, with males gaining the mating advantage at 8-9 days of age, seems practicable.

Despite cracking issue of suitable male age for treatment with ME, the ME delivery system must be practical to allow mass application at emergence and release facilities. As noted above, feeding ME to hundreds of thousands or even millions of sterile males daily is not logistically feasible. Considering ongoing SIT operational programmes, we compared ME-aromatized males at 5 days of age for mating success with ME-fed and untreated males. Our results indicated that males aromatized at 5 days of age (sexually immature) achieved higher mating success than ME-fed and untreated males after attaining sexual maturity. Thus, aromatherapy resolves not only the

problem of a practical ME-delivery system, but also provides the possibility of treating males at 5 days of age, which is compatible with the existing protocols adopted in operational programmes. Such treated males would gain a mating advantage after reaching their sexual maturity age. Thus, it appears that fewer ME-aromatized sterile males of *B. zonata* would be required to induce sterility in wild females compared to untreated sterile males, which would lead to enhanced cost-effectiveness of SIT application.

Traditionally, *Bactrocera* fruit fly control programmes deployed MAT initially to suppress the male population and then implemented SIT to achieve the eradication of the wild population (Steiner et al., 1970; Koyama et al., 1984). Sequential application of MAT and SIT necessitates the removal of traps installed over a large area before deployment of SIT, otherwise, many released males would be attracted to and killed in ME-baited traps. Removal of ME traps before SIT application is logistically difficult and it would incur a financial cost and may lead to the resurgence of the wild population, which may undermine the efficiency of SIT application. Therefore, because of the possibility of significantly reducing the number of sterile males required, simultaneous application of MAT and SIT is advisable. A prerequisite for combining MAT with SIT is that sterile males treated with lure before release should not visit the male lure traps/baits in the field. Shelly (1994) reported that, after initial feeding on ME, *B. dorsalis* males showed a two to three times reduced tendency to revisit ME-baited traps as compared to untreated males. However, before the present study, it was not known whether *B. zonata* males treated with ME by feeding or aromatherapy would show a reduced tendency to visit ME-baited traps. Here, we demonstrated that ME-aromatized and ME-fed males of *B. zonata* were captured significantly less frequently in ME-baited traps than untreated males when tested at different intervals after initial exposure given at sexual maturity. The effect of ME treatment on sexually mature (15 days old) males persisted up to 15 days after initial exposure to the lure, which clearly indicates a long-lasting effect of ME treatment. As noted above, to reduce financial costs, SIT programmes typically release sterile males before they attain sexual maturity. Therefore, we evaluated males by treating them with ME when immature, at 5 days of age for their tendency to visit ME-baited traps at varying intervals up to 25 days post-treatment. ME-aromatized and ME-fed males displayed a similar significant reduction in their response to ME, considerably less than untreated

males, and this effect was evident for as long as 25 days after initial exposure. In contrast to *B. zonata*, ME-aromatherapy did not suppress the re-feeding of *B. dorsalis* males (Shelly, 2020).

The present results indicate that *B. zonata* males can be treated with ME through aromatherapy, and such treated males can subsequently be released when still immature for the simultaneous application of MAT and SIT. The combined MAT and SIT application will deplete the population of wild males, thereby increasing the ratio of sterile: to wild males and the effectiveness of the SIT. By accelerating population reduction of the pest population, a combined MAT and SIT approach would result in significant cost savings for the control programme.

### *3.4.1 Conclusions*

A system for delivering ME by aromatherapy, which appears practical in SIT emergence and release facilities, was developed for treating *B. zonata* males. ME-aromatherapy at 5 days (immature) of age enhanced male mating success after attaining sexual maturity. The ME-aromatized males exhibited reduced attraction to ME-baited traps, which enables the application of MAT and SIT at the same time. The simultaneous application of both techniques will enhance the cost-effectiveness of *B. zonata* management programmes.

# Chapter 4: Effect of Methyl Eugenol and Dietary Protein on Mating Performance of Peach Fruit Fly, *Bactrocera zonata* Saunders (Diptera: Tephritidae) Males


## ABSTRACT

This section investigated the dietary protein and ME effect on the copulatory success of *B. zonata* males. The males were provided with i) dietary protein and treated with ME (ME+P+) ii) dietary protein and not treated with ME (ME-P+) iii) sugar-only diet and treated with ME (ME+P-) and iv) untreated (ME-P-). Firstly, after emergence, the protein-fed males had access to their respective diets till sexual maturity. The ME+P+ and ME+P- males were fed on ME at 15 days of age and competed for mating success with ME-P+ and ME-P- males 1-day post-treatment (DPT) in field cages. The results showed that mature ME+P+ *B. zonata* males had a mating advantage over other competing males. Secondly, ME+P+ and ME+P- males had access to dietary protein for 5 days and after that provided a sugar-only diet. The ME+P+ and ME+P- males were fed on ME at 5 days of age and tested for mating success with ME-P+ and ME-P- males at sexual maturity (15 days of age). The ME+P+ males gained elevated copulatory success than other males. The study proved that the inclusion of dietary protein is necessary with ME treatment for the enhancing mating success of males. The study indicated that prerelease feeding on dietary protein and treatment with ME can enhance the mating performance of *B. zonata* males, consequently increasing the effectiveness of SIT.


## 4.1 INTRODUCTION

In the sterile insect technique (SIT) the target insects are raised on a large scale. The females present in the wild produce no offspring after copulation with the sterile males, resulting in a reduction of the target insect population, thus sustained and planned releases of sterile males can eventually eradicate the target population (Knipling, 1955). The application of SIT as a component of regional management programmes has been used successfully for suppressing the population of fruit flies and other insect pests (Hendrichs & Robinson, 2009). However, due to

long-term rearing under controlled conditions and procedures involved in SIT, the mating competitiveness of the males is compromised (Cayol, 2000; Lance et al., 2000). The reduced mating competitiveness can be improved through effective management of the mother insect colony (Robinson & Hendrichs, 2005). Supplementary diets and exposure to certain chemicals can enhance the sexual effectiveness of the males (Shelly et al., 2007; Pereira et al., 2013).

Tephritid male and female fruit flies are generally anautogenous and need nutrition for their sexual development (Drew & Yuval, 2000). The fruit flies forage for carbohydrates and protein to sustain their metabolic activities and sexual development (Hendrichs & Prokopy, 1994). In nature, nutrition comes from a variety of sources such as plant exudates, bird feces, bacteria, yeast, honeydews and floral products (Hagen, 1958; Neilson & Wood, 1966; Drew et al., 1983; Drew & Lloyd, 1987; Hendrichs & Hendrichs, 1990; Hendrichs et al., 1991, 1993). While a prerelease diet may provide sterile males with nutritional resources to increase mating success, treatment with semiochemicals such as Methyl eugenol (ME) can further enhance their mating competitiveness.

Owing to its quarantine pest status, *B. zonata* infers with intranational trade of horticultural crops. Its management has generally been relied on insecticide application. However, due to the harmful side effects of insecticide application on the environment and human health, demand for effective environment-friendly alternatives is increasing. Therefore, SIT which has been proven very successful in suppressing/eradicating many species of fruit flies, can potentially be applied to manage *B. zonata* populations. Furthermore, knowledge of dietary protein supplements, either alone or in combination with ME for enhancing the sexual performance of *B. zonata* males is scarce. The objective of the present study was to investigate the effect of ME and dietary protein on the mating performance of *B. zonata* males.

## 4.2 MATERIALS AND METHODS

### *4.2.1 Study insects*

At the time of the experiments, the flies were ~ 39 generations under laboratory rearing. Eggs were collected and flies were reared according to previously described conditions under the heading “2.3.1 Study insects”.

### *4.2.2 Treatments*

There were four treatments:

1) The males were fed on dietary protein (P) and treated with ME (ME+P+)
2) The males were fed on dietary protein (P) and not treated with ME (ME-P+)
3) The males were fed on sugar-only diet and treated with ME (ME+P-)
4) The males were fed on sugar-only diet and not treated with ME (ME-P-)

### *4.2.3 ME-feeding*

ME-feeding was performed according to the previously described protocol under the heading "3.3.2. ME-feeding". The males were provided with their respective diets and water *ad libitum*.

### *4.2.4 ME-aromatherapy*

ME-aromatherapy of the males was performed according to the previously described protocol under the heading "3.3.3. ME-aromatherapy". The males were provided with their respective diets and water *ad libitum*.

### *4.2.5 No-ME treatment*

Same as the previously described protocol under the heading "3.3.4". The males were provided with their respective diets and water *ad libitum*.

### *4.2.6 Field cages*

Semi-circular walk-in screened-cloth field cages (2 m high and 1.5 m in diameter) were used for the experiments. Eight field cages (90 cm apart) were placed inside two adjacent glass houses of same size (3.96 × 3.96 × 3.81 m). A potted artificial tree mimicking a citrus tree (height 1.8 m; canopy 1 m in diameter) was placed in each field cage. The glass houses were fitted with exhaust fans for ventilation and 26±2°C temperature with 60±5% R.H. was maintained during the experiments. The roof of glasshouses and glass side walls provided natural illumination, making the environment semi-natural. The experimental setup allowed to run eight replications per day simultaneously.

### *4.2.7 Effect of ME and dietary protein feeding till sexual maturity on mating success of B. zonata males*

After eclosion, the males had access to their respective diets till sexual maturity (15 days of age). ME-feeding of the ME+P+ and ME+P- males was performed at 15 days of age. The ME+P+ and ME+P- males were tested 1-day post-treatment (DPT) for mating success with ME-P+ and ME-P- males in field cages. The males (n= 20 each) from all treatments were released at the same time in each field cage, 90 min before sunset and were allowed to settle down. Sexually mature virgin females (n= 20) were introduced in each field cage 15 min later. The age of all flies was the same (16 days) at the time of the experiment. After mating the couples were collected in vials with screened lids. The couples were brought to the laboratory for identification. Eight replications were performed.

### *4.2.8 Effect of ME and dietary protein feeding on mating success of sexually immature B. zonata males*

Two experiments were performed. In the first experiment, the same experimental procedures were adopted as above except after the emergence ME+P+ and ME-P+ males were provided dietary protein for 5 days (sexually immature) and then given a sugar-only diet. The ME+P+ and ME+P- males were fed on ME at 5 days of age. The males competed for mating success in field cages at sexual maturity (15 days of age). The age of all flies was the same (15 days) at the time of the experiment. In the second the experiment same experimental procedures were followed except ME+P+ and ME-P+ males competed for mating success in the absence of ME+P- and ME-P- males. The experiments were replicated eight times.

### *4.2.9 Effect of ME-aromatherapy and dietary protein feeding on mating success of B. zonata males*

ME-fed and ME-aromatized males competed for mating success in field cages. After emergence, the males were fed on dietary protein for 5 days and then switched to a sugar-only diet. The males were exposed to ME at 5 days of age. The males were tested for mating success at sexual maturity. The age of all flies was the same (15 days) at the time of the experiment. The experiment was replicated eight times.

### *4.2.10 Statistical analyses*

The data were expressed as a percentage of mating success. The data were distributed normally. Data were analyzed by paired Student's t-test and one-way ANOVA and upon detection of significant difference, pairwise comparisons of the means were done by Tukey's test. For analysis significance level was 95%, $P \leq 0.05$. GraphPad Prism version 8.0.2 was used to perform analyses.

## 4.3 RESULTS

### *4.3.1 Effect of ME and dietary protein feeding till sexual maturity on mating success of B. zonata males*

There was significant a difference among all treatments ($F_{3, 28} = 269.4$, $P < 0.001$). The ME+P+ males achieved maximum copulatory success followed by ME-P+ males. The ME+P- and ME-P- males achieved very few mattings and did not differ significantly from each other (Figure 4.1).

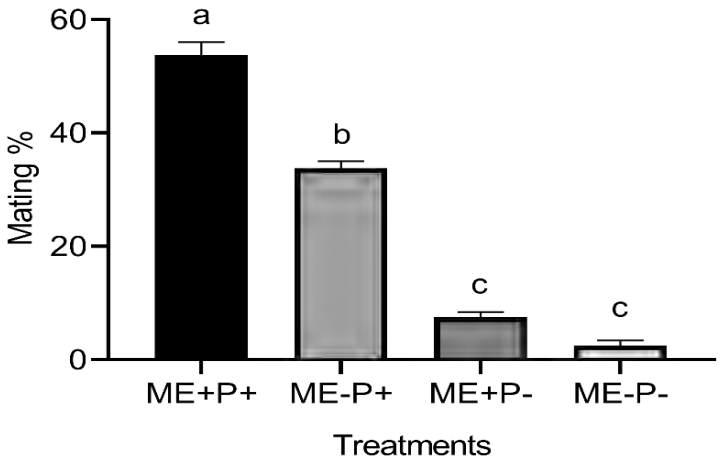


Figure 4.1 Mating % of ME+P+, ME+P-, ME+P- and ME-P- sexually mature *B. zonata* males. The males were treated with ME at 15 days of age and tested for mating success 1-day post-treatment. Mean ± SE is represented by horizontal lines for eight replicates.

### *4.4.2 Effect of ME and dietary protein feeding on mating success of sexually immature B. zonata males*

There was a significant difference among all treatments ($F_{3, 28} = 244.7$, $P< 0.001$). The ME+P+ males attained maximum matings followed by ME-P+ males. The ME+P- and ME-P- males achieved very few mattings and did not differ significantly from each other (Figure 4.2). Similarly, ME+P+ males and ME-P+ males differed significantly in terms of copulatory success (t= 3.31, d.f= 7, $P$=0.01). ME+P+ males achieved more matings than ME-P+ males (Figure 4.3).

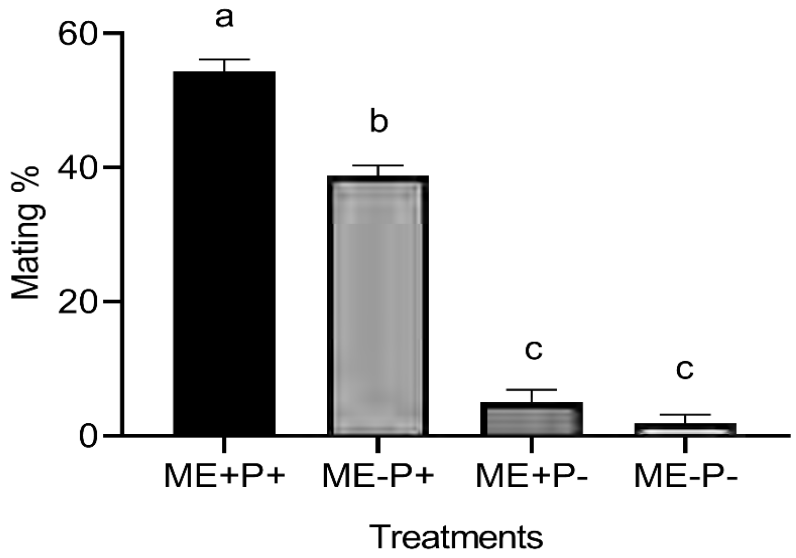


Figure 4.2 Mating % of ME+P+, ME+P-, ME+P- and ME-P- sexually immature *B. zonata* males. The males were treated with ME at 5 days of age, allowed to feed on dietary protein for 5 days and then switched to a sugar-only diet. The males were tested for mating success at sexual maturity (15 days of age). Mean ± SE is represented by horizontal lines for eight replicates.

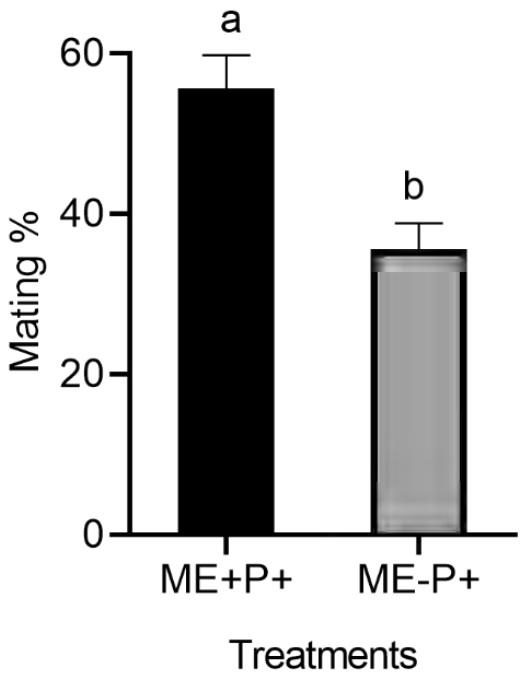


Figure 4.3 Mating % of ME+P+ and ME-P+ sexually immature *B. zonata* males. The males were treated with ME at 5 days of age, allowed to feed on dietary protein for 5 days and then switched to a sugar-only diet. The males were tested for mating success at sexual maturity (15 days of age). Mean ± SE is represented by horizontal lines for eight replicates.

### *4.3.3 Effect of ME-aromatherapy and dietary protein feeding on mating success of B. zonata males*

ME-aromatized and ME-fed males did not differ significantly and had the same level of matings (t= 0.21, d.f= 7, *P*= 0.84; Figure 4.4).

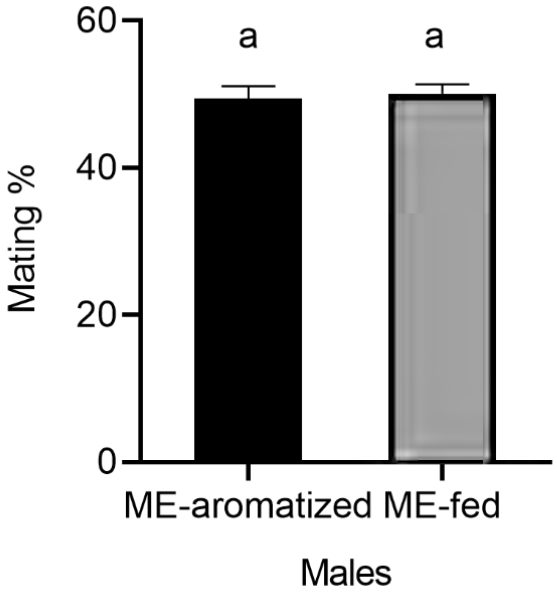


Figure 4.4 Mating % of ME-aromatized and ME-fed sexually immature *B. zonata* males. The males were treated with ME at 5 days of age, allowed to feed on dietary protein for 5 days and then switched to a sugar-only diet. The males were tested for mating success at sexual maturity (15 days of age). Mean ± SE is represented by horizontal lines for eight replicates.

## 4.4 DISCUSSION

The present study provided insight into dietary protein and ME effect on the mating performance of *B. zonata* males. The study indicated that the provision of dietary protein and ME had a visible effect on the mating success of *B. zonata* males. The males who had continuous access to dietary protein until sexual maturity and were allowed to feed on ME secured more copulatory success as compared to competing males who were either protein or ME-deprived. Our results correspond to Shelly et al., (2005) who reported a similar effect of protein diet and exposure to ME in *Bactrocera dorsalis*. Similar findings on the effect of dietary protein and ME for enhancing mating success were also reported in *B. dorsalis* and *Bactrocera correcta* (Obra & Resilva, 2013; Orankanok et al., 2013). The ME-P+ males although achieved less mating success than ME+P+, obtained significantly more mating success than ME+P- and ME-P-males. The ME+P- and ME-P- males were sexually non-competitive and achieved very few copulations.

Likewise, Ndzana et al., (2016) observed mating advantage in protein-fed *B. zonata* males. Effect of inclusion of protein dietary protein on mating performance enhancement has been reported in various fruit fly species such as *Ceratitis capitata* (Blay & Yuval, 1997; Kaspi & Yuval, 2000; Shelly & Kennelly, 2002; Shelly et al., 2003; Yuval et al., 2003), *Bactrocera tryoni* (Taylor et al., 2013), *B. dorsalis* (Obra & Resilva, 2013), *Zeugodacus cucurbitae* (Haq et al., 2010) and several *Anastrepha* species (Aluja et al., 2001; Liedo et al., 2013).

In most tephritid fruit flies sexual development is dependent on the availability of a protein diet (Drew & Yuval, 2000). The protein diet has a significant effect on the mating success of males as these males are capable of producing pheromones for longer periods. The males after ME ingestion produce more powerful pheromones which are more attractive to females. ME+P+ males had more mating success than ME+P- shows the ingestion of ME triggers metabolism in males that requires a sustained supply of energy in the form of protein. The low copulatory success in ME+P- males may also be related to the toxicity of ME in males (Chang et al., 2009) which increases the energy costs of metabolizing ME. Therefore, after ingestion of ME, the males require nutrition in the form of protein. The results indicated that dietary protein is essential for mating success enhancement of males after ingestion of ME.

Due to space and cost constraints in most of the operational SIT programs, the sterile males are retained for 4-5 days in sterile male holding and release facilities and generally released as immature males. For example, in the eradication program for melon fly, *Z. cucurbitae*, Okinawa islands, Japan, the sterile males were released at the age of 3-4 days though the males required more than one week to attain sexual maturity (Nakamori & Kuba, 1990). Holding the sterile males for more than 4-5 days adds costs to SIT application. In previous experiments in the current study, we have demonstrated that ME treatment of *B. zonata* males at 5 days of age can enhance the mating success of the males at the onset of sexual maturity. In the current experiment when the males were provided dietary protein initially for 5 days and then switched to a sugar-only diet, the results indicated that the males which were provided dietary protein and ME (ME+P+) achieved higher mating success than other competing males. The results were similar to those obtained in males who had access to dietary protein *ad libitum* until sexual maturity. Furthermore, ME+P+ males attained higher mating when competed only with ME-P+ males after initially feeding on

dietary protein for 5 days. The results showed that initial feeding on dietary protein for 5 days and then treatment with ME can effectively increase the mating success of the males at sexual maturity. The present study indicated that the inclusion of a protein diet was essential for achieving mating success with ME exposure. Providing males with dietary protein for 5 days following ME treatment is sufficient for enhancing mating success of *B. zonata* males and can be incorporated into release protocols for SIT.

ME-aromatherapy at 5 days of age might have triggered the metabolism in the males that required additional dietary resources in the form of protein. As the ME-aromatized males were held for 10 days post aromatherapy and the protein reserves acquired during feeding on dietary protein for 5 days might have been consumed early due to high metabolic activity. However, the mating success achieved by ME-aromatized males was at par with ME-fed males. Moreover, released sterile males can forge for nutritional resources available in the field (Barry et al., 2003; Shelly & McInnis, 2003; Maor et al., 2004; Yuval et al., 2007). ME-aromatherapy of the males after initial feeding on dietary protein for 5 days enhances the mating success and can boost the effectiveness of SIT programme.

### *4.4.1 Conclusion*

The current study provides information on the effect of dietary protein in post-teneral adult diet and ME on the mating competitiveness of *B. zonata* males. The results indicated that dietary protein and exposure to ME act synergistically for mating performance enhancement of *B. zonata* males. Prerelease feeding of males on dietary protein for 5 days after emergence is sufficient to increase male mating competitiveness for enhancing the success of SIT. ME-aromatherapy can serve as a delivery method for ME treatment of sterile males in fly emergence and release facilities. However, further investigations on the starvation survival of the males after feeding on dietary protein and ME treatment are needed.

# Chapter 5: Effect of Methyl Eugenol and Dietary Protein on Survival of Peach Fruit Fly, *Bactrocera zonata* Saunders (Diptera: Tephritidae) Males


## ABSTRACT

This section of the current study investigated the effect of ME and dietary protein on the survival of *B. zonata* males. The ME+P+, ME-P+, ME+P- and ME-P- males were allowed to feed for 5 days on their respective diets and were assessed under extreme scenarios of the absence of food and water and then with access to water. The results showed that sugar-fed males (ME-P-) had significantly more survival than other males. The protein-fed males (ME-P+) had the lowest survival. The present study also indicated that although ME+P+ males had less survival than sugar-fed males, they performed better than the males of other treatments. The current study indicated that ME-aromatized males had better survival than ME-fed males. ME-aromatherapy can be applied as a ME delivery system in sterile male emergence and release facilities with enhanced male mating success and no adverse effect on male survival.


## 5.1 INTRODUCTION

Generally, both male and female tephritid fruit flies are anautogenous requiring nutrition for their sexual development (Drew & Yuval, 2000). An adult diet comprising yeast hydrolysate and sugar is used in fruit fly rearing colonies, for optimum sexual development and production (Perez-Staples et al., 2007; Fanson & Taylor, 2012). The role of dietary protein in mating performance enhancement has been demonstrated in several species of fruit flies (Pereira et al., 2021). However, supplementing the males with a protein diet for a certain number of days and then cutting its supply may adversely affect the survival of males. Kaspi & Yuval (2000) reported high mortality in protein fed *Ceratitis capitata* males.

Releasing the ME-fed sterile males can enhance the effectiveness of SIT application (Shelly, 2020, Rasool et al. 2023). Even though ME-feeding enhanced male mating success, pre-release feeding on ME has limited practicality at present, because feeding ME to hundreds of thousands or even millions of sterile males daily is not logistically feasible. To overcome the

limitation of ME-feeding, the system delivering ME by aromatherapy was developed and tested on *Bactrocera carmbolae* and *Bactrocera dorsalis* (Haq et al., 2014, 2018). In Chapter 3, we have determined that ME-aromatherapy enhances the sexual performance of *B. zonata* males parallel to ME-feeding.

In the previous Chapter, it has been reported that a protein diet enhances *B. zonata* male mating success. ME can further enhance the sexual performance of the males feeding on dietary protein but ME does not enhance the copulatory success of the males in the absence of a protein diet. Enhanced sexual activities have been reported to cost males their survival (Johansson et al., 2005; Papadopoulos et al., 2010). Thus, the elevated mating performance of the males as a result of access to ME and protein supplements may cost the *B. zonata* males in terms of survival. The present study investigated the survival of *B. zonata* males by feeding them on a protein diet or sugar-only diet for 5 days, treating them with ME-feeding or ME-aromatherapy at 5 days of age and then depriving them of food. The males were fed for 5 days, as the sterile males are kept in sterile males holding and release facility for 4-5 days (Nakamori & Kuba, 1990; McInnis et al., 2013).

## 5.2 MATERIALS AND METHODS

### *5.2.1 Study insects*

At the time of the experiments, the flies were ~ 39 generations under laboratory rearing. The flies were reared according to previously described conditions under the heading "2.3.1 Study insects".

### *5.2.2 ME-feeding*

ME-feeding was performed according to the procedure described in "3.3.2 ME-feeding".

### *5.2.3 ME-aromatherapy*

The ME-aromatherapy of the males was performed by following the protocol described under the heading "3.3.3. ME-aromatherapy".

### *5.2.4 Effect of ME-feeding and dietary protein on survival of B. zonata males*

There were four treatments:

1) The males were ME-fed and provided with dietary protein (ME+P+)
2) The males were not ME-fed and provided with dietary protein (ME-P+)
3) The males were ME-fed and provided with sugar-only diet (ME+P-)
4) The males were not ME-fed and provided with sugar-only diet (ME-P-)

Two sets of experiments were conducted. In the first set of experiments males were fed on the respective dietary regime for 5 days, fed on ME and the next morning the males were deprived of food and water. The experiment was conducted under controlled conditions with the temperature at 25±2 ℃, 60±5% R.H, and 14L: 10D photoperiod. One hundred males from each treatment were shifted to 5 clean screened cages (20 × 15 × 15 cm) by keeping 20 males in each cage without food and water. The dead males were counted and removed every 12 h from the cages. The experiment was continued till the mortality of all males. In the second set of experiments, the same experimental protocols were adopted except that males were provided with water.

### *5.2.5 Effect of ME-aromatherapy on survival of B. zonata males*

. Two sets of experiments were conducted. In the first set of experiments males were fed on a protein diet for 5 days, treated with ME by feeding or aromatherapy and the next morning the males were deprived of food and water. One hundred males from each treatment were shifted to 5 clean screened cages (20 × 15 ×15 cm) by keeping 20 males in each cage without food and water. The experiment was performed under controlled conditions with temperature at 25±2 ℃, 60±5% R.H. and 14L: 10D photoperiod. The dead males were counted and removed every 12 h from the cages. The experiment was continued till the mortality of all males. The second set of experiments was conducted following similar experimental procedures except the males had access to water.

### *5.2.6 Statistical analyses*

The data were analyzed by Cox regression model (95% confidence interval) and Kaplan-Meier test was applied for pairwise comparisons by using SPSS software version 26.

## 5.3 RESULTS

### *5.3.1 Effect of ME-feeding and dietary protein on survival of B. zonata males*

There was no significant difference among treatments on male survival when they were not provided water ($\chi^2$= 4.96, d.f= 3, *P*=.175; Figure 5.1). However, there was a significant difference among treatments on male survival when water was provided ($\chi^2$= 50.24, d.f= 3, *P* < 0.001; Figure 5.2).

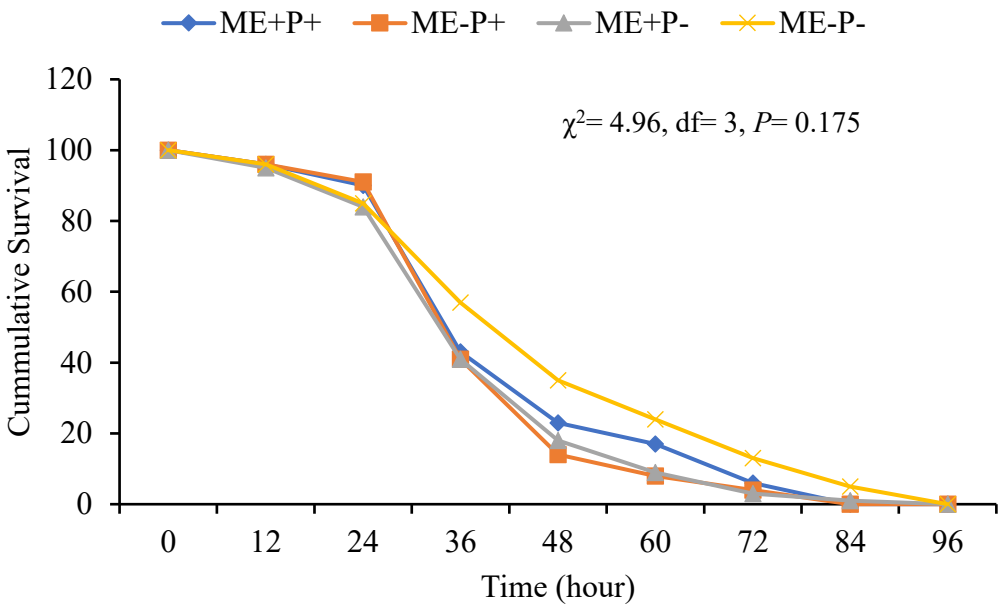


Figure 5.1 Effect of ME-feeding and dietary protein on survival of *B. zonata* males without water

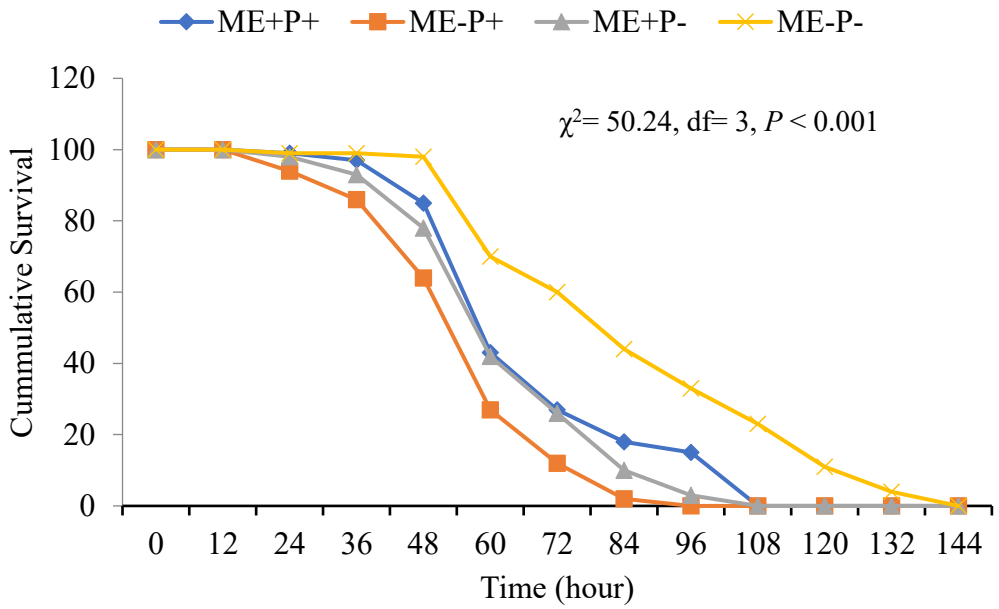


Figure 5.2 Effect of ME-feeding and dietary protein on survival of *B. zonata* males with water

### *5.3.2 Effect of ME-aromatherapy on survival of B. zonata males*

A significant difference in survival of ME-aromatized and ME-fed males was observed when the treated males were starved without water ($\chi^2$= 4.45, d.f= 1, $P$= 0.035; Figure 5.3). Similarly, a significant difference was observed between ME-aromatized and ME-fed males when they were deprived of food but had access to water ($\chi^2$= 12.22, d.f= 1, $P$ < 0.001; Figure 5.4).

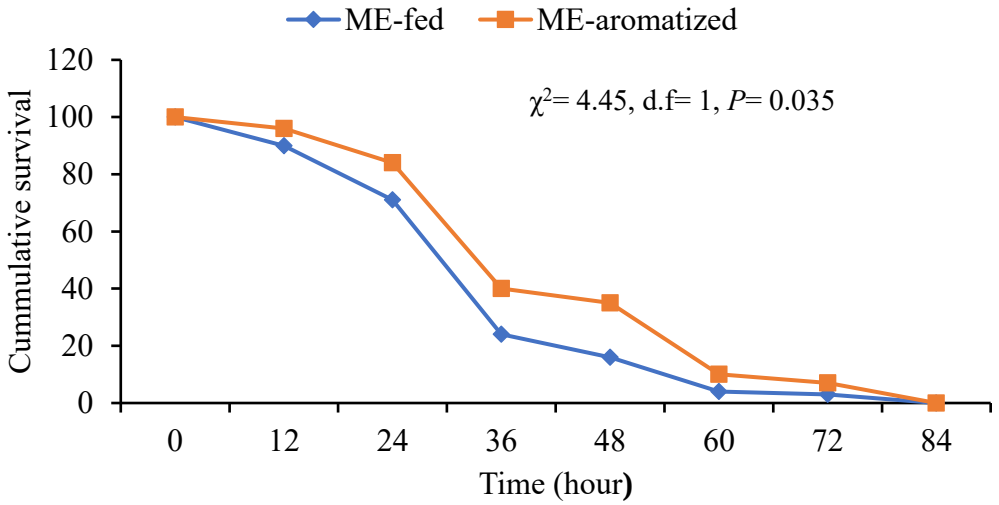


Figure 5.3 Effect of ME-aromatherapy on survival of *B. zonata* males without water

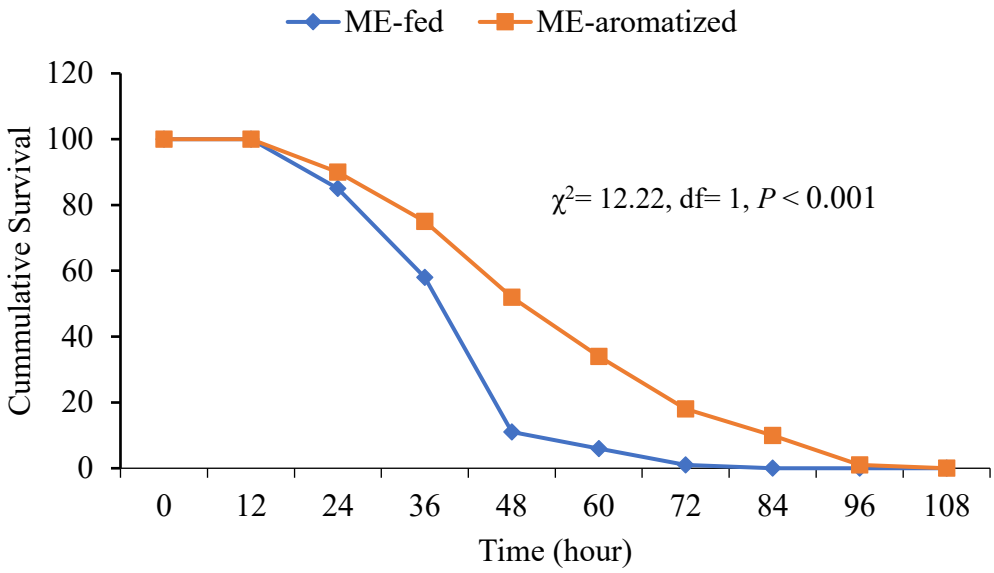


Figure 5.4 Effect of ME-aromatherapy on survival of *B. zonata* males with water

## 5.4 DISCUSSION

In the current study, we explored ME and dietary protein effect on the starvation survival of *B. zonata* males, the first time known to the best of our knowledge. The males of different treatments were given access to their respective diets for 5 days and were either ME-fed or not ME-fed. In the absence of food and water, no significant difference in the survival of males of different treatments was observed. After the field releases the sterile male may have access to water and switching them off completely from food and water may be unnatural as at least water may be available in the wild in the form of dew and other sources. However, considering the extreme scenarios of the non-availability of food and water in the field, male starvation survival was assessed. No difference in starvation survival of differently treated males may be due to the desiccation of males rather than the treatment effect. However, when males were provided with water, males treated differently had different survival. The males which had a sugar-only diet (ME-P-) survived longer than other males. Similar results were reported by Maor et al. (2004) in *C. capitata* where sugar-fed males survived longer than protein-fed males. The current study indicated that protein-fed males (ME-P+) males had a low survival rate as compared to other

treatments. These results correspond with Kaspi & Yuval (2000) who reported that protein-fed males were short-lived when they had no access to protein diet. Negative effect of starvation survival in protein-fed males has also been reported the Levy et al. (2005) and Utgés et al. (2013).

The tephritid males need protein for their sexual development, and access to dietary protein commits males toward sexual development and mating activities such as sexual signalling (Carey et al., 2002; Yuval et al., 2007). Food deprivation of males affects the metabolism of males which has adverse consequences on their survival. Haq et al. (2013) reported that cutting off the protein supply until the *Z. cucurbitae* males reached the developmental threshold may cost their survival, but protein diet deprivation after males had attained the developmental threshold did not affect their survival. The present study indicated that although ME+P+ males had less survival than sugar-fed males, they performed better than the males of other treatments. Treatment with ME increases the fitness of males as it has been reported that ME enhances the protein content of ME-treated males (Reyes-Hernández et al., 2019). Although sugar-fed males had a better survival rate, they were sexually uncompetitive as has been reported previously in this study. The males have a limited supply of sperm, and they can inseminate only a few females during their lifetime, therefore the males that are sexually competitive with wild males are more desirable than long-surviving uncompetitive sterile males. A pre-release diet can be productive for the successful application of SIT as there is evidence that the released males forage for food resources available in nature irrespective of their prerelease diet (Shelly & Kennelly, 2002; Barry et al., 2003; Maor et al., 2004; Yuval et al., 2007; Gavriel et al., 2010).

In most AWP-IPM programmes involving SIT the males are held for 4-5 days in sterile males emergence and release facilities and during this period the males may be supplemented with a protein diet and treated with ME. In the current study, the males had access to dietary protein for 5 days and were treated with ME on day 5. Holding the males for 5 days and feeding on dietary protein may be enough for *B. zonata* males to achieve a certain developmental threshold and such a male holding period is also compatible with the current system of sterile male holding and release system. However optimum days required for protein feeding and their effect on survival may further be investigated.

The present study also indicated that ME-aromatized males had a significantly better survival rate than ME-fed males. For comparison prior studies are not available on ME treatment affecting the survival of *B. zonata* males. Low survival of ME-fed males may be attributed to the toxic effect of ME-feeding. Steiner (1952) observed that the males kill themselves by overfeeding ME while Chambers et al., (1972) also described ME as rather toxic for *B. dorsalis* males. Haq et al. (2018) observed 10-15% mortality in *B. dorsalis* ME-fed males than in ME-aromatized males. Similarly Wee & Tan (2007) reported high mortality in *B. carambolae* ME-fed males. The males overconsume ME due to abundant availability during ME-feeding that induces toxicity. The cost of detoxifying ME is metabolically high and deprivation of food may have resulted in low survival of the males. However, the ME-aromatized males might have received only the required quantity of ME to achieve satiation. The study showed that ME-aromatherapy can increase the cost-effectiveness of SIT due to better survival of ME-aromatized males.

The study was conducted under controlled conditions on a small scale, further studies may be carried out in larger field cages to assess the survival of the released males under seminatural conditions.

### *5.4.1 Conclusion*

Access to a protein diet is necessary for males to gain the mating advantage. ME application by aromatherapy is practicable in SIT operational programmes and enhances *B. zonata* male mating success parallel to ME feeding. Feeding the males on dietary protein for 5 days of age, treating them with ME by aromatherapy and the next morning depriving them of food and water had no adverse effect on male survival compared to non-ME-treated males. ME-aromatized males showed better survival than ME-fed males, which supports the release of ME-aromatized males in the field.

# Chapter 6: Conclusion

In the current study, necessary parameters for enhancing the efficiency of SIT application were studied and the package developed demonstrated that SIT applications will have enhanced cost-effectiveness.

The results indicated that

1. The tendency of a proportion of the males to visit ME source before mating initiation age has implications for the success of MAT for *B. zonata* management. ME treatment of the males at the immature age of 5 days indicated that the effect of ME persisted, and males gained the mating advantage after reaching sexual maturity. Treating males with ME at 5 days of age is compatible with the current sterile male emergence and release system and can enhance the effectiveness of SIT.
2. A system for delivering ME by aromatherapy, which appears practical in SIT facilities, was developed for treating *B. zonata* males. ME application by aromatherapy enhances *B. zonata* male mating success parallel to ME-feeding. ME-aromatherapy at 5 days (immature) of age enhanced male mating success after attaining sexual maturity. The ME-aromatized males exhibited a reduced tendency to revisit ME-sourced traps, which enables the application of MAT and SIT together. The combined application of both techniques will enhance the cost-effectiveness of *B. zonata* management programmes.
3. The study indicated that access to a protein diet is necessary for males to gain mating advantage. The male treatment with ME and dietary protein act synergistically for mating performance enhancement of *B. zonata* males. Prerelease feeding of males on dietary protein for 5 days after emergence and ME treatment can enhance their mating success.
4. Feeding the males on dietary protein for 5 days of age, treating them with ME by aromatherapy and depriving them of food and water did not affect male survival adversely as compared with ME-fed males. ME-aromatized males showed better starvation survival compared with ME-fed males which supports the release of ME-aromatized males in the field.

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# PUBLICATIONS FROM THESIS

1. Rasool A, Munis MFH, Shah SH, Fatima S, Irshad A & Haq Iu (2023) Age-dependent effect of methyl eugenol on male mating success of the peach fruit fly, *Bactrocera zonata*. Entomologia Experimentalis et Applicata 171: 838–845.
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Entomologia Experimentalis et Applicata

ORIGINAL ARTICLE

# Age-dependent effect of methyl eugenol on male mating success of the peach fruit fly, *Bactrocera zonata*

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**Abstract**

The peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae), is a pest of economic importance, endemic to South and South-East Asia. It is highly invasive and due to its quarantine pest status, it interferes with horticultural trade. Males of many *Bactrocera* species are strongly attracted to methyl eugenol (ME) [1,2-dimethoxy-4-(2-propenyl) benzene], a phenylpropanoid compound naturally occurring in many plant species. Feeding on ME is also reported to enhance male mating success in several *Bactrocera* species. Such an effect can increase the effectiveness of the sterile insect technique (SIT). The current study was designed to identify the sexual maturity age of males of *B. zonata*, the relation between male age and attraction to ME, and the effect of ME on male mating success. The results showed that males initiated their mating at 8 days of age and attained the highest mating success at 16 days of age. The percentage of immature males responding to ME increased with age and the maximum response was observed during the age of sexual maturity. Methyl eugenol treatment at sexual maturity significantly enhanced male mating success over untreated males. The males treated with ME at a sexually immature age (5 days old) achieved significantly higher mating success after reaching sexual maturity. These findings suggested that *B. zonata* males can be treated with ME at the age of 5 days in sterile-male 'holding and release' facilities and thereafter released in the field, where they are expected to achieve higher mating success after attaining sexual maturity. The results are discussed as a valid approach for enhancing the effectiveness of SIT application against *B. zonata*.

**KEYWORDS**

attraction, *Bactrocera zonata*, Diptera, invasive, mating success, methyl eugenol (ME), peach fruit fly, phenylpropanoid, quarantine pest, SIT, sterile insect technique, Tephritidae

## INTRODUCTION

Methyl eugenol (ME) [1,2-dimethoxy-4-(2-propenyl) benzene], a phenylpropanoid compound found in more than 450 plant species (Tan & Nishida, 2012), is a strong attractant for males of several tropical tephritid fruit fly species of the genera *Bactrocera* and *Dacus* (Howlett, 1912; Drew, 1974, 1989; Cunningham & Suda, 1985; White & Elson-Harris, 1992; Shelly et al., 2010). Methyl eugenol mixed with insecticide has been extensively used for managing *Bactrocera* fruit flies populations by luring and killing the males, which is known as the male annihilation technique (MAT) (Steiner et al., 1965; Vargas et al., 2010). In certain cases, where the MAT failed to eradicate the wild population, the sterile insect technique (SIT) has been applied (Steiner et al., 1970; Koyama et al., 1984, 2004; Pérez-Staples et al., 2021).

The SIT is an environmentally benign technique that involves the mass-rearing of male insects, their sterilization by ionizing radiation, and release over the target area in numbers large enough to outcompete their wild counterparts. Wild females that mate with sterile males produce no offspring and the sustained releases of sterile males in adequate numbers can reduce the wild population (Knipling, 1955). The application of SIT as a component of area-wide integrated pest management (AW-IPM) programs has been proven successful in suppressing the

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# Methyl eugenol aromatherapy: a delivery system facilitating the simultaneous application of male annihilation and sterile insect technique against the peach fruit fly

**Awais Rasool,[a,b] Sehar Fatima,[b] Said Hussain Shah,[b] Muhammad Farooq Hussain Munis,[a*] Afshan Irshad,[c] Todd E. Shelly[d] and Ihsan ul Haq[b*]**

## Abstract

**BACKGROUND: The peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) is an economically important polyphagous, quarantine pest endemic to South and South-East Asia. The male annihilation technique (MAT) and the sterile insect technique (SIT) are environmentally benign techniques used to suppress fruit fly populations on an area-wide basis. The MAT and SIT are typically used sequentially to avoid killing released sterile males; however, MAT and SIT potentially could be used simultaneously and thereby increase the overall efficiency of control programmes. Mating competitiveness of sterile males against wild counterparts is critical for the success of the SIT. Feeding on a semiochemical, methyl eugenol (ME) has been reported to enhance the male mating performance of many *Bactrocera* spp., including *B. zonata*, but its use in SIT operational programmes is limited owing to the absence of a viable delivery system.**

**RESULTS: In the present study, we demonstrated that ME aromatherapy, a practical method for large-scale delivery of ME olfactorily, enhances the mating success of treated *B. zonata* males. ME aromatherapy application to 5-day-old immature males for a duration of 5 h resulted in increased mating success of males tested when sexually mature, compared to untreated males. The ME-aromatized males also exhibited reduced attraction to ME-lure.**

**CONCLUSION: A practical delivery system for applying ME by aromatherapy to mass-reared males was developed. ME-aromatherapy enhanced male mating success and suppressed their subsequent attraction to ME, thus enabling the application of MAT and SIT at the same time.**


**Keywords:** peach fruit fly; Semiochemical; methyl eugenol; simultaneous application of MAT and SIT

## 1 INTRODUCTION

Tephritid fruit flies (Diptera: Tephritidae) are devastating insect pests of horticultural crops that cause damage by feeding on fruits and interfere with horticultural trade as a consequence of their quarantine pest status.[1–5] Few insect species impact international trade in horticultural products more than tephritid fruit flies.[6] The application of synthetic insecticides has largely failed to suppress fruit fly populations. Furthermore, increased restrictions on the use of synthetic insecticides as a result of their harmful effects on human health and the environment warrant the exploration of alternative methods that are effective as well as environmentally friendly.

The male annihilation technique (MAT) is an environmentally benign technique used against *Bactrocera* spp. that involves luring males to certain chemicals, that is, methyl eugenol (ME) mixed with a toxicant. Subsequent ingestion of this mixture is lethal to the males. ME (1,2-dimethoxy-4-(2-propenyl) benzene) is a phenylpropanoid compound that occurs naturally in >450 plant species and is highly attractive to males of certain *Bactrocera* spp.[7,8] Owing to this powerful attraction, ME has been used for population monitoring and population suppression.[9,10] For example, ME-based MAT was used to eradicate populations of *Bactrocera dorsalis* (Hendel),

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