Oviposition deterrents for the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae) from fly faeces extracts

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Abstract

After oviposition, females of the Mediterranean fruit fly *Ceratitis capitata* Wiedemann deposit a host-marking pheromone on the fruit surface that deters oviposition by conspecifics. Methanolic extracts of fruit fly faeces elicit a similar deterrent effect. The results of laboratory and field experiments using raw methanolic extracts of *C. capitata* faeces as an oviposition deterrent are reported. Laboratory bioassays revealed a significant positive relationship between concentration of faeces and the inhibition of oviposition responses by *C. capitata*. Treatment of halves of coffee bushes with methanolic extracts containing 0.1, 1.0 and 10 mg faeces ml^{-1} resulted in a significant reduction of infestation only at the highest concentration ($P = 0.03$). Treatment of blocks of coffee bushes with an extract of 10 mg faeces ml^{-1} resulted in an 84% reduction in infestation by *C. capitata* in sprayed plants and a 56% reduction in adjacent untreated coffee bushes surrounding treated plots, probably due to the deterrent effect of host-marking pheromone on fly oviposition. We conclude that faeces contain oviposition deterrent substances that effectively reduce fruit infestations by *C. capitata*, suggesting a clear potential for the use of this infochemical in integrated management programmes targeted at this pest.

Keywords: *Ceratitis capitata*, Tephritidae, oviposition deterrence, infochemicals

Introduction

After ovipositing in a plant, many phytophagous insects leave behind chemical markers. These markers, known as host-marking pheromones (HMP) (Prokopy, 1981; Nufio & Papaj, 2001), indicate to other females of the same species that the oviposition resource has already been occupied. Females landing on such a resource usually reject it as a site for their own eggs. These chemical signals may be secreted at the time of oviposition, as seen in some Lepidoptera and in the anthomyiid fly *Hylemya* sp. (Zimmerman, 1979;

Schoonhoven *et al.*, 1990; Gabel & Thiery, 1994), after depositing the eggs, as seen in tephritid flies (Averill & Prokopy, 1989), or are emitted by some larval secretions or the larval frass, as seen in some lepidopterans and chrysomelids (Williams *et al.*, 1986; Hilker, 1989; Hilker & Klein, 1989).

Host-marking pheromones and their oviposition-deterring activity have been studied in several tephritid species of economic importance (Roitberg & Prokopy, 1987; Averill & Prokopy, 1989). This infochemical (*sensu* Dicke & Sabelis, 1988) induces a complex series of responses in female fruit flies that include oviposition deterrence (Averill & Prokopy, 1987), reduction of clutch size and the number of clutches per fruit (Papaj *et al.*, 1989a, 1990), and movement away from heavily infested oviposition resource patches (Roitberg *et al.*, 1982, 1984). Such characteristics make the HMP an attractive tool for fruit fly control (Prokopy, 1981).

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The use of these infochemicals as a method of reducing fruit infestation by tephritid flies was first tested by Katsoyannos & Boller (1976, 1980). Using semi-purified pheromone extracts of the European cherry fruit fly *Rhagoletis cerasi* (Linnaeus), these authors observed a ten-fold reduction in fruit infestation in cherry orchards. Later, Boller & Hurter (1985) reported that faeces of *R. cerasi* females resulted in similar behavioural and electrophysiological responses as those produced by HMP extracts. Using behavioural bioassays, electrophysiological tests and chemical purification and separation, the main component of the HMP of *R. cerasi* from female fly faeces was identified and synthesized (Hurter *et al*., 1987; Ernst & Wagner, 1989). Further studies revealed that female flies responded in a comparable way to fruit treated with raw extracts or with a synthetic derivative (Boller & Aluja, 1992; Aluja & Boller, 1992a). Field tests also proved that the synthetic pheromone effectively reduced fruit infestation in cherry trees (Aluja & Boller, 1992b).

Until now, the use of synthetic HMP as a novel pest management tool has been successfully tested only in the stenophagous species, *R. cerasi* (Aluja & Boller, 1992b). The discovery of a host-marking pheromone that deters oviposition in the cosmopolitan and polyphagous Mediterranean fruit fly (medfly), *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Prokopy *et al*., 1978a), has stimulated studies to develop a similar control method for this important pest (Boller *et al*., 1994). However, the use of this type of infochemical for control of *C. capitata* has been questioned, especially because females reuse oviposition sites and use HMP marks as a cue to localize the oviposition puncture (Papaj *et al*., 1989b; Papaj, 1994; Díaz-Fleischer *et al*., 2000).

In the present study, the effect of methanolic faecal extracts on the oviposition behaviour of *C. capitata* was examined. The first objective was to determine a dose of faecal extracts that effectively reduced the degree of infestation in coffee berries. In comparison to earlier studies, a more extensive trial was conducted by treating entire plots of plants instead of branches or single plants. This was done in an attempt to better understand the foraging and oviposition decision making by female flies in response to plants treated with oviposition deterrents and its implications in fruit fly control.

Materials and methods

Study sites

The study was undertaken at the laboratories of the MoscaMed/MoscaFrut Rearing Facilities, Metapa de Domínguez, Chiapas State, Mexico. Insects were maintained at a temperature of $25 \pm 1°C$ and a relative humidity of $60 \pm 10\%$, and exposed to a 12:12 h light/darkness cycle. Field experiments were carried out at the San José plot of the 'Mujuliá' coffee plantation located at Colomba Costa Cuca, Department of Quetzalténango, Guatemala. The plantation is located at 1400 m above sea level (14°4'N, 91°47'W). Experiments were performed during the coffee fruiting season. The dose-response experiment was conducted in November 1994, and the trial using whole plots of plants took place in November and December 1996.

Host-marking pheromone extracts

Faeces of *C. capitata* adult females from the MoscaMed mass rearing facility were used to obtain raw extracts. Extraction was performed according to the method described by Díaz-Fleischer *et al*. (2004) for the HMP of *Anastrepha ludens* (Loew) (Diptera: Tephritidae). The method was slightly modified from that of Boller & Hurter (1985). Both methods have successfully used fly faeces to obtain, isolate and identify the HMPs of *R. cerasi* and *A. ludens* (Hurter *et al*., 1987; Ernst & Wagner, 1989; Aluja *et al*., 2003). These methods consist of introducing large numbers of flies ($\sim$3000 individuals) into $30 \times 30 \times 30$ cm glass cages. After four weeks, when most of the flies were dead, the faeces were scraped from the cage walls using razor blades. Extracts were prepared using absolute methanol (J.T. Baker, Xalostoc, Mexico) as solvent. This mixture was manually shaken for 20 min and then centrifuged at 12,000 rpm for another 20 min. After this process, the active liquid fraction was decanted from the solids. For this study, a standard solution of 100 mg of fly faeces ml^{-1} methanol was obtained. This solution was diluted in water to the equivalent of 10, 1.0 and 0.1 mg ml^{-1} of solvent before it was applied to plants. Pheromone solutions were applied by means of a manual knapsack sprayer.

Study insects

Wild *C. capitata* flies were obtained from infested coffee berries (*Coffea arabica* L.) collected at the 'Mujuliá' coffee plantation, Guatemala, and taken to the laboratory. All fruits were placed in baskets containing vermiculite to provide adequate pupation substrate (Aluja *et al*., 1987). Upon eclosion, females were held together with males in $30 \times 30 \times 30$ cm screened cages with access to water and food *ad libitum* until they were 15–20 days old. Food consisted of a mixture of three parts sugar to one part hydrolysed yeast protein (ICN Biochemicals, Aurora, Ohio, USA). Laboratory conditions were $26 \pm 1°C$, $60 \pm 15\%$ relative humidity, and 12 h light/12 h darkness. Details of fly holding conditions for laboratory and seminatural HMP experiments are described in Boller *et al*. (1994).

Laboratory bioassay

To evaluate faecal extracts, we used the bioassay designed by Boller & Aluja (1992). This involved a $30 \times 30 \times 40$ cm plexiglas cage with an open side that faces the operator. The opening is closed with a clear Kleen-Pack$^®$ film that adheres to the cage wall. Fresh coffee branches with approximately 15 leaves were placed on both lateral sides of the cage in order to provide resting sites for the flies. A wooden platform ($27 \times 27 \times 2$ cm) was placed in the cage bearing 30 holes (13 mm diameter) drilled in a hexagonal arrangement (4 cm apart) to support 6 cm tall water-filled glass vials. A single three-quarters ripe coffee berry was placed on each vial. Fifteen berries were treated with one of the fly faeces solutions whereas the remaining berries (control) were treated with methanol–water solutions alone.

Each bioassay lasted 60 min. Landings on fruits, oviposition attempts and aculeus draggings (indicating ovipositions) were registered. The biological activity was evaluated by calculation of the discrimination coefficient (DC) (Boller & Hurter, 1985; Boller & Aluja, 1992). The total number of
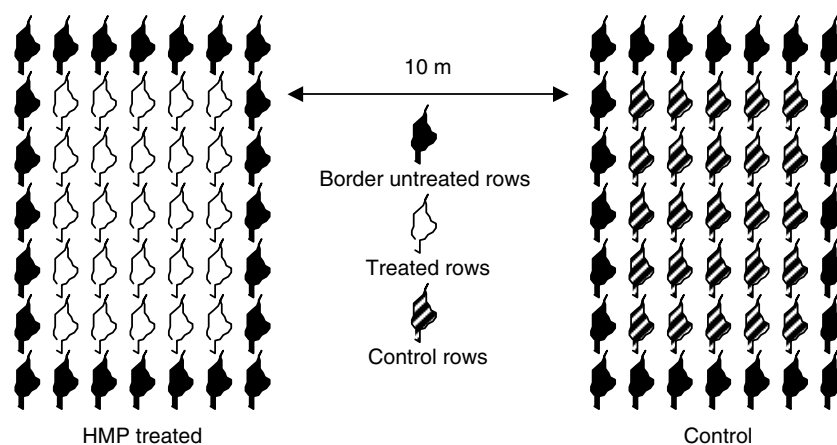
Fig. 1. Layout of coffee plant plots and distribution of treatments.

successful ovipositions observed was divided by the total number of landings on the respective category of fruits (i.e. treated or untreated berries) to correct for an unequal number of landings on the two fruit types. The resulting values were directly compared by using the DC as follows:

$$DC = \frac{A-B}{A+B} \times 100$$

where A = ovipositions into control fruits and B = ovipositions into treated fruits. DC values vary between 0 and 100. DC = 100 indicates high biological activity of the extract and full discrimination. DC = 0 indicates complete absence of biological activity (Boller & Hurter, 1985).

Effect of methanol on plant foliage

To determine whether methanol damages plant foliage and in consequence affects fly oviposition behaviour, 28 coffee plants were chosen at random within a plot of 100 plants (10×10 plants). Fourteen plants were sprayed twice at a ten-day interval with a $10\,\text{mg ml}^{-1}$ methanol–water solution. Five days after the second spraying, the 28 plants were covered with cages made of nylon gauze (1.0 m in diameter by 1.5 m in length). Each plant received five artificial hosts. Half of the hosts (70 spheres) were treated with a $10\,\text{mg ml}^{-1}$ methanolic pheromone solution; the other 70 hosts were treated with absolute methanol. Hosts and plants were distributed in a factorial design (treated plant × treated host) to obtain seven replicates of each combination. Artificial hosts consisted of agar spheres (Bacteriological agar, Sigma Chemical Co., St Louis, Missouri, USA) wrapped in Parafilm™ (American National Can, Neenah, Wisconsin, USA) (Boller, 1968). Agar was coloured with green food dye (McCormick, Herdez, Mexico). These oviposition devices simulated a natural host, but without the chemical and morphological variation usually found among fruits (Freeman & Carey, 1990). Twelve-day-old, sexually mature C. capitata flies were released into each enclosure at a density of two male + female pairs per host and left for 24 h with food and water. After this time, artificial hosts were taken to the laboratory where they were dissected to determine the number of eggs per host.

Treatment of plant halves

Twenty coffee plants within a plot of 100 plants (10×10 plants), were selected at random for the experiment. Each treatment was applied to five plants. These plants were sprayed twice, at an interval of ten days. Half of the branches were treated with one of three faecal extract solutions (10, 1.0 and 0.1 mg faeces ml^{-1}). Five plants were left unsprayed as a control treatment. Application was performed when most of the coffee berries were three-quarters ripe (turning yellow). This stage of fruit ripeness is the most susceptible for fly oviposition (Abasa, 1972). Coffee berries were collected 20 days later when they were mature (turned red) and taken to the laboratory for dissection to determine the number of C. capitata larvae per fruit. Control plants were not sprayed with an equivalent water–methanol solution because the field-cage experiment demonstrated that spraying tree foliage with the highest $10\,\text{mg ml}^{-1}$ methanol–water mixture did not induce significant changes in the oviposition behaviour of C. capitata flies. Additionally, previous studies reported that, once evaporated, methanol has no effect on the behaviour or on the receptor cell activity of fruit flies (Boller & Hurter, 1985; Boller et al., 1994; Städler et al., 1992; Díaz-Fleischer et al., 2004).

Treatment of plots of coffee plants

Eight plots containing 49 coffee plants each, in a square arrangement (7×7 bush rows), were selected at random. Four plots were treated with a $10\,\text{mg ml}^{-1}$ faecal extract solution, the other four plots were used as controls. To test the effect of HMP on neighbouring plants, only the 25 plants in the centre of the treated plot, rows 2 to 5, were sprayed. Control and treated plots were 10 m apart (approximately 10 rows) (fig. 1).

As in the first experiment, plants were sprayed twice in a ten-day interval. The first application was performed when berries were three quarters ripe. Samples of 50 fruits per plant were harvested 20 days later once they reached full ripeness. Fruits were taken to the laboratory, counted and weighed. Coffee berries from rows and columns 1 and 7 (borders rows) were also sampled. After one week, all fruits were dissected to count C. capitata larvae.
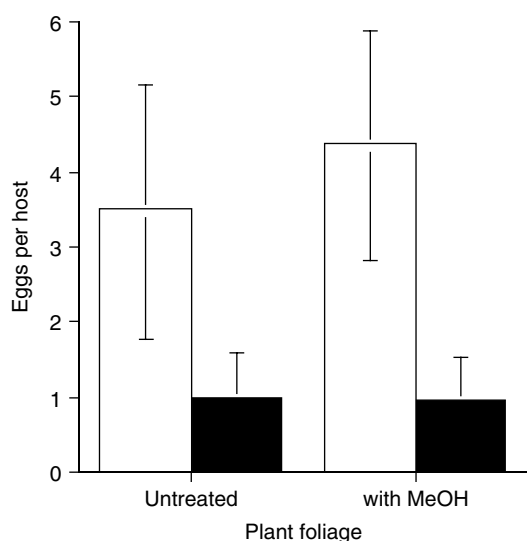
Fig. 2. Average eggs per host ($\pm$ SE) of artificial hosts (agar spheres) treated with $10\,mg\,ml^{-1}$ raw extracts of host-marking pheromone of *Ceratitis capitata* (■) and an untreated control (□) exposed on coffee plants whose foliage was sprayed or not with a $10\,mg\,ml^{-1}$ methanolic solution.

Adult fly population density was monitored during the entire experimental period with Jackson traps (one per plot) baited with trimelure. Traps were checked and lures replaced on a weekly basis.

Statistical analyses

A Z-test (one sample t-test) was used to determined whether discriminant coefficient values, calculated from the laboratory bioassay results, were significantly different from zero (Zar, 1984). For the experiment in which half of a plant was treated, data were subjected to a one-way ANOVA. Fruit infestation within plants (sprayed and unsprayed halves) was compared by means of paired t-tests. Data of the effect of 10% methanol-water solution on plant foliage experiment were analysed using a two-way ANOVA. A complete random block ANOVA was used to analyse the whole-plot experiment (Abacus Concepts, Inc., 1991). Data on infestation (i.e. larvae per fruit) were transformed to arcsin $\sqrt{x+0.5}$ for statistical analysis (Zar, 1984). To facilitate interpretation, all tables and figures show untransformed values. Post-hoc analyses were performed using Fisher's LSD Test. To measure the efficacy of a treatment, the Abbot index was used (Abbot, 1925).

Results

Laboratory bioassay

The $10\,mg\,ml^{-1}$ faeces solution exhibited high biological activity, with a mean DC of 69 ± 9.5 (mean $\pm$ SE) and this was significantly different from zero (Z test, t = 7.3; df = 4; P = 0.001). The $1\,mg\,ml^{-1}$ faeces solution showed a lower biological activity, DC = 38.8 ± 2.5 which was significantly greater than zero (Z test, t = 15.6; df = 4; P = 0.0001). The $0.1\,mg\,ml^{-1}$ solution displayed the lowest biological activity,
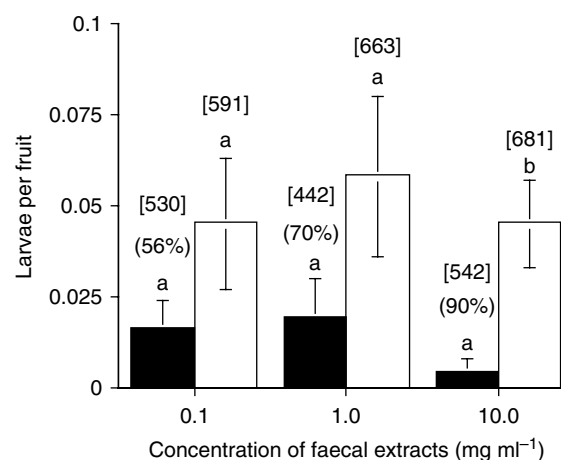

Fig. 3. Effect of treating with three concentrations of host-marking pheromone, sprayed on one side of the plant, on coffee berry infestation by *Ceratitis capitata* (■, treated part; □, untreated part). Columns represent mean ($\pm$ SE) infestation within plants. Columns headed by different letters are significantly different (paired t-test, $P < 0.05$)). The Abbott index of effectiveness for each treatment is given in parenthesis. Total number of collected fruits appears in square brackets.

DC = 27.6 ± 3.4 but this was also significantly different from zero (Z test, t = 7.8; df = 4; P = 0.001).

Effect of methanol solution on plant foliage

No significant differences were observed in the number of eggs oviposited by flies in plants treated with the $10\,mg\,ml^{-1}$ methanol–water solution compared to untreated plants ($F_{1,24} = 0.13$; P = 0.73). However, significant differences were found between HMP treated and untreated hosts ($F_{1,24} = 5.87$; P = 0.02). Control hosts received 3.9 ± 1.1 eggs (mean $\pm$ SE), whereas HMP-treated hosts received 0.9 ± 0.4 eggs. The interaction was not significant, indicating that plant treatment did not affect host treatment ($F_{1,24} = 0.131$; P = 0.72) (fig. 2).

Treatment of plant halves

As observed in the laboratory bioassay, the highest ($10\,mg\,ml^{-1}$) HMP concentration was the only treatment that resulted in significant effects when sprayed and unsprayed halves of the plants were compared (t = 3.4; df = 4; P = 0.03). This treatment reduced the mean number of larvae per fruit by a factor of 10 (fig. 3). No significant differences were detected for the $0.1\,mg\,ml^{-1}$ (t = 1.3; df = 4; P = 0.26) or the $1.0\,mg\,ml^{-1}$ (t = 2.7; df = 4; P = 0.06) treatments.

At the level of whole plants (both treated and untreated halves), the reduction of infestation in treated plants was only around twofold with respect to control plants. No statistical differences between treatments were detected in this case ($F_{3,16} = 0.42$; P = 0.74) (fig. 4).

The Abbot index indicated that the $10\,mg\,ml^{-1}$ solution exhibited a 90% effectiveness in reducing fruit infestation, when comparing treated and untreated parts of the plants. Nevertheless, the effectiveness of this solution was reduced
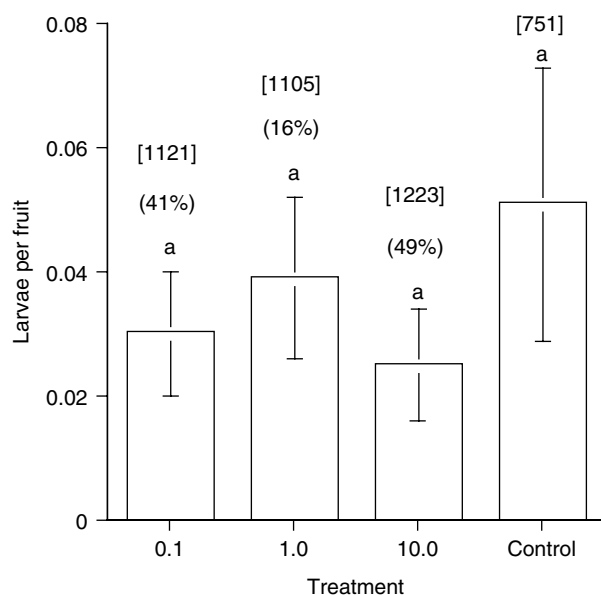
Fig. 4. Effect of treating with three concentrations of host-marking pheromone, sprayed on one side of the plant, on infestation of coffee berries by *Ceratitis capitata* (mean ± SE). Bars represent infestation in the whole plants (both sides) of the same treatment. Means accompanied by different letters are significantly different (Fisher's LSD, $P < 0.05$). The Abbott index of effectiveness for each treatment is given in parenthesis. Total number of collected fruits appears in square brackets.

to 49% when half-treated plants were compared to control plants (figs 3 and 4).

Treatments of plots of coffee plants

A total of 160 plants were sampled (76 treated and 84 control). Despite high pest densities during the study (0.654 flies per trap per day) (fig. 5), a five-fold reduction in the prevalence of infestation of HMP-treated coffee berries was observed (fig. 6). The number of larvae per fruit differed significantly between treated and untreated plots ($F_{1,141} = 7.06$; $P = 0.009$). However, no statistical differences were detected among blocks of each treatment ($F_{3,141} = 0.19$; $P = 0.91$). The Abbot index indicated that the $10\,mg\,ml^{-1}$ solution had an effectiveness of 84% in reducing fruit infestation (fig. 6).

In the case of the plants on the plot perimeter (border rows), a two-fold reduction in the degree of fruit infestation was observed in plants around the treated plots compared with plants that surrounded the control plots ($F_{1,100} = 4.29$; $P = 0.041$), and no significant differences were detected among blocks of the same treatment ($F_{3,100} = 1.99$; $P = 0.12$) (fig. 6).

Discussion

The present study provides clear evidence that methanolic extracts of *C. capitata* faeces deter oviposition resulting in a significant reduction in fruit infestation. Fruit infestation in the faeces-treated plants remained at a very low level despite the high pest population. A greater reduction in the degree of infestation of coffee berries and
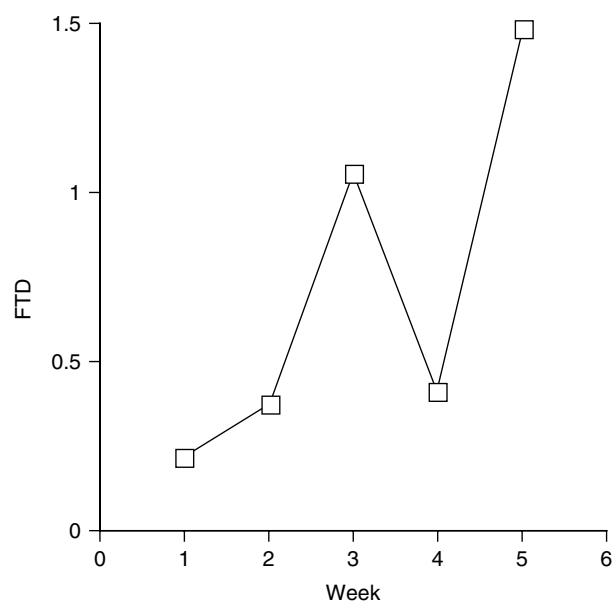

Fig. 5. FTD values (flies per trap per day) for *Ceratitis capitata* trapped during each week of the study.
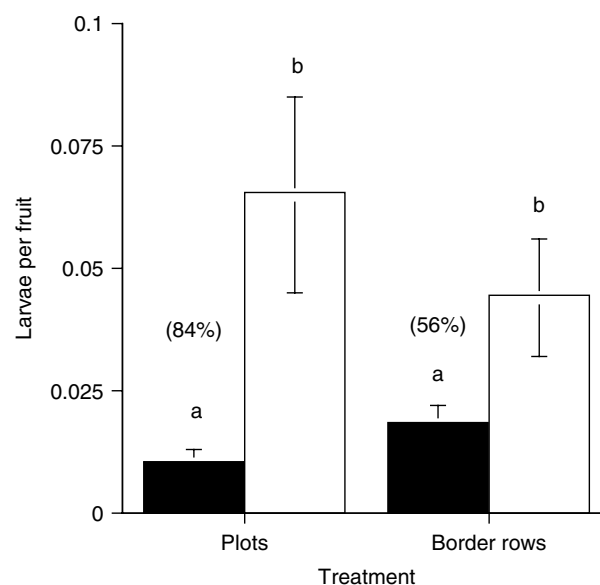

Fig. 6. Average infestation (mean larvae per fruit ± SE) of coffee berries treated with $10\,mg\,ml^{-1}$ raw extracts of host-marking pheromone of (HMP) of *Ceratitis capitata* (■) and untreated control (□) berries from plants in treated and adjacent untreated rows. Means accompanied by different letters are significantly different (Fisher's LSD, $P < 0.05$). The Abbott index of effectiveness for HMP-treated plants in plots and borders are given in parenthesis.

higher Abbot index values were obtained with the highest faeces concentration.

Discrimination coefficient values reported here are quite similar to those obtained with raw HMP extracts of female *C. capitata* natural marks (Boller *et al.*, 1994). These results are

not surprising, since extracts of *R. cerasi* faeces generate oviposition deterrent effects similar to those produced by isolated HMP (Boller & Hurter, 1985). Our field results parallel those of the European cherry fruit fly when cherries were sprayed with raw methanolic extracts of conspecific HMP (Katsoyannos & Boller, 1980). However, in contrast to *R. cerasi*, treatment of half of the coffee plant with medfly faecal extracts did not generate a significant reduction in the level of infestation of fruits over the entire plant (Aluja & Boller, 1992b). This may be attributed to differences in foraging behaviour between stenophagous species, like *R. cerasi*, and the polyphagous *C. capitata*. For example, the average duration of intra-tree flights in the stenophagous *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) tend to be far lower than observed in *C. capitata* (Prokopy *et al*., 1978b). Canopy can therefore be searched effectively by *C. capitata* foraging flies with fewer, but longer, flights than do *R. pomonella* females, and thereby increase their probability of finding untreated fruit. In the case of flies of the genus *Bactrocera*, the stenophagous species tend to exhibit more selective host oviposition behaviour than their polyphagous relatives, even after long periods of host deprivation (Fitt, 1986). This was attributable to differences in the control of oogenesis; stenophagous species control oocyte maturation, whereas polyphagous species do not appear to inhibit oocyte development (Fitt, 1986). Such differences in oogenesis lead to females with divergent egg loads and in consequence to different foraging and oviposition behaviours (Minkenberg *et al*., 1992).

Intriguingly, the degree of infestation of fruits in treated plots (0.01 ± 0.003 larvae per berry) was double that observed in treated parts of the plants in the experiment involving treating halves of plants (0.004 ± 0.004). This pattern was also observed in *R. cerasi* when comparing the degree of infestation of entire sprayed cherry trees with the degree of infestation of treated halves of trees (Aluja & Boller, 1992b). It seems that, even in the field, females of both fly species may develop a certain degree of habituation to their HMP when they experience continuous exposure to the info-chemical (Aluja & Boller, 1992a). Differences in the pattern of infestation were observed between the untreated side of the plant, in the trial involving treatment of halves of plants, and the untreated border plants that surrounded treated plants in the treated plot experiment. Fruit infestation in the neigh-bouring untreated part of the plants was comparable to the infestation in the control plants (figs 2 and 3). However, in the experiment involving treated plots, the untreated border plants (i.e. those that were close to the treated plot) exhibited fewer larvae per fruit than the control plants, indicating that the deterrent effect of the HMP was not solely confined to the plants treated with HMP. It may be that flies that visited treated plants flew far away from treated blocks. Female flies that find a high rate of infested and marked fruit exhibit a greater propensity for engaging in long distance flights i.e. ≥1000 m (Roitberg *et al*., 1984). This difference can be explained in terms of fly search and host selection behaviour. Once a female fly has landed on a host tree, she begins searching for fruits within the tree canopy and generally oviposits into unmarked fruits (Roitberg *et al*., 1982; Aluja *et al*., 1989). When most of the fruits are pheromone marked she will tend to move to a neighbouring plant in the search for unmarked hosts. However, if unmarked hosts are also scarce in the second plant she will fly farther than just to the next neighbouring plant (Roitberg *et al*., 1982, 1984).

The oviposition-deterring activity of faeces may be common among insects. For example, faeces of many lepidopteran larvae exhibit high oviposition deterrence activity (Williams *et al*., 1986; Hilker & Klein, 1989) and, comparable to the case of *R. cerasi*, it has been possible to isolate the compounds that provoke female oviposition deterrence (Anderson *et al*., 1993). Indeed, many chemical marks used in animal communication originated from digestion by-products (Bradbury & Vehrencamp, 1998). The fact that HMP is produced in the fly midgut makes it a likely product of digestion (Prokopy *et al*., 1982). However, female fruit flies only produce the active compound upon reaching sexual maturity and not before (Boller & Hurter, 1985; Städler *et al*., 1992). Additionally, males do not produce HMP (Städler *et al*., 1992). Therefore, the active compound in tephritid flies should be related to the oviposition process in an analogous way to the HMP of some moths and anthomyiids that are released at the moment of egg deposition (Zimmerman, 1979; Gabel & Thiery, 1994, 1996).

The results of the present study indicate that an oviposition-deterrent component with a biological activity similar to that of HMP methanolic raw extracts is present in faeces of *C. capitata*. Also, it can be concluded that the use of HMP can be an effective means of reducing infestations by *C. capitata*. The magnitude of reductions in infestation and the effectiveness of faeces extracts treatments (Abbot index) observed in this study are similar to those reported for the European cherry fruit fly (Katsoyannos & Boller, 1976, 1980; Aluja & Boller, 1992b). Moreover, the results of applying the extracts to whole plants and blocks of plants indicated a clear potential for the use of this infochemical in integrated pest management programmes directed at *C. capitata*. Nevertheless, additional studies are necessary to further improve effectiveness of the technique. For example, HMP must be tested on fruits larger than coffee berries since oviposition pheromone deterrence is inversely related to fruit size (Averill & Prokopy, 1989). As 50 cages of flies yielded just 250 g of faeces per month, large-scale trials will require an improved method for obtaining HMP extracts, for example, by synthesis of the active compounds, as achieved for *R. cerasi* and *A. ludens*. Unfortunately, the possibility of obtaining large quantities of HMP from female flies at the Metapa mass-rearing facility has disappeared following the introduction of a male-only strain of *C. capitata*.

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