Resistance to Malathion in Field Populations of *Ceratitis capitata*

Author(s): C. Magaña, P. Hernández-Crespo, F. Ortego, and P. Castañera

Source: Journal of Economic Entomology, 100(6):1836-1843. 2007.

Published By: Entomological Society of America

DOI: [http://dx.doi.org/10.1603/0022-0493\(2007\)100\[1836:RTMIFP\]2.0.CO;2](http://dx.doi.org/10.1603/0022-0493(2007)100[1836:RTMIFP]2.0.CO;2)

URL: [http://www.bioone.org/doi/](http://www.bioone.org/doi/full/10.1603/0022-0493%282007%29100%5B1836%3ARTMIFP%5D2.0.CO%3B2)

[full/10.1603/0022-0493%282007%29100%5B1836%3ARTMIFP%5D2.0.CO%3B2](http://www.bioone.org/doi/full/10.1603/0022-0493%282007%29100%5B1836%3ARTMIFP%5D2.0.CO%3B2)

BioOne (www.bioone.org) is a nonprofit, online aggregation of core research in the biological, ecological, and environmental sciences. BioOne provides a sustainable online platform for over 170 journals and books published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Web site, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/page/terms_of_use.

Usage of BioOne content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

Resistance to Malathion in Field Populations of *Ceratitis capitata*C. MAGAÑA, P. HERNÁNDEZ-CRESPO, F. ORTEGO, AND P. CASTAÑERA¹Departamento de Biología de Plantas, Centro de Investigaciones Biológicas, CSIC,
Ramiro de Maeztu 9, 28040 Madrid, Spain

J. Econ. Entomol. 100(6): 1836–1843 (2007)

ABSTRACT The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is considered one of the most economically damaging pests of citrus orchards in Spain. Insecticide treatments for the control of this pest are mainly based on aerial and ground treatments with malathion bait sprays. However, the frequency of insecticide treatments has been increased in some areas of the Comunidad Valenciana in the last years, because of problems with the control of *C. capitata*. We have found that field populations from citrus and other fruit crops from different geographical areas in Spain showed lower susceptibility to malathion (6- to 201-fold) compared with laboratory populations. More importantly, differences in susceptibility could be related to the frequency of the field treatments. A resistant strain (W), derived from a field population, and a susceptible laboratory strain (C) were maintained in the laboratory. The W strain showed cross-resistance to the organophosphate fenthion (10-fold) but not to spinosad. Enzymatic assays showed that acetylcholinesterase activity was less inhibited in vivo by malathion and in vitro by malaoxon (active form of malathion) in adult flies from the W-resistant strain. Experiments to evaluate the effects of synergists revealed that the esterase inhibitor S,S,S-tributyl phosphorotriothioate (DEF) partially suppressed malathion resistance. Thus, target site insensitivity and metabolic resistance mediated by esterases might be involved in the loss of susceptibility to malathion in *C. capitata*. Nonetheless, additional biochemical and molecular studies will be required to confirm this hypothesis.

KEY WORDS insecticides, citrus, acetylcholinesterase, synergists, esterases

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most destructive pests of fruits in the world, attacking >250 species of fruits and vegetables (Liquido et al. 1991). In Spain, this fly is considered one of the most economically damaging pests of citrus orchards. Direct losses result from the oviposition in fruits, larval activity, and eventual infection by fungi (Metcalf and Metcalf 1993). In addition, quarantine measures are required for exportation to fly-free areas. The control of *C. capitata* in the Comunidad Valenciana, which is the main area of citrus production in Spain, is mainly based on field monitoring of the population levels and aerial and ground treatments with malathion bait sprays (Primo-Millo et al. 2003).

Resistance to malathion in the field has been reported for a number of insects, including some dipteran pests (Hughes et al. 1984, Hemingway 1985, Raghavendra et al. 1998, Hsu and Feng 2000). However, no resistance to malathion has been reported yet for field populations of *C. capitata* (Wood and Harris 1989, Viñuela 1998), despite being the most widely used insecticide for the control of this pest. It has been suggested that this may be because of incomplete

selection pressure of the insecticide being used, caused by the mobility and broad host range of *C. capitata* (Georghiou 1972, Orphanidis et al. 1980). Nonetheless, Koren et al. (1984) reported the potential of *C. capitata* in developing resistance to malathion after 18 generations of selection in the laboratory. Besides, the frequency of insecticide treatments, in the last years, has been increased in some areas of the Comunidad Valenciana to maintain the effectiveness, which may be interpreted as the result of increased tolerance to malathion.

The aim of this study was to find the susceptibility to malathion of Spanish field populations of *C. capitata* that have been subjected to different selection pressures during the last years. Effects of synergists, acetylcholinesterase activity, and cross-resistance to other insecticides were also evaluated in a resistant and a susceptible strain.

Materials and Methods

Field Sampling. *Ceratitis capitata* adults were obtained from larvae-containing infested fruits collected in fruit orchards in 2004 or 2005. Sampling was performed at seven localities in the Comunidad Valenciana (Burriana, Castellón, Villareal, Carlet, Alcudia,

¹ Corresponding author, e-mail: castan@cib.csic.es.

Fig. 1. Collection sites of *C. capitata* in the Comunidad Valenciana (Burriana, Castellón, Villareal, Carlet, Alcudia, Serra, and Orihuela), Cataluña (Bajo Ampurdan), and La Rioja (Albelda).

Serra, and Orihuela), one in Cataluña (Bajo Ampurdan), and one in La Rioja (Albelda) (Fig. 1). They were chosen based on representative hosts (citrus and stone fruits in the Comunidad Valenciana and apples in Cataluña and La Rioja), and the insecticide regimens that were applied in these areas (Table 1).

The infested fruits collected in the fields were placed in plastic trays inside 51 ventilated containers, both with a layer of vermiculite or arlite. They were kept in an environmentally controlled rearing room, at a photoperiod of 16-h light and 8-h dark, and a temperature of $26 \pm 3^\circ\text{C}$, until pupation occurred. Emerging adults from field-collected fruits (F_0) were used for testing their susceptibility to malathion.

Laboratory Populations. Two laboratory populations were analyzed, Lab-INIA and Lab-IVIA. Lab-INIA was initially established at Instituto Nacional de Investigaciones Agrarias (INIA; Madrid, Spain) and has been maintained for ≈ 40 yr in laboratory conditions. Lab-IVIA was established at Instituto Valenciano de Investigaciones Agrarias (IVIA; Valencia, Spain) from wild *C. capitata* collected at nontreated experimental fields in 2001.

According to results obtained in susceptibility bioassays and the availability of insects, the susceptible strain Lab-IVIA (C) and a resistant strain established from the field population collected in Castellón (W) were maintained in the laboratory for further studies.

Lab-IVIA (C). The C strain has been maintained in our laboratory for the last 3 yr. The adults were kept in rearing cages containing $\approx 2,000$ individuals and fed with adult diet (4:1:0.1 glass sucrose: hydrolyzed yeast: water). Water was supplied through a sponge wick fed from a water reservoir. The cages were cubical in shape (20 by 20 by 20 cm) with gauze on one side, through which the flies laid eggs that were collected in a try with water located below the cage. The cages were kept in an environmentally controlled rearing room at the conditions indicated above. The collected eggs were seeded at a density of 0.15 ml of eggs in a container with 250 g of larval rearing diet (Albajes and Santiago-Álvarez 1980). The diet was partially covered with aluminum foil to avoid desiccation and placed in 21 plastic containers with vermiculite at the bottom to allow third-instar larvae to emerge from the food medium and pupate. Six days before the eclosion (~ 14 d after seeding the eggs), pupae were sieved from the vermiculite and placed in the adult rearing cages described above.

Castellón Strain (W). The W strain has been maintained in our laboratory for the last 3 yr. Adults were reared as described for the C strain, but apples were regularly introduced (one or two per day) to the adult rearing cages as substrate for oviposition. Apples were taken from the cages every 1–2 d and held on trays as described above. Every generation, except the sixth, the W strain was treated with malathion to maintain the selection pressure. Approximately 1,000–2,000 adults (3–5 d old) were treated with a concentration of malathion between 1,000 and 3,000 ppm for 24–48 h to obtain $\approx 50\%$ mortality.

Bioassays. The arena for the bioassays consisted of ventilated plastic dishes (89 mm diameter, 23 mm high). Ten to 20 pupae were confined per plastic dish, and the emerged adults were fed with water and adult diet for 3–5 d before testing. The insecticide was tested as follow: mixed with the diet (feeding bioassay), applied directly on the insect (topical application), or in combination with synergists (bioassays with synergists). Three to six different concentrations of insecticide with a partial response (mortality $>0\%$ and $<100\%$) were used, and two to four replicates were performed for each concentration, depending on the availability of insects. Adults flies were kept in an environmentally controlled rearing room during the

Table 1. Year of sampling, host, and spraying regimens of the fruit orchards where *C. capitata* infested fruits were collected

Area	Locality	Year	Host	Malathion treatments against <i>C. capitata</i>
Comunidad Valenciana	Burriana (Castellón)	2004	Medlar	8–9 a.t. per year in 2003 and 2004
	Castellón (Castellón)	2004	Citrus	8–9 a.t. and 2–4 g.t. per year in 2003 and 2004
	Villareal (Castellón)	2005	Khaki	Urban area, nontreated in the last 2–3 yr
	Carlet (Valencia)	2004	Peach	8 a.t. in 2002, 6 a.t. in 2003, and 6 a.t. in 2004 ^a
	Alcudia (Valencia)	2004	Peach	8 a.t. in 2002, 6 a.t. in 2003, and 6 a.t. in 2004 ^a
	Serra (Valencia)	2005	Citrus	Nontreated in 2001, 2002, 2003, and 2004, 1 g.t. in 2005
	Orihuela (Alicante)	2005	Citrus	9 a.t. and 4 g.t. in 2005
Cataluña	Bajo Ampurdan (Girona)	2004	Apple	1 g.t. in 2004
La Rioja	Albelda	2004	Apple	Nontreated field but close to other fruit crops treated

^a Two to three additional ground treatments with fenitrothion.

a.t., aerial treatment (every 5–7 d during the maturation period); g.t., ground treatment (every 10 d during the maturation period).

tests, under the environmental conditions indicated above. Mortality was recorded after 48 h. Flies were considered dead if they were ataxic (remained on their backs, unable to walk, with no further sign of movement).

Feeding Bioassays. Feeding bioassays were used to assess the susceptibility of field and laboratory populations to malathion and to examine cross-resistance to fenthion and spinosad in the C and W strains. The insecticides used are commercial formulations approved for the control of *C. capitata* for citrus crops in Spain: Malafin 50 EC (Agrodan, Madrid, Spain), an emulsifiable concentrate of malathion 50% (wt:vol); Lebaycid LE (Bayer CropScience, Valencia, Spain), an emulsifiable concentrate of fenthion 50% (wt:vol); and Spintor Cebo (Dow AgroSciences Ibérica, Madrid, Spain), spinosad 0.024% (wt:vol). They were diluted in water and mixed with the diet to obtain a range of concentrations. Three to 5 d after emergence of adults, the rearing diet was replaced by diet containing the appropriate concentration of insecticide. The control consisted of diet mixed with water.

Topical Application. Topical application was used to determine contact toxicity to malathion in the C and W strains. Malafin 50 EC was diluted in acetone to obtain a range of doses. Adult flies (3–5 d old) were maintained at 4°C for 30 min; thereafter, a 0.5- μ l drop of acetone solution of Malafin or acetone alone (control) was applied to the dorsal thorax of each fly by using an automatic microapplicator 900 $\times$ (Burkard Manufacturing Co., Hertfordshire, United Kingdom). The treated insects were placed in the ventilated plastic dish containing water and the rearing diet. Thirty randomly selected adults were weighed per bioassay to calculate the doses in relation to fresh weight (μ g insecticide/g fresh weight).

Synergists. The synergists used were as follows: S,S,S-tributyl phosphotriethioate (DEF; 98% technical) as an esterase inhibitor, obtained from Chem Service (West Chester, PA); triphenyl phosphate (TPP; 98% technical) as an inhibitor of ali-esterases, obtained from Fluka (Madrid, Spain); piperonyl butoxide (PBO; 90% technical) as a cytochrome P450 (mooxygenases) inhibitor, obtained from Aldrich (Milwaukee, WI); and diethyl maleate (DEM; 97% technical) as an inhibitor of glutathione-S-transferase, obtained from Aldrich. Three micrograms of PBO, 1 μ g of DEM, 1 μ g of DEF, or 5 μ g of TPP were used per insect. These were the highest doses of the synergists that showed no mortality on adults from the C strain. Synergists were diluted in acetone and topically applied as described above. Acetone was used as control. After 2 h, the flies were treated with malathion as described in the feeding bioassay.

Data Analysis. Mortality data were used to determine their susceptibility to malathion (LC_{50} with a 95% CI) by probit analysis using the computer program POLO-PC (LeOra Software 1997), which automatically corrected for control mortality by Abbott's transformation. The significance of differences in susceptibility was tested by the 95% CL of lethal concentration ratios (LCRs) at the LC_{50} (Robertson and

Preisler 1992). The relative toxicity of malathion with respect to malathion plus the synergist (synergistic ratio) was calculated using the computer program POLO-PC, and the significance of the effects was tested by the 95% CL of the synergistic ratios at the LC_{50} .

Enzymatic Assays. Heads from adults (3–5 d old) from C and W strains were homogenized with a glass homogenizer in 300 μ l of 0.15 M NaCl containing 1% (vol:vol) of Triton X-100. Solubilized protein was isolated by centrifugation at 12,000 rpm for 5 min in a UNIVERSAL 32 R (Hettich International, Kirchlingern, Germany) centrifuge at 4°C. The supernatants were collected and used as enzyme source.

Acetylcholinesterase (AChE) activity was determined by the spectrophotometric method described by Ellman et al. (1961), and assay conditions were optimized for *C. capitata*. The reaction mixture consisted of 10 μ l of tissue homogenate, 2 μ l of 200 mM acetylthiocholine iodide (ASChI; Sigma, St. Louis, MO), 20 μ l 10 mM 5'-dithio-bis (2-nitrobenzoic acid) (DTNB; Sigma), and potassium phosphate buffer, pH 7.2, up to 200 μ l. The reaction was started by the addition of the substrate (ASChI) and the reagent (DTNB), and the increment in absorbance at 412 nm was recorded for 5 min. Blanks were used to account for spontaneous breakdown of the substrate. The specific activity of AChE was measured as nanomoles of acetylthiocholine iodide hydrolyzed per minute and per milligram of protein, assuming a molar extinction coefficient of $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the Ellman's product (Ellman 1959).

AChE activity was examined in untreated flies from the C and W strains and after diet exposure to malathion (50 ppm). The feeding treatments were performed as explained above, and the AChE activity of single individuals (eight from each of the four groups) was determined after 48 h.

AChE in vitro inhibition assays were performed by preincubating pooled head extracts (30 adult heads from C or W strains) with 1×10^{-7} , 2.5×10^{-7} , 5×10^{-7} , 7.5×10^{-7} , 1×10^{-6} , 2.5×10^{-6} , or 5×10^{-6} M of malaoxon (active form of malathion) during 3 min at 30°C, before addition of substrate and reagent. Malaoxon was provided by Cromlab (Barcelona, Spain). A stock solution (0.1 M) was prepared in ethanol, and further dilutions in buffer were made immediately before use. The concentration of ethanol in the enzyme-inhibitor mixture was 1% (vol:vol) or less. Control samples contained no inhibitor but the same alcohol concentration. Three experimental replicates were carried out for each concentration, and AChE activity was measured as described above.

Protein concentration was determined according to the procedure of Bradford (1976) with bovine serum albumin (Sigma) as the protein standard. Protein standards were prepared in the proper buffer; maintaining the same concentration of Triton X-100 as in the samples (Friedenauer and Berlet 1989).

Table 2. Susceptibility to malathion of field and laboratory populations of *C. capitata*

Population	Field treatments with malathion	n	Slope ± SE	LC ₅₀ ^a (95% CL)	χ ²	df	LCR (LC ₅₀) ^b (95% CL)
Lab-IVIA	—	641	2.7 ± 0.3	16 (11–19)	88.6	33	1
Lab-INIA	—	367	2.9 ± 0.3	12 (10–14)	29.8	18	0.8 (0.6–1.0)
Burriana	5–10/yr	185	0.8 ± 0.1	1,376 (668–3,126)	27.1 ^c	18	88 (47–166) ^d
Carlet	5–10/yr	148	1.7 ± 0.3	3,137 (2,182–4,850)	7.4 ^c	18	201 (129–314) ^d
Orihuela	5–10/yr	136	0.8 ± 0.1	1,636 (763–3,629)	8.5 ^c	16	105 (46–241) ^d
Alcudia	5–10/yr	95	1.2 ± 0.2	1,029 (503–2,074)	14.5 ^c	13	66 (34–127) ^d
Castellón	5–10/yr	208	0.9 ± 0.1	1,874 (1,190–3,300)	20.5 ^c	22	120 (69–209) ^d
Bajo Ampurdan	1/yr	97	0.8 ± 0.2	519 (130–1,169)	23.0 ^c	23	33 (11–100) ^d
Serra	1/yr	95	0.9 ± 0.2	938 (278–2,735)	15.2 ^c	10	60 (25–143) ^d
Villareal	Nontreated	251	1.6 ± 0.2	102 (44–160)	47.7	28	6 (4–11) ^d
Albelda	Nontreated	66	1.2 ± 0.3	472 (163–1,084)	10.5 ^c	10	30 (13–72) ^d

^a Concentrations expressed in ppm of malathion in the diet.

^b LCR at LC₅₀ level of each population with respect to the laboratory Lab-IVIA population.

^c Good fit of the data to the probit model ($P > 0.05$).

^d Lethal concentration is significantly different ($P < 0.05$) if the LCR 95% CI does not include 1.

Results

Susceptibility to Malathion of Field and Laboratory Populations. The mortality of the controls was always <5%. All field populations were significantly less susceptible to the insecticide (LC₅₀ > 1,000 ppm of malathion active ingredient) than the laboratory populations Lab-IVIA (LC₅₀ = 16 ppm) and Lab-INIA (LC₅₀ = 12 ppm; Table 2). More importantly, differences in susceptibility were obtained between populations sampled in fields subjected to different frequency of treatments. Those populations collected from fields with 5–10 aerial treatments per year showed the higher levels of resistance (LC₅₀ between 1,000 and 3,000 ppm), with an LCR, with respect to the Lab-IVIA population, in the range 66–201. Populations from fields with only one treatment per year, Bajo Ampurdan, and Serra, showed LC₅₀ between 500 and 1,000 ppm, and LCR values between 33 and 60. Populations from untreated fields, Villareal and Albelda, showed LC₅₀ between 100 and 500 ppm and LCR values between 6 and 30, respectively (Table 2).

Based on their relative LCR values, field populations can be grouped according to the frequency of the treatments. The populations with the higher number

of treatments (Burriana, Carlet, Orihuela, Alcudia, and Castellón) were not significantly different between themselves, except Carlet with Burriana and Alcudia. A second group included the populations with only one treatment (Bajo Ampurdan and Serra) and the population from Albelda (collected from a nontreated field but close to other treated fields). They were not significantly different from each other and presented LCR values < 1 with respect to the populations from the first group, although these differences were not always significant. Finally, the population from Villareal, obtained from an urban area that has not been treated in the last years, was significantly different from all other populations.

Susceptibility of the Susceptible and Resistant Strains to Malathion and Other Insecticides. The susceptibility to malathion of the susceptible (C) and resistant (W) strains was assayed by ingestion and topical application (Table 3). Differences in susceptibility were obtained between both strains, independently of how the insecticide was applied. In both cases, the W strain was more resistant to malathion than the C strain, although the difference was higher by ingestion (LCR = 79-fold) than by contact application (22-fold).

Table 3. Susceptibility of the susceptible (C) and the resistant (W) strains of *C. capitata* to malathion, fenthion, and spinosad

Insecticide	Assay	Strain ^a	n	Slope ± SE	LC ₅₀ ^b (95% CL)	χ ²	df	LCR (LC ₅₀) ^c (95% CL)
Malathion	Feeding	C	162	2.3 ± 0.4	18 (11–23)	16.7 ^d	16	1
		W	210	0.9 ± 0.1	1,406 (664–3,150)	62.1	32	79 (44–141) ^e
	Topical	C	153	1.3 ± 0.2	1.2 (0.6–2.1)	24.4 ^d	15	1
		W	159	6.4 ± 1.4	27 (18–33)	39.5	17	22 (14–32) ^e
Fenthion	Feeding	C	108	1.8 ± 0.3	1.3 (0.6–2.2)	18.6 ^d	14	1
		W	144	2.6 ± 0.4	12 (10–15)	15.5 ^d	19	9 (6–16) ^e
Spinosad	Feeding	C	278	2.3 ± 0.1	0.4 (0.3–0.5)	13.7 ^d	10	1
		W	164	1.9 ± 0.4	0.7 (0.4–0.9)	27.1 ^d	18	2 (1–3)

^a The susceptible Lab-IVIA (C) and the resistant Castellón (W) strains.

^b Concentrations expressed in ppm of malathion, fenthion, or spinosad in the diet for the feeding bioassays and as micrograms of malathion per gram fresh weight for topical assays.

^c LCR at LC₅₀ level of the resistant strain with respect to the susceptible strain.

^d Good fit of the data to the probit model ($P > 0.05$).

^e Lethal concentration is significantly different ($P < 0.05$) if the LCR 95% CI does not include 1.

Table 4. Effect of synergists on the toxicity of malathion to the susceptible (C) and the resistant (W) strains of *C. capitata*

Strain	Insecticide	Synergist	n	Slope ± SE	LC ₅₀ ^a (95% CL)	χ ²	df	SR ^b (95% CL)
C	Malathion	—	319	2.4 ± 0.3	15 (11–19)	49.7	31	1
		+TPP	148	2.1 ± 0.4	6.8 (2–11)	24.8	13	2 (1.3–3.4) ^d
		+DEF	59	5.0 ± 1.6	16 (9–22)	1.0 ^c	4	1.14 (0.6–2)
		+PBO	309	2.0 ± 0.3	18 (8–29)	96.6	30	0.8 (0.5–1.2)
		+DEM	117	2.8 ± 0.5	25 (19–33)	7.1 ^c	10	0.6 (0.4–0.9) ^d
		—	568	0.9 ± 0.1	1,303 (909–1,881)	77.0 ^c	69	1
W	Malathion	+TPP	133	0.9 ± 0.2	407 (177–1,038)	17.5 ^c	13	3.2 (1.5–6.8) ^d
		+DEF	108	0.8 ± 0.2	149 (25–407)	8.8 ^c	10	8 (3–21) ^d
		+PBO	107	0.7 ± 0.2	5,022 (1,072–1,037,280)	20.6 ^c	12	0.3 (0.1–0.8) ^d
		+DEM	49	0.8 ± 0.2	3,491 (923–206,410)	0.7 ^c	4	0.5 (0.1–1.6)
		—	568	0.9 ± 0.1	1,303 (909–1,881)	77.0 ^c	69	1
		+TPP	133	0.9 ± 0.2	407 (177–1,038)	17.5 ^c	13	3.2 (1.5–6.8) ^d

^a Concentrations expressed in ppm of malathion.
^b Synergistic ratio (SR) at LC₅₀ of malathion with respect to malathion plus the synergist in the same strain.
^c Good fit of the data to the probit model ($P > 0.05$).
^d SR is significantly different ($P < 0.05$) if the confidence interval does not include 1.

In addition, the susceptibility of the C and W strains to spinosad and fenthion were tested by ingestion (Table 3). Both spinosad and fenthion were more toxic for the C and W strains than malathion. However, the W strain (LC₅₀ = 12 ppm) was nine-fold significantly more tolerant to fenthion than the C strain (LC₅₀ = 1.3 ppm), whereas no significant differences were found with respect to spinosad (LC₅₀ = 0.4 ppm for the C strain and 0.7 ppm for the W strain).

Bioassays with Synergists. Adults flies of the C and W strains were pretreated with synergists (DEF, PBO, DEM, or TPP) 2 h before being treated with malathion (Table 4). In the W strain, DEF (8.0-fold) and TPP (3.2-fold) significantly enhanced the effect of malathion, whereas no significant differences were obtained with DEM. When adults of the W strain were pretreated with PBO, the susceptibility to malathion was reduced (0.3-fold). Because the methodology used for testing the insecticide did not allow concentrations >10,000 ppm, the mortalities obtained at the highest concentrations tested were closed to the LC₅₀ (5,022 ppm), resulting in a large upper LC₅₀ 95% CL (1,072–1,037,280). When the assay was continued for another 12 h, the results were more consistent, with an LC₅₀ of 6,022 ppm (95% CL = 2,907–10,651) and a synergistic ratio of 0.2 (95% CL = 0.1–0.4). In the C strain, neither PBO nor DEF had any effect on the toxicity of malathion, DEM was antagonistic (0.6-fold), and TPP was moderately synergistic (2.0-fold).

AChE Activity and In Vitro Inhibition by Malaoxon. Adult flies from the C strain treated with 50 ppm malathion presented a significant reduction in AChE activity (79.0 ± 10.1 nmol of ASChI hydrolyzed per minute and per milligram of protein) compared with untreated flies from the same strain (158.8 ± 15.4; $P < 0.05$, Student-Newman-Keuls test). On the contrary, no significant differences were obtained between treated (136.7 ± 21.2) and untreated (124.5 ± 24.2) flies from the W strain.

In vitro inhibition of AChE activity was higher in the C strain than in the W strain in the presence of increased concentrations of malaoxon (Fig. 2). A concentration of 7.5×10^{-7} M malaoxon produced almost complete inhibition of AChE activity in the C strain (95% inhibition), whereas AChE inhibition was

greatly reduced at this concentration in the W strain (46% inhibition); a concentration of 5×10^{-6} M malaoxon was necessary to obtain an inhibition >80% in the W strain. The AChE activity of the control group for the W strain (110.6 ± 3.8) was lower than that of the C strain (144.4 ± 3.8).

Discussion

In this study, *C. capitata* field populations from citrus and other fruit crops from different geographical areas in Spain showed lower susceptibility to malathion (6- to 201-fold) compared with laboratory populations. Moreover, differences in susceptibility could be correlated with the frequency of the field treatments. Accordingly, the five populations from the Comunidad Valenciana, subjected to the high selection pressure (5–10 aerial treatments/yr), showed the highest levels of resistance (LC₅₀ between 1,000 and 3,000 ppm and LC₉₀ >10,000 ppm). This finding is of great relevance for the control of this pest in the Comunidad Valenciana, the main citrus growing area of Spain, because the concentration of malathion in the protein baits used in aerial treatments is 7,500 ppm. In fact, control failures may be already occurring, which may have contributed to the increase in the

Fig. 2. In vitro inhibition of AChE activity in the susceptible (C) and resistant (W) strains of *C. capitata*. Three experimental replicates (using a pool of heads from 20 individuals) were carried out for each malaoxon concentration. SE was always <3%.

frequency of the insecticide treatments experienced in the last years. Other operational factors that may have contributed to the development of resistance are the higher amounts of active ingredient used in the formulations (triplicated in the last 10–15 yr), the expansion of extra early varieties of citrus extremely sensitive to the attack of this insect, and the switch from control measures based on malathion and fenitrothion to only malathion. Low levels of resistance to malathion (LC_{50} between 100 and 500 ppm) were also detected in field populations subjected to only one ground treatment per year or collected from fields that have not been treated, suggesting that resistant flies may have already dispersed from areas with high selection pressure to untreated areas. Different studies indicate that *C. capitata* is a species in which the majority of adults do not move large distances when hosts are available, but when hosts are absent or become unavailable, they disperse rapidly and cover quite large distances in search of hosts (Fletcher 1989).

A resistant strain (W), derived from a field population from Castellón, and a susceptible strain (C), from Lab-IVIA, were selected to compare the activity of malathion by ingestion and topical application. The W strain was more resistant to malathion than the C strain in both feeding (79-fold) and topical (22-fold) bioassays. However, topical application seems to be more effective than ingestion, which may indicate that resistance evolved linked to current control strategies, because field exposure to malathion baits is mainly by ingestion. These results contrast with those obtained ~20 yr ago by Viñuela and Arroyo (1982), who found no differences in susceptibility to malathion by topical application between laboratory and field populations of *C. capitata* from citrus crops from the Comunidad Valenciana. Our results with the C strain in topical application bioassays ($LC_{50} = 1.2 \mu\text{g/g}$) are comparable to those obtained by Viñuela and Arroyo (1982) with laboratory populations ($LC_{50} \approx 2 \mu\text{g/g}$). However, Viñuela and Arroyo (1982) reported that doses of 5 and 10 $\mu\text{g/g}$ resulted in 100% mortality in the field populations tested, whereas these doses produced no mortality in our resistant strain (data not shown). These findings suggest that, the detected resistance most probably developed recently because of the continuous use of malathion, as has been reported for the Oriental fruit fly, *Bactrocera dorsalis* Hendel, in Taiwan (Hsu and Feng 2000).

Other insecticides approved for the control of *C. capitata* for citrus crops in Spain include fenitrothion, spinosad, fosmet, trichlorfon, lambda cyhalothrin, and lufenuron (<http://www.mapa.es/es/agricultura/pags/fitos/fitos.asp>). Our results showed that the W strain was nine-fold more resistant to fenitrothion than the C strain. It may indicate that the mechanism that confers resistance to malathion confers also moderate cross-resistance to fenitrothion (Hsu et al. 2004). However, because fenitrothion was the most widely used insecticide for *C. capitata* control in Spain during the 1960s, 1970s, and 1980s (Lloréns-Climent and Gilabert-Artiges 1997), different mechanisms could have been selected

independently to confer resistance to both insecticides. With respect to spinosad, we have found no differences in susceptibility when the W and C strains were compared. Nevertheless, Hsu and Feng (2006) showed that a laboratory colony of *B. dorsalis* selected for resistance to malathion showed cross-resistance to spinosad. Thus, the potential for resistance development must be considered if spinosad and malathion are used jointly in resistance management programs. The role of the other insecticides in the evolution to resistance to malathion is unlikely, because their use is limited to a few areas. In any case, it is necessary to reduce the use of insecticides for the control of *C. capitata* and replace them for more environmental friendly strategies. In this sense, the Comunidad Valenciana has initiated pilot programs to control *C. capitata* based on the release of sterile males and the use of insecticides with a chemosterilant action (Navarro-Llopis et al. 2004).

The resistance to malathion and other organophosphorous insecticides (OPs) could be caused by mutations on the target site, the acetylcholinesterase (Mutero et al. 1994, Oakeshott et al. 2005), or the detoxification of the insecticide by metabolic enzymes (Ranson et al. 2002, Feyerherzen 2005). We found that AChE activity of adult flies from the W-resistant strain of *C. capitata* was less sensitive to be inhibited in vivo by malathion and in vitro by malaoxon, suggesting that target site insensitivity may be associated with malathion resistance. Modified AChE, showing a decreased in the sensitivity by OPs, has been linked with OP resistance in some insect species (Li and Han 2004, Russell et al. 2004) including tephritids (Vontas et al. 2002, Hsu et al. 2006). Zahavi and Tahori (1970) reported that *C. capitata* adults from a field population in Israel possessed an AChE less sensitive to phosphamidon than flies from other field or laboratory strains, but they did not associate these differences with changes in the susceptibility to OPs insecticides. Moreover, we found a loss of AChE activity on the W strain compared with the C strain, as has been reported for other cases of target site resistance (Vontas et al. 2002, Hsu et al. 2006).

Using synergists, we showed that the esterase inhibitor DEF partially suppressed the resistance to malathion (eight-fold) in the resistant W strain, indicating that esterases may also play a significant role in the resistance to malathion in this species. Increased detoxification of malathion by specific malathion carboxylesterase activity (MCE) is a well-studied resistant mechanism in many malathion resistant strains of different species (Townsend and Busvine 1969, Hemingway 1982, Hughes et al. 1984). This kind of resistance has been often diagnosed on the basis of its synergism by TPP. Hughes et al. (1984) indicated that TPP completely eliminated the resistance to malathion in a resistant strain of the housefly *Lucilia cuprina* (Wiedemann), and the synergistic ratios exceeded 200-fold. Hemingway (1985) also indicated that a resistant strain of *Anopheles arabiensis* Patton showed high levels of suppression of resistance by TPP. We found that the toxicity of malathion was only slightly

increased by TPP in both C (2-fold) and W (3.2-fold) strains. Thus, a hydrolytic mechanism mediated by esterases, but probably different from the MCE, seems to be involved in this resistance. However, inferences from the results with the synergists must be made cautiously because they are not entirely specific (Ahmad and Hollingworth 2004). Indeed, esterase activity (1-naphthyl acetate as substrate) is reduced by 90% in adults of *C. capitata* treated with DEF, whereas MCE activity (*S*-methyl thiobutanoate as substrate) is only reduced by 26% when the flies are treated with TPP (unpublished results). Additional biochemical and molecular studies will be needed to confirm this hypothesis.

Metabolic resistance to malathion mediated by cytochrome P450s (Maitra et al. 2000) and glutathione-S-transferases (Taskin and Kence 2004) has also been described in dipteran species. However, our results with synergists suggest that these enzymes are not involved in conferring resistance to malathion in *C. capitata*. In fact, the oxidase inhibitor PBO enhanced, rather than suppressed, the resistance to malathion in the W strain. This result may have been caused by a block at the activation step, from the "thion" form of malathion to the activated "oxon" form of malafoxon, mediated by cytochrome P450s (Eto 1974). Ringo et al. (1995) showed that chlorpyrifos resistance was enhanced in *Drosophila melanogaster* (Meigen) by PBO, and Hemingway (1982) indicated that PBO had a slightly antagonist effect on a malathion-resistant strain of *Anopheles stephensi* (Liston). On the contrary, no significant effects were obtained when PBO was tested on the C strain. It may be because of two reasons: (1) malathion, although it is not the active form of the insecticide, is also toxic and it may have enough activity on the C strain, but not on the W strain, which is ≈ 100 -fold more resistant; and (2) if esterases are involved in resistance in the W strain, an antagonistic effect would be expected when the activation of malathion is inhibited, because malafoxon is a potent inhibitor of esterases, whereas malathion is a poor inhibitor of them (Oakeshott et al. 2005). Interestingly, an antagonist effect on malathion toxicity was also obtained with DEM in the C strain. It has been pointed out that DEM is not a completely specific inhibitor of glutathione-S-transferases and that it can inhibit cytochrome P450s (Anders 1978, Welling and De Vries 1985). In fact, an inhibition of 26% was obtained for cytochrome P450s when adults were pretreated with DEM (unpublished results). Thus, the antagonism of malathion by DEM could be also attributed to the inhibition of the metabolic activation of malathion.

In conclusion, resistance to malathion has been found, for the first time, in field populations of *C. capitata*. Enzymatic assays and experiments with synergists suggest that both target site insensitivity and metabolic resistance might be involved in the loss of susceptibility to this insecticide. Nevertheless, the resistance mechanisms need to be studied further to confirm this hypothesis and provide the necessary

information for the design of a suitable resistance management strategy.

Acknowledgments

We thank A. Escudero (IRTA, Girona), R. Argiles (TRAGSA, Valencia), V. Navarro (Universidad Politécnica de Valencia, Valencia), J. M. Llorens (Conselleria de Agricultura, Pesca y Alimentación, Alicante), M. Vendrell (Cooperativa de Alcudia, Valencia), and V. Marco (Universidad de la Rioja, LA Rioja) for assistance in field sampling and F. Beitia (IVIA, Valencia) for providing the laboratory populations. This work received financial support from CICYT (AGL2004-05169).

References Cited

- Ahmad, M., and R. M. Hollingworth. 2004. Synergism of insecticides provides evidence of metabolic mechanisms of resistance in the obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae). *Pest Manag. Sci.* 60: 465–473.
- Albajes, R., and C. Santiago-Álvarez. 1980. Efectos de la densidad larvaria y de la alimentación en la proporción de sexos de *Ceratitis capitata* (Diptera: Tephritidae). *An. INIA Ser. Agric.* 13: 175–182.
- Anders, M. W. 1978. Inhibition and enhancement of microsomal drug metabolism by diethyl maleate. *Biochem. Pharmacol.* 27: 1098–1101.
- Bradford, M. M. 1976. A rapid and sensitive method from quantitation of micrograms quantities protein utilizing the principle of dye binding. *Anal. Biochem.* 72: 248–254.
- Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82: 70–77.
- Ellman, G. L., K. D. Courtney, V. J. Andres, and R. M. Feather-Stone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7: 88–95.
- Eto, M. 1974. Organophosphorus pesticides: organic and biological chemistry. CRC, Cleveland, OH.
- Feyereisen, R. 2005. Insect cytochrome P450, pp. 1–77. In L. I. Gilbert, K. Iatrou, and S. S. Gill (eds.), *Comprehensive molecular insect science*. Elsevier, Oxford, United Kingdom.
- Fletcher, B. S. 1989. Movements of tephritid fruit flies, pp. 209–217. In A. S. Robinson and G. Hooper (eds.), *World crop pests. Fruit flies: their biology, natural enemies and control*, vol. 3B. Elsevier, Amsterdam, The Netherlands.
- Friedenauer, S., and H. H. Berlet. 1989. Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Anal. Biochem.* 178: 263–268.
- Georghiou, G. P. 1972. The evolution of resistance to pesticides. *Annu. Rev. Ecol. Syst.* 3: 133–168.
- Hemingway, J. 1982. The biochemical nature of malathion resistance in *Anopheles stephensi* from Pakistan. *Pest. Biochem. Physiol.* 17: 149–155.
- Hemingway, J. 1985. Malathion carboxylesterase enzymes in *Anopheles arabiensis* from Sudan. *Pest. Biochem. Physiol.* 23: 309–313.
- Hsu, J. C., and H. T. Feng. 2000. Insecticide susceptibility of the Oriental Fruit Fly (*Bactrocera dorsalis* (Hendel)) (Diptera: Tephritidae) in Taiwan. *Chinese J. Entomol.* 20: 109–118.
- Hsu, J. C., and H. T. Feng. 2006. Development of resistance to spinosad in oriental fruit fly (Diptera: Tephritidae) in laboratory selection and cross-resistance. *J. Econ. Entomol.* 99: 931–936.

- Hsu, J. C., H. T. Feng, and W. J. Wu. 2004. Resistance and synergistic effects of insecticides in *Bactrocera dorsalis* (Diptera: Tephritidae) in Taiwan. *J. Econ. Entomol.* 97: 1682–1688.
- Hsu, J. C., D. S. Haymer, W. J. Wu, and H. T. Feng. 2006. Mutations in the acetylcholinesterase gene of *Bactrocera dorsalis* associated with resistance to organophosphorus insecticides. *Insect Biochem. Molec. Biol.* 36: 396–402.
- Hughes, P. B., P. E. Green, and K. G. Reichmann. 1984. Specific resistance to malathion in laboratory and field populations of the Australian sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae). *J. Econ. Entomol.* 77: 1400–1404.
- Koren, B., A. Yawetz, and A. S. Pery. 1984. Biochemical properties of characterizing the development of tolerance to malathion in *Ceratitis capitata*, Wiedmann (Diptera: Tephritidae). *J. Econ. Entomol.* 77: 864–867.
- LeOra Software. 1997. POLO-PC user's guide to probit or logit analysis. LeOra Software, Berkeley, CA.
- Li, F., and Z. J. Han. 2004. Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, *Aphis gossypii* Glover. *Insect Biochem. Molec. Biol.* 34: 397–405.
- Liquido, N. J., L. A. Shinoda, and R. T. Cunningham. 1991. Host plants of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae): an annotated world review. *Misc. Publ. Entomol. Soc. Am.* 77: 1–52.
- Lloréns-Climent, J. M., and J. Gilibert-Artiges. 1997. Eficacia de dos insecticidas en aplicación aérea contra la mosca del mediterráneo (*Ceratitis capitata*). *OILB/WPRS Bull.* 20: 165–174.
- Maitra, S., S. M. Dombrowski, M. Basu, O. Raustol, L. C. Waters, and R. Ganguly. 2000. Factors on the third chromosome affect the level of Cyp62 and Cyp6a8 expression in *Drosophila melanogaster*. *Gene* 248: 147–156.
- Metcalf, R. L., and R. A. Metcalf. 1993. Destructive and useful insects. Their habits and control. McGraw Hill, New York.
- Mutero, A., M. Pralavorio, J. Bride, and D. Fournier. 1994. Resistance associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl. Acad. Sci. U.S.A.* 91: 5922–5926.
- Navarro-Llopis, V. N., J. Sanchis-Cabanes, I. Ayala, V. Casanaginer, and E. Primo-Yufera. 2004. Efficacy of lufenuron as chemosterilant against *Ceratitis capitata* in field trials. *Pest. Manag. Sci.* 60: 914–920.
- Oakeshott, J. G., C. Claudianos, P. M. Campbell, R. Newcomb, and R. J. Russell. 2005. Biochemical genetics and genomics of insect esterases, pp. 309–381. *In* L. I. Gilbert, K. Iatrou, and S. S. Gill (eds.), *Comprehensive molecular insect science*. Elsevier, Oxford, United Kingdom.
- Orphanidis, P. S., B. Kalmoukos, B. Betzios, and E. Kapetanakis. 1980. Development of resistance in *Ceratitis capitata* Wied. in laboratory under intermittent pressure of organophosphorus and chlorinated insecticides. *Ann. Inst. Phytopathol. Benaki.* 12: 198–207.
- Primo-Millo, E., F. Alfaro Lassala, and R. Argilés Herrero. 2003. Plan de actuación contra la mosca de las frutas (*Ceratitis capitata*) en la Comunidad Valenciana. *Phytoma España.* 153: 127–130.
- Raghavendra, K., S. K. Subbarao, M.K.K. Pillai, and V. P. Sharma. 1998. Biochemical mechanisms of malathion resistance in Indian *Anopheles culicifacies* (Diptera: Culicidae) sibling species A, B, and C: microplate assays and synergistic studies. *Ann. Entomol. Soc. Am.* 91: 834–839.
- Ranson, H., C. Claudianos, F. Ortelli, C. Abgrall, J. Hemingway, M. V. Sharakhova, M. F. Unger, F. H. Collins, and R. Feyereisen. 2002. Evolution of supergene families associated with insecticide resistance. *Science* 298: 179–181.
- Ringo, J., G. Jona, R. Rockwell, D. Segal, and E. Cohen. 1995. Genetic variation for resistance to chlorpyrifos in *Drosophila melanogaster* (Diptera: Drosophilidae) infesting grapes in Israel. *Insect J. Econ. Entomol.* 88: 1158–1163.
- Robertson, J. L., and H. K. Preisler. 1992. Pesticide bioassays with arthropods. CRC, Boca Raton, FL.
- Russell, R. J., C. Claudianos, P. M. Campbell, I. Horne, T. D. Sutherland, and J. G. Oakeshott. 2004. Two major classes of target site insensitivity mutations confer resistance to organophosphate and carbamate insecticides. *Pest. Biochem. Physiol.* 79: 84–93.
- Taskin, V., and M. Kence. 2004. The genetic basis of malathion resistance in housefly (*Musca domestica* L.) strains from Turkey. *Russ. J. Genet.* 40: 1215–1222.
- Townsend, M. G., and J. R. Busvine. 1969. The mechanism of malathion resistance in the blowfly *Chrysomya putoria*. *Entomol. Exp. Appl.* 12: 243–267.
- Viñuela, E. 1998. La resistencia a insecticidas en España. *Bol. San. Veg. Plagas.* 24: 487–496.
- Viñuela, E., and M. Arroyo. 1982. Resultados del ensayo de la susceptibilidad de 6 poblaciones españolas de *Ceratitis capitata* Wied., para detección de su posible resistencia de los insecticidas. *An. INIA/Ser. Agric.* 21: 183–192.
- Vontas, J. G., M. J. Hejazi, N. J. Hawkes, N. Cosmidis, M. Loukas, and J. Hemingway. 2002. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. *Insect Mol. Biol.* 11: 329–336.
- Welling, W., and J. M. De Vries. 1985. Synergism of organophosphorus insecticides by diethyl maleate and related compounds in house flies. *Pest. Biochem. Physiol.* 23: 358–369.
- Wood, R. J., and D. J. Harris. 1989. Artificial and natural selection, pp. 19–31. *In* A. S. Robinson and G. Hopper (eds.), *Fruit flies: their biology, natural enemies and control*, vol. 3B. Elsevier, Amsterdam, The Netherlands.
- Zahavi, M., and A. S. Tahori. 1970. Differences in acetylcholinesterase sensitivity to phosphamidon in Mediterranean fruit fly strains. *Israel J. Entomol.* 5: 185–191.

Received 9 March 2007; accepted 16 July 2007.