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# Mechanisms of resistance to organophosphates in *Tetranychus urticae* (Acari: Tetranychidae) from Greece

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**Abstract**

We investigated the mechanisms conferring resistance to methyl-parathion (44-fold) and to methomyl (8-fold) in *Tetranychus urticae* from Greece by studying the effect of synergists on the resistance and the kinetic characteristics of various enzymes in a resistant strain (RLAB) and a susceptible reference strain (SAMB). It is shown that *S*,*S*,*S*-tributyl phosphorotrithioate, a synergist that inhibits esterases and glutathione *S*-transferases, and piperonyl butoxide, a synergist that inhibits cytochrome P450 mediated monooxygenases, did not affect the level of methyl-parathion or methomyl resistance in RLAB and that resistance ratios to both insecticides did not change significantly in the presence of either synergist. Isoelectric focusing of esterase allozymes on single mites revealed no differences in staining intensity and glutathione *S*-transferase activity was not significantly different in the two strains. The activity of two cytochrome P450 monooxygenase groups was compared. No significant difference of 7-ethoxyresorufin-*O*-diethylase activity was observed between strains that were two-fold higher in RLAB than in SAMB. The kinetic characteristics of acetylcholinesterase, the target enzyme of organophosphates and carbamates, revealed that acetylcholinesterase in RLAB was less sensitive to inhibition by paraoxon and methomyl in comparison with SAMB. $I_{50}$, the inhibitor concentration inducing 50% decrease of acetylcholinesterase activity was greater (119- and 50-fold with paraoxon and methomyl, respectively) and the bimolecular constant $k_i$ was lower (39- and 47-fold with paraoxon and methomyl, respectively) in RLAB compared to SAMB. 

*Keywords:* *Tetranychus urticae*; Insecticide resistance; Acetylcholinesterase; Detoxifying enzymes

## 1. Introduction

*Tetranychus urticae* Koch is a phytophagous mite with worldwide distribution and a large number of host plants. The species has been heavily exposed to insecticides and acaricides, and organophosphate-resistant populations have been reported in more than 40 countries in both greenhouses and open field crops (Georghiou and Lagunes-Tejeda, 1991). The most common mechanism of resistance to organophosphates (OP) in *T. urticae* is reduced sensitivity of the target enzyme acetylcholinesterase (AChE) to the inhibitory action of toxicants; this has been reported in *T. urticae* from several countries (Smissaert, 1964; Smissaert et al., 1970; Voss and Matsumura, 1964; Zahavi and Tahori, 1970; Tag El-Din, 1990). Another type of OP resistance reported in *T. urticae* is based on an increase in detoxification capacity resulting from increased carboxy-esterase and phosphatase activities (Matsumura and Voss, 1964; Herne and Brown, 1969). Identifying the physiological mechanisms of resistance is the first step in the characterization of resistance genes and is a prerequisite for understanding the evolution of insecticide resistance and for resistance management. In a recent study, we reported the occurrence of resistance to two OPs (methyl-parathion and methydathion) and a carbamate (methomyl) in two localities of Greece by analyzing strains derived from mites collected on different host plant species in greenhouses and open field crops

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(Tsagkarakou et al., 1996). Resistance ratios varied considerably with methidathion and with methomyl. In the presence of methyl-parathion, the Greek strains displayed a striking similarity with a clear plateau at 20% mortality.

The objective of the present study was to use bioassays and biochemical analyses to investigate the physiological mechanisms responsible for the resistance to methyl-parathion and methomyl in one of the Greek strains examined previously (Tsagkarakou et al., 1996). For this, we: (a) studied the effect of two synergists that inhibits detoxifying enzymes on dose–mortality responses to methyl-parathion and methomyl; and (b) compared the activity of enzymes known to be involved in OP and carbamate resistance (i.e. the target enzyme AChE and the detoxifying enzymes cytochrome P450 mediated monooxygenases, esterases and glutathione *S*-transferases).

## 2. Materials and methods

### 2.1. Mite strains

Two *T. urticae* strains were used: SAMB, a susceptible reference strain from the Netherlands, initially collected on *Sambucus nigra* (L) and obtained from the Laboratory of the Applied Entomology at Amsterdam, and RLAB derived from the Greek LABOAT strain studied by Tsagkarakou et al. (1996) after selection for 23 generations with methyl-parathion, with doses killing 70–90% of mites.

### 2.2. Bioassays

The insecticides used were commercial formulations of methyl-parathion (Methyl Bladan 40 EC, 400 g/l; Bayer, Puteaux, France) and methomyl (Lannate 20 SL 200 g/l; Dupont, Paris, France).

For the bioassays, a 35 mm diameter disc of bean leaf was placed in plastic Petri dishes containing moistened cotton wool. Twenty adult females were transferred onto each leaf disc with a fine brush. Individual Petri dishes were then sprayed with 1.5 ml aqueous solutions of insecticide using a precision Potter Spray Tower (Burkard, Rickmansworth, Hertfordshire, UK) (Potter, 1952). Five replicates of at least four concentrations causing 0–100% mortality after 24 h were tested with each insecticide.

Two synergists were used to investigate the effect of inhibition of detoxifying enzymes on resistance. The synergists used were PBO (piperonyl butoxide, Fluka AG St Quentin, France), an inhibitor of cytochrome P450 mediated monoxygenases, and DEF (*S,S,S*-tributyl phosphorotrithioate, Interchim), an inhibitor of esterases and glutathione-*S*-transferases (GST). Maximum sublethal doses of the two synergists were determined on susceptible females. These doses were 100 and 30 mg/l for PBO and DEF, respectively. Glass microscope slides were dipped in acetone solutions of synergists and left to dry. Mites were then transferred onto one slide and covered by a second one. The two slides were separated by a piece of thin cardboard placed in such a way that mites could walk and remain in contact with the synergists. Four hours later, the mites were placed on a bean leaf disc and sprayed with methyl-parathion or methomyl.

Mortality data were analyzed by probit regression (Raymond et al., 1993) based on Finney (1971). Resistance ratios (RR) or synergism ratios (SR) were considered to be significantly different from 1 ($P<0.05$) when their 95% confidence limits (95% CL) did not include this value.

### 2.3. Biochemical analyses

The in vitro activities of detoxifying esterases, GST, cytochrome P450 monooxygenases and the target enzyme AChE were studied in the two strains.

Esterases were analyzed on single *T. urticae* female homogenates subjected to isoelectric focusing on cellulose acetate membranes using alpha- and beta-naphthyl acetates as substrates (Tsagkarakou et al., 1996). This electrophoretic method is an appropriate technique for analyzing genetic variations in single mites, overcoming the problem of the minute size of this species, allowing to identify both variations in allelic frequencies and differences in staining intensity (i.e. differences in activity). *Est* alleles were named in relation to their isoelectric point (*pI*), and there frequency was estimated with the GENEPOP package using the Maximum likelihood program when null alleles were presented.

For the measurement of cytochrome P450 monooxygenase and GST activity, extracts of total proteins were prepared by homogenizing 10,000 adult females in 11 ml 0.05 M phosphate buffer, pH 7.2, containing 0.1 mM dithithreitol (DTT), 1 mM tetra-acetic ethylenediamine acid (EDTA) and 0.4 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 15,000*g* for 10 min at 4°C. The supernatant was then centrifuged at 100,000*g* for 1 h at 4°C to separate the microsomal pellet from the soluble fraction. The microsomal pellet was resuspended in the same phosphate buffer containing 30% glycerol and was used to determine cytochrome P450 monooxygenase activity. The supernatant was used as a source of soluble proteins to determine GST activity. Total protein concentrations in the supernatant and the microsomal fractions were determined using the method of Lowry et al. (1951).

GST activity was evaluated according to Habig et al. (1974) using 1-chloro-2.4 -dinitrobenzene (CDNB) and 3.4-dichloro-nitrobenzene (DCNB) as substrates. The

reaction mixture (final volume: 1 ml) consisted of a volume of homogenate equivalent to 50 μg protein, 50 μl substrate solution at varying concentrations (final concentrations 0.05, 0.1, 0.25, 0.5, and 1 mM), 10 μl 100 mM GSH (reduced glutathione) and 0.01 M phosphate buffer pH 7.0. Variations in optical density were recorded at 340 nm using as a blank the same reaction mixture but without protein extract. Kinetic parameters ($K_m$ = Michaelis constant and $V_m$ = maximum velocity) were determined according to Lineweaver and Burk (1934).

The activities of two cytochrome P450 monooxygenase groups, 7-ethoxycoumarin-*O*-deethylase (ECOD) and 7-ethoxyresorufin-*O*-deethylase (EROD), were measured in microtitration plate wells on a spectrofluorimeter (Fluorite 1000, Dynatech) using 7-ethoxycoumarin (7EC) and 7-ethoxyresorufin (7ER) as substrates for ECOD and EROD, respectively (de Sousa et al., 1995). The reaction mixture for measuring ECOD activity (final volume: 1 ml) contained 20 μl 1 M 7EC, 50 μl 0.04 M NADPH and 930 μl 0.05 M phosphate buffer pH 7.2. Fifty microliters reaction mixture and a volume of microsomal suspension equivalent to 100 μg proteins were placed in each well with phosphate buffer (100 μl final volume). After 2 h incubation at 30°C, the reaction was stopped by adding 100 μl glycine/ethanol buffer (v/v) pH 10.4. The release of fluorescent 7-hydroxycoumarin (7OHC) was measured at 390 nm excitation and 450 nm emission (filter 3, intensity 2500, energy 5). The activity of each strain was measured in five wells (corresponding to five replicates) and two controls, where the reaction was stopped immediately to correct for non-enzymatic activity. EROD activity was estimated by measuring the release of 7-hydroxyresorufin (7OHR) which is fluorescent at all pH values and thus enables direct kinetic analysis. The reaction mixture consisted of 40 μl 0.2 mM 7ER, 100 μl 0.04 M NADPH and 1860 μl phosphate buffer. One hundred microliters of reaction mixture and a volume of microsomal suspension equivalent to 100 μg proteins were placed in each well together with phosphate buffer (up to 200 μl). Fluorescence was measured at 535 nm excitation and 600 nm emission every 5 min for 35 min. Five replicates of each strain were performed and controls consisted of four wells per strain without NADPH.

Regression lines for GST and EROD activities in the two strains were compared by covariance analysis using the GLIM computer package (Baker and Nelder, 1985). Differences in ECOD activities between the two strains were investigated by two-factor analysis of variance.

AChE activity was measured by spectrophotometry using acetylthiocholine (AcSCh) as substrate as described by Ellman et al. (1961). One hundred adult females were mass homogenized with a Teflon glass homogenizer in 100 μl extraction buffer containing 1 M NaCl, 10 mM Tris pH7.0, and 0.4% Triton X100. The supernatant obtained after centrifugation for 10 min at 10,000*g* was used as the enzyme source. The reaction was conducted with 90 μl homogenate and 10 μl ethanol in 900 μl substrate–reagent solution (final concentrations: 1.7 mM DTNB and 1 mM AcSCh). Activity was estimated after 3 min incubation, by measuring the variation in optical density at 412 nm with a spectrophotometer (Kontron-Uvikon 930, Paris, France). Four replicates were performed for each strain.

Residual AChE activities were measured with increasing concentrations of the inhibitors carbamate methomyl (commercial formulation) and oxidized OP paraoxon (Bayer, Leverkusen Germany). The inhibitor concentration inducing 50% inhibition of AChE ($I_{50}$), was determined by incubating 90 μl homogenate with 10 μl ethanol inhibitor solutions at various concentrations for 10 min before adding 900 μl substrate–reagent solution. Final inhibitor concentrations varied between $10^{-7}$ and $10^{-2}$ M for paraoxon and between $6.2\times10^{-6}$ and $1.2\times10^{-2}$ M for methomyl. The inhibitory action of methomyl and paraoxon was also analyzed by calculating the bimolecular rate constant, $k_i$, using the method of Aldridge (1950). Briefly, 90 μl supernatant was incubated with 10 μl inhibitor solution at a given concentration for various times (0–40 min) before the addition of 900 μl substrate–reagent solution. The slope of the curve of ln residual activity against time divided by inhibitor concentration gives $k_i$. The constant $k_i$ was estimated for each strain at two concentrations of each insecticide (paraoxon and methomyl). Blanks without homogenate or substrate were used to correct for non-enzymatic activity.

## 3. Results

### 3.1. Resistance characteristics of the RLAB strain

After 23 generations of selection with methyl-parathion, the dose–mortality response of RLAB had become linear. The plateau observed at 20% mortality before selection (Tsagkarakou et al., 1996) had disappeared, indicating that susceptible genotypes had been eliminated. The linearity of concentration–mortality curves was accepted ($P>0.05$) for the susceptible reference strain SAMB tested with methomyl and for the RLAB strain tested with methyl-parathion. The resistance ratio of RLAB as compared to the susceptible SAMB was 44-fold with methyl-parathion and 8-fold with methomyl at $LC_{50}$ (Table 1).

### 3.2. Analysis of detoxifying mechanisms in RLAB

The detoxifying mechanisms were investigated in vivo by study of the effect of synergists on resistance and in vitro by comparison of the activity of esterases,

Table 1
Responses of SAMB and RLAB strains of *T. urticae* to methyl-parathion and to methomyl with and without synergists (*n*, number of mites tested, CI of $LC_{50}$, $SR_{50}$ and $RR_{50}$ are given in parenthesis)

| Treatement | *n* | Slope±SE | $LC_{50}$[a] | $\chi^{2}$[b] | df | $SR_{50}$[c] | $RR_{50}$[d] |
|---|---|---|---|---|---|---|---|
| *SAMB* | | | | | | | |
| Methyl-parathion | 673 | 2.6±0.5 | 64 (41–99) | 22.3 | 4 | – | – |
| Methyl-parathion+DEF | 443 | 2.8±0.3 | 28 (23–32) | 1.1 | 2 | 2.5 (1.4–3.3) | – |
| Methyl-parathion + PBO | 512 | 4.7±1.1 | 120 (72–203) | 9.4 | 2 | 0.5 (0.3–1.1) | – |
| *RLAB* | | | | | | | |
| Methyl-parathion | 358 | 3.8±0.3 | 2831 (2495–3170) | 4.0 | 3 | – | 44 (27–71) |
| Methyl-parathion+DEF | 487 | 4.2±2.0 | 4677 (1812–12,062) | 44.8 | 2 | 0.6 (0.2–1.7) | 170 (54–536) |
| Methyl-parathion+PBO | 659 | 2.5±0.7 | 4055 (2171–7569) | 45.7 | 4 | 0.7 (0.4–1.3) | 34 (14–78) |
| *SAMB* | | | | | | | |
| Methomyl | 379 | 3.1±0.3 | 182 (159–210) | 7.6 | 3 | – | – |
| Methomyl+DEF | 735 | 1.7±0.3 | 148 (78–281) | 42.0 | 5 | 1.3 (0.8–2.0) | – |
| Methomyl+PBO | 722 | 1.7±0.4 | 190 (78–465) | 33.0 | 4 | 1.0 (0.5–1.7) | – |
| *RLAB* | | | | | | | |
| Methomyl | 771 | 2.2±0.6 | 1389 (730–2649) | 48.0 | 4 | –8 | (4–13) |
| Methomyl+DEF | 697 | 2.4±0.2 | 1659 (1460–1915) | 2.3 | 3 | 0.8 (0.5–1.4) | 11 (7–18) |
| Methomyl+PBO | 719 | 1.9±0.5 | 1429 (707–2899) | 58.0 | 5 | 1.1 (0.4–3.3) | 7 (3–16) |

[a] LC expressed in mg/l.
[b] Chi-square testing linearity.
[c] SR, synergism ratio = LC observed in absence of synergist/LC observed in presence of synergist.
[d] RR, resistance ratio = $LC_{RLAB}/LC_{SAMB}$.

GST and cytochrome P450 monooxygenases in the resistant (RLAB) and susceptible (SAMB) strains.

In the presence of the synergist PBO, linearity of concentration–mortality curves was rejected ($P<0.05$) in both strains for both methyl-parathion and methomyl. In the presence of the synergist DEF, linearity of concentration–mortality curves was accepted ($P>0.05$) for the susceptible reference strain SAMB tested with methyl-parathion and for the RLAB strain tested with methomyl and rejected ($P<0.05$) for the SAMB strain tested with methomyl and for the RLAB strain tested with methyl-parathion.

The in vivo analyses showed that the addition of PBO to biossays had no significant effect on methomyl and methyl-parathion dose–mortality responses in RLAB and SAMB (95% CI of synergism ratios contained the value 1). The addition of DEF to bioassays did not modify the dose–mortality response to methomyl in RLAB and SAMB or to methyl-parathion in RLAB. In contrast, it significantly decreased tolerance to methyl-parathion in SAMB. These results showed that neither esterases (or GST) inhibited by DEF nor P450 cytochrome mediated monooxygenases inhibited by PBO played a role in the observed resistance of RLAB. This conclusion was confirmed by the observation that the resistance ratios to both insecticides did not change significantly in the presence of either synergist. Table 1 shows that the CIs of the RRs with or without addition of synergists overlapped in all cases.

The in vitro activity of esterases was compared in the two strains after the electrophoretic separation of single mite homogenates. Two loci were identified by their preferential hydrolysis capability with regard to α- (*Est-1* locus) and β- (*Est-2* locus) naphthyl acetate. *Est-1* had two phenotypes corresponding to the presence or absence of a band. *Est-2* displayed one band (homozygous genotypes) or two bands (heterozygous genotypes). The frequency of *Est-1* phenotypes and *Est-2* band staining intensity did not vary during the selection of RLAB (Table 2). However the number of *Est-2*

Table 2
Allelic frequencies observed at EST locus in the susceptible strain (SAMB) and in the resistant strain before (LABOAT) and after selections (RLAB) (*N*, number of tested mites)

| | SAMB | LABOAT | RLAB |
|---|---|---|---|
| *Est-1*[a] | | | |
| (*N*) | 174 | 45 | 28 |
| A | 0.276 | 0.933 | 0.965 |
| P | 0.724 | 0.067 | 0.035 |
| *Est-2* | | | |
| (*N*) | 173 | 41 | 28 |
| 115 | 0.038 | 0.098 | 0.554 |
| 100 | 0.665 | 0.207 | 0.446 |
| 95 | 0 | 0.024 | 0 |
| 90 | 0.298 | 0.110 | 0 |
| 80 | 0 | 0.085 | 0 |
| 70 | 0 | 0.268 | 0 |
| 60 | 0 | 0.206 | 0 |

[a] Two phenotypes observed at *Est-1* locus: (A) corresponds to absence of isozymes (P) to its presence. Phenotype frequencies are given here.

alleles decreased from seven to two alleles; this could be the result of selection or genetic drift. If selection was involved it is unlikely to be the result of methyl-parathion selection pressure because *Est-2* $^{100}$ and *Est-2* $^{115}$ were also present in the susceptible SAMB strain.

GST activity using DCNB as substrate was very weak in RLAB and SAMB strains and could not be further analyzed. Kinetic parameters of GST activities estimated with CDNB as substrate revealed no difference between the two strains: $V_{max}$ was 1.3 and 2 μmol $mg^{-1}$ $min^{-1}$ and $K_m$ was 0.6 and 1.2 mM for RLAB and SAMB, respectively. The intercept and slopes of the Lineweaver–Burk plots were not significantly different ($P>0.05$) in the two strains (Fig. 1).

Similarly, there was no significant difference ($P>0.05$) in EROD activity between the two strains: 0.75 and 0.85 pmol of 7OHR $min^{-1}$ $mg^{-1}$ of protein for RLAB and SAMB, respectively. However, ECOD activity was significantly higher ($P<0.05$) in RLAB than in SAMB, with 1.05 and 0.47 pmol 7OHC $min^{-1}$ $mg^{-1}$ protein, respectively.

### 3.3. Target enzyme activity

AChE activity was studied using 0.9 mM of AcSCh as a substrate and was found to be similar in the RLAB and SAMB strains, with 12.1±1.3 and 12.7±0.5 pmol $min^{-1}$ per individual, respectively.

AChE from RLAB was less sensitive than AChE from SAMB to inhibition by paraoxon and methomyl, as is shown by the $I_{50}$ values and the bimolecular constant $k_i$. The $I_{50}$ for paraoxon was $10^{-4}$ M and $8.4\times10^{-7}$ M for RLAB and SAMB, respectively, and the $I_{50}$ for methomyl was $8\times10^{-4}$ M and $1.6\times10^{-5}$ M for RLAB and SAMB, respectively. Residual AChE activity at increasing incubation times with a given inhibitor concentration enabled the calculation of the bimolecular rate constant $k_i$. Plots of the ln of residual activity against time for a given paraoxon or methomyl concentration were linear, showing that only one type of AChE is present in each strain (Fig. 2). The $k_i$ values for RLAB were identical at both concentrations of each inhibitor ($k_i$ $=1\times10^3$ $M^{-1}$ $min^{-1}$ and $k_i$ $=0.034\times10^3$ $M^{-1}$ $min^{-1}$ for paraoxon and methomyl, respectively). The $k_i$ values for SAMB were very similar at both concentrations of paraoxon ($k_i$ $=57\times10^3$ $M^{-1}$ $min^{-1}$ and $=20\times10^3$ $M^{-1}$ $min^{-1}$) and of methomyl $k_i$ $=1.8\times10^3$ $M^{-1}$ $min^{-1}$ and $=1.4\times10^3$ $M^{-1}$ $min^{-1}$). The $k_i$ of AChE was thus 39- and 47-fold higher in SAMB than in RLAB for paraoxon and methomyl, respectively. These results show clearly that AChE in the resistant strain is less sensitive to the inhibitor tested than in the susceptible strain.

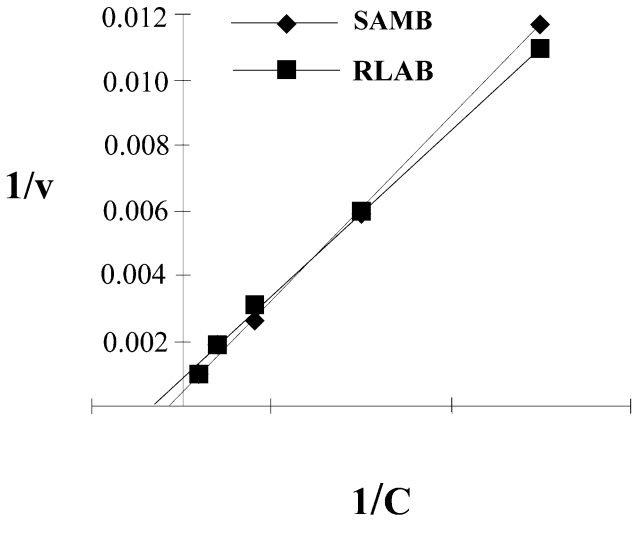


Fig. 1. Lineweaver–Burk plot showing the kinetics of glutathione S-transferase in RLAB and SAMB. V in nmoles $mg^{-1}$ $min^{-1}$ and C in mM.

## 4. Discussion

The RLAB strain, derived from *T. urticae* collected in Greece and selected for 23 generations with methyl-parathion, displayed 44-fold resistance to this insecticide and 8-fold resistance to methomyl when compared to the susceptible strain SAMB. Investigation of the physiological mechanisms of resistance showed that: (a) resistance was not synergized by DEF, an inhibitor of esterases and GST, or by PBO, an inhibitor of some cytochrome P450 monooxygenases; (b) comparison of GST, esterase and EROD activities revealed no difference between the two strains although ECOD activity was twice as high in the RLAB strain as in the susceptible strain; (c) AChE activity was similar in the two strains and; (d) however, AChE from RLAB was less sensitive to inhibition by paraoxon and methomyl.

The results of bioassays using the synergist DEF are in agreement with those obtained by analyzing esterase and GST activities. Both in vivo and in vitro experiments showed that there was no difference between the two strains, indicating that detoxication by esterases or GST does not play an important role in the observed resistance to methyl-parathion and methomyl.

The synergist PBO had no significant effect on resistance to methyl-parathion or methomyl in the resistant and susceptible strains, indicating absence of involvement of cytochrome P450 monooxygenases. So far, there have been no reports of resistance to OP or carbamates due to oxidative detoxification in *T. urticae*. However, in our study, the in vitro analysis showed a two-fold higher ECOD activity in the RLAB strain. In general, the results of OP toxicity using PBO as a synergist are difficult to interpret because of the combined action of oxidases in insecticide activation and degradation. Moreover, it has been shown that when both detoxification

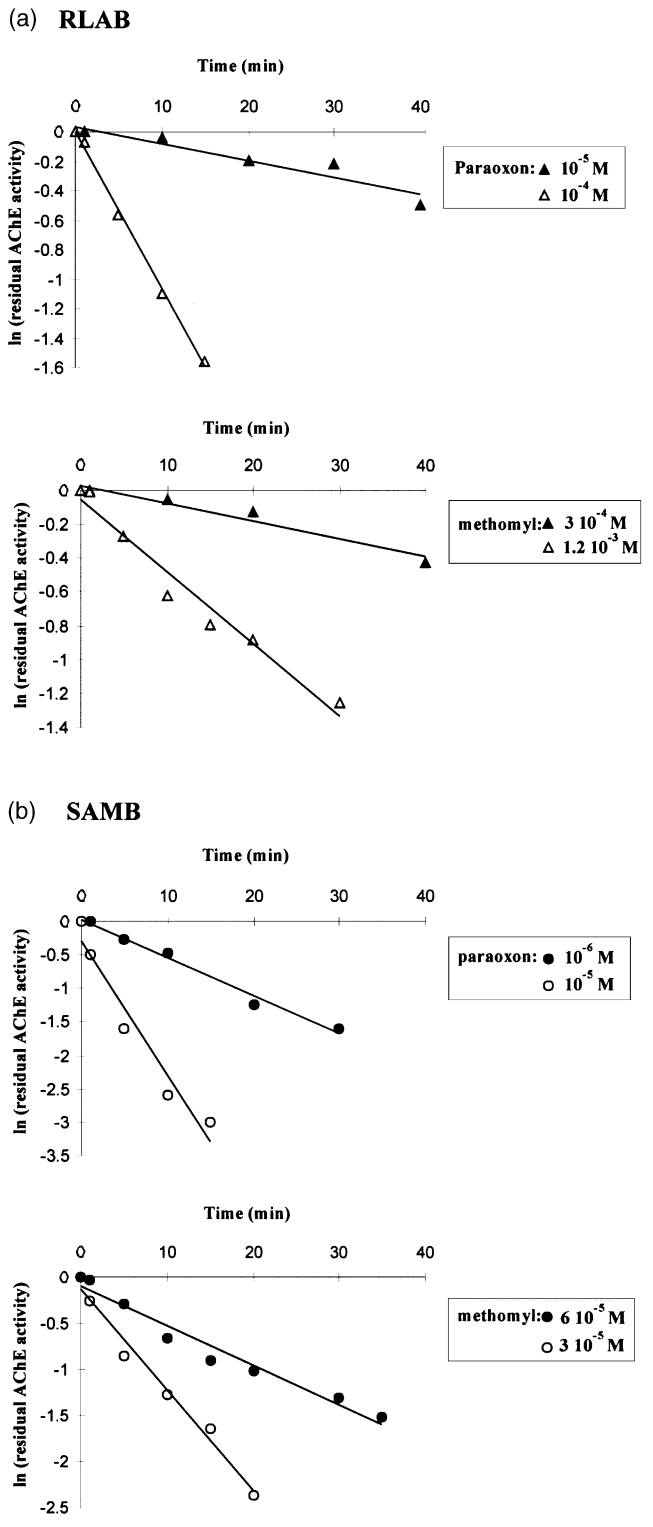


Fig. 2. Plots of the residual AChE activity from RLAB and SAMB against the inhibition time at two paraoxon and methomyl concentrations.

and target alteration are involved in resistance, these two mechanisms have an additive interaction (Raymond et al., 1989). It is therefore difficult to show the role of detoxifying enzymes with bioassays using synergists unless they contribute more to the total resistance than the insensitive target. It therefore seems possible that in our investigations the use of PBO did not reveal the role of cytochrome P450 monooxygenases in the resistance of RLAB because it was masked by the insensitive AChE. However, it is also possible that ECOD activity is not inhibited by PBO or that it is not involved in the resistance of RLAB as was found in *Drosophila melanogaster* (Brun, 1996).

The kinetics of AChE clearly showed that this enzyme was different in the two strains. Both inhibitors had a higher $I_{50}$ and a lower $k_i$ in the RLAB strain compared to the SAMB strain. This decreased AChE affinity in RLAB explains the cross resistance between an OP and a carbamate. The AChE alteration present in the RLAB strain did not decrease its capacity to hydrolyze ACSh, as is often observed (Smissaert, 1964; Voss and Matsumura, 1964; Zahavi and Tahori, 1970; Tag El-Din, 1990). It is noted that, as in our study, there was no difference in AChE activity between resistant and susceptible *T. urticae* from Israel (Zahavi and Tahori, 1970).

So far, the most common mechanism of resistance to OP in *T. urticae* is the insensitivity of the target enzyme, AchE. This has been reported in strains from the Netherlands, Germany, the USA, New Zealand, Israel, Egypt (Zahavi and Tahori, 1970; Helle, 1984; Cranham and Helle, 1985; Tag El-Din, 1990) and Greece (this study). In a given region, the resistance genes may have derived from mutation events occurring in the region or may have been introduced. In previous studies, we showed that gene flow is high between *T. urticae* populations which are separated by a few meters but gene flow decreases sharply as geographic distances increase (Tsagkarakou et al. 1997, 1998). These observations, and the fact that resistance in *T. urticae* appeared rapidly and simultaneously in different regions of the world after the first use of insecticides (Cranham and Helle, 1985), suggest that mutations conferring resistance may have appeared independently in the different localities. In order to verify this hypothesis, it would be necessary to compare susceptible and resistant AChEs from various regions. Molecular studies have shown that the decreased sensivity of AChE to insecticide inhibition results from a single point mutation or a combination of point mutations in the AChE gene in *Drosophila* (Mutéro et al., 1994) and in the Colorado potato beetle *Leptinotarsa decemlineata* (Zhu et al., 1996). However other studies have shown that this was not the case in the cattle tick *Boophilus microplus* (Hernandez et al., 1999), and in the green rice leafhopper *Nephotettix cincticeps* (Tomita et al., 2000). All these studies were

done on acetylcholinesterase genes that were cloned using sequence homologies with torpedo acetylcholinesterase. The demonstration that such an acetylcholinesterase is not the gene involved in *Culex pipiens* resistance (Malcolm et al., 1998) raises the question on whether the studied sequences were those of the target enzyme involved in insecticide resistance in insects. At present the only biochemical tests available for monitoring insensitive acethylcholinesterase in the field are based on inhibition characteristics (Bourguet et al., 1996). It seems that such type of biochemical tests will be difficult to develop for mites due to their minute size, and progress in the molecular characterization of the target AchE gene is needed to develop tests based in molecular biology techniques, specially PCR.

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