Bulletin of Entomological Research (2009) **99**, 23–31
doi:10.1017/S0007485308006081

First published online 1 July 2008
Printed in the United Kingdom

Resistance mechanisms to mitochondrial electron transport inhibitors in a field-collected strain of *Tetranychus urticae* Koch (Acari: Tetranychidae)

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Abstract

A Belgian field strain (MR-VP) of *Tetranychus urticae* (Koch) (Acari: Tetranychidae) exhibits different levels of resistance to four frequently used METI (mitochondrial electron transport inhibitor)-acaricides, i.e. tebufenpyrad, fenpyroximate, pyridaben and fenazaquin. Resistance factors for these compounds were 184, 1547, 5971 and 35, respectively. A 23.5-fold increase in 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation activity suggested that metabolic resistance through elevated levels of cytochrome P450 dependent monooxygenase-activity is a possible resistance mechanism.

However, synergism studies with different metabolic inhibitors revealed some contrasting resistance mechanisms between the METI-acaricides. Tebufenpyrad resistance could only be synergized after pre-treatment with the monooxygenase inhibitor piperonyl butoxide (PBO), whereas pyridaben resistance was strongly synergized both by PBO and the esterase inhibitor S,S,S-tributylphosphorotrithioate (DEF). Resistance levels to fenpyroximate could neither be suppressed by PBO nor by DEF. Although METI-acaricides are structurally related, these findings probably reflect a different role of esterases and monooxygenases in metabolic detoxification between these compounds. The overall lack of synergism by diethylmaleate (DEM) suggests that glutathione-*S*-transferases are not an important factor in resistance to METIs.

Reciprocal crosses between susceptible females and resistant males showed no maternal effect, and resistance to METI-acaricides was inherited generally as a dominant trait. Backcrosses with F_1 females revealed striking differences in the mode of inheritance. Although resistance to fenpyroximate and pyridaben was under monogenic control, resistance to tebufenpyrad was under control of more than one gene.

Keywords: METI, *Tetranychus urticae*, acaricide resistance, synergism, inheritance, detoxification

(Accepted 15 February 2008)

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Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is a worldwide pest of many plant species including several economically important agricultural crops (Jeppson *et al.*, 1975; Van de Vrie *et al.*, 1985). *T. urticae* is often difficult to manage because of its ability to quickly develop resistance to acaricides. The high reproductive potential, extreme short lifecycle and arrhenotokous parthenogenesis, combined with frequent acaricide applications, facilitates establishment of resistance. Genetically fixed mechanisms of pesticide resistance in spider mites were similar to those found in pest insects and include enhanced metabolic detoxification of acaricides through cytochrome P450-dependent monooxygenases, esterases or glutathione-*S*-transferases, and/or an altered target site conferring target site resistance (Knowles, 1997).

Depending on the chemistry and number of applications, a high level of resistance to acaricides can develop and is often associated with cross-resistance. Failures in the chemical control of spider mites caused by resistance have been reported for various compounds, such as organophosphates (Herron *et al.*, 1998; Stumpf *et al.*, 2001), carbamates (Cranham & Helle, 1985), dicofol (Unwin, 1971; Van Leeuwen *et al.*, 2005), organotins (Goodwin *et al.*, 1995), hexythiazox, clofentezine (Herron *et al.*, 1993), abamectin (Campos *et al.*, 1995; Stumpf & Nauen, 2002), bifenthrin (Herron *et al.*, 2001; Van Leeuwen & Tirry, 2007) and chlorfenapyr (Herron & Rophail, 2003).

Since 1994, several cases of resistance have been described against the METI (mitochondrial electron transport inhibitors)-acaricides in strains of *Tetranychus* spp. from Japan, Korea, Belgium, Australia and England (Ozawa, 1994; Cho *et al.*, 1995; Bylemans & Meurrens, 1997; Herron & Rophail, 1998; Devine *et al.*, 2001; Nauen *et al.*, 2001). The METIs fenazaquin, fenpyroximate, pyridaben and tebufenpyrad, which are now in widespread use globally, were developed in the 1990s and inhibit complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial respiratory pathway, probably by binding to a subunit of the associated electron transport particles (Hollingworth & Ahammadsahib, 1995). However, the underlying resistance mechanism(s), the patterns of cross-resistance and the inheritance of the resistance trait in *T. urticae* have only been investigated in a few strains (Devine *et al.*, 2001; Stumpf & Nauen, 2001).

In the present study, a field-collected resistant strain of *T. urticae* from Belgium was investigated. A preliminary screening with several commercially important acaricides revealed, amongst others, a striking resistance to several METI acaricides. Therefore, this strain was put under continuous pressure of tebufenpyrad in order to reach homogeneity and was named MR-VP. Resistance to tebufenpyrad, fenpyroximate and pyridaben was studied in detail. The effect of synergists, known to inhibit important detoxification routes, was investigated to gain insight in METI-detoxification in the resistant strain. Piperonyl butoxide (PBO), S,S,S-tributyl-phosphorotrithioate (DEF) and diethylmaleate (DEM) were used to inhibit cytochrome P450 monooxygenases, esterases and glutathione-*S*-transferases, respectively (Van Leeuwen *et al.*, 2004). These data were backed up by analysis of enzyme activities in crude homogenates. Finally, the number of genes involved, their dominance and a possible maternal effect in resistance were investigated by crossing susceptible and resistant mites.

Materials and methods

Acaricides and chemicals

The acaricides were commercial formulations (Fyto Vanhulle, Belgium) of fenazaquin (200 g a.i. l^{-1} Suspension Concentrate [SC]), fenpyroximate (50 g a.i. l^{-1} SC), pyridaben (150 g a.i. l^{-1} SC) and tebufenpyrad (200 g a.i. l^{-1} SC).

All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Belgium).

Strains

Two strains of *T. urticae* were used in this study. The German susceptible strain (GSS) has been maintained in laboratory culture without acaricide treatment since 1965 (Nauen *et al.*, 2001). The METI-resistant strain (MR-VP) was originally collected from different cultivars of bean plants in a greenhouse at the national botanical garden (Brussels, Belgium) in September 2005. The spray history revealed that spider mites were controlled in the last ten years by applying mainly Pyranica (tebufenpyrad 200 g a.i. l^{-1} SC) and Sanmite (pyridaben 150 g a.i. l^{-1} SC) and occasionally Apollo (clofentezine 500 g a.i. l^{-1} SC), Nissorun (hexythiazox 100 g a.i. kg^{-1} WP), Talstar (bifenthrin 8 g a.i. l^{-1} SC), Torque (fenbutatin oxide 550 g a.i. l^{-1} SC), Vertimec (abamectin 18 g a.i. l^{-1} SC) and Vydate (oxamyl 250 g a.i. l^{-1} SC). Since 2004 the application of these products appeared to be unsatisfactory for complete control of mite outbreaks. The strain was further pressurised in the laboratory with 1000 mg l^{-1} tebufenpyrad to reach homogeneity.

Mites of the GSS and MR-VP strain were reared on potted kidney bean (*Phaseolus vulgaris* L. cv. Prelude) plants in a climatically controlled room at 26 ($\pm$0.5)°C, 60% relative humidity (RH) and 16/8 h light/dark photoperiod. The strains were changed weekly. MR-VP was maintained on bean plants sprayed with a hand-held spraying device (Birchmeier, Switzerland) until runoff with 1000 mg l^{-1} tebufenpyrad to avoid contamination. Six months after the start of the selection with 1000 mg l^{-1} tebufenpyrad, mites were used in the experiments.

Toxicity bioassays

Adulticidal bioassays were conducted using a standard method described recently (Van Leeuwen *et al.*, 2004). Briefly, 20–30 young adult female mites were transferred to the upper side of 9 cm^2 square-cut kidney bean leaf discs on wet cotton wool, which had been sprayed with 0.75 ml of spray fluid at 1 bar pressure in a Cornelis spray tower (1.58 $\pm$ 0.06 mg aqueous acaricide deposit cm^{-2}) (Van Laecke & Degheele, 1993). The plates were then placed in a climatically controlled room at 26 $\pm$ 0.5°C, 60% RH and 16/8 h (L/D) photoperiod. Four replicates of six concentrations of each acaricide plus a control (de-ionised water) were tested. Mortality was assessed after one day, except for fenazaquin, where mortality was assessed after three days. Mites were scored as being alive if they could walk normally after they were prodded with a camel's hair brush. All control mortalities were lower than 10%. LC_{50}-values, slopes and 95% confidence limits were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA) (Robertson & Preisler, 1992a).

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Synergism studies

In order to check for metabolic resistance through synergistic ratios, female mites were placed onto leaf discs which had been sprayed with PBO, DEF or DEM. Twenty-four hours later, living mites were collected and used in toxicity experiments with tebufenpyrad, fenpyroximate or pyridaben. Based on preliminary tests, the concentrations of PBO, DEF and DEM were chosen as the concentration that caused maximum 5–10% mortality (GSS: 1000 mg l^{-1} PBO, 500 mg l^{-1} DEF, 2000 mg l^{-1} DEM; MR-VP: 5000 mg l^{-1} PBO, 2000 mg l^{-1} DEF, 2000 mg l^{-1} DEM). Before use, PBO (~90% purity), DEF (98% purity) and DEM (97% purity) were dissolved in a mixture of *N*,*N*-dimethylformamide and emulsifier W (3 : 1 by weight) and subsequently diluted with de-ionised water (100-fold). Synergism ratios and their confidence limits were calculated using the formula and statistics of dose ratios (Robertson & Preisler, 1992b). If the 95% confidence interval includes 1, then the LC_{50} of the acaricide alone is not significantly different from the LC_{50} of the acaricide + synergist.

Crossing experiments

In an attempt to estimate the dominance of the resistance, individuals of the susceptible (GSS; genotype SS) and resistant (MR-VP; genotype RR) strain were reciprocally crossed to produce hybrid F_1 females (SR, RS). This was achieved by placing 50 female teleiochrysalids of one strain and 100 adult males of the other strain on the upper side of a primary bean leaf on wet cotton wool in a Petri dish (four replicates) as described previously (Van Leeuwen *et al*., 2004). Directly after moulting, the diploid females were fertilised by the haploid males. After three days, fertilised females were collected and placed on fresh bean leaves and were allowed to lay eggs for 14 days. Every day, the egg laying females were collected and placed on a fresh leaf. The resulting F_1 females were collected ten days after hatching and were used after maturation (1–3 days) in a bioassay with the appropriate concentrations of tebufenpyrad, fenpyroximate or pyridaben with at least four replicates per concentration. The degree of dominance (*D*) was estimated for the F_1 females using the formula (Stone, 1968):

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

in which X_1 is the log of the LC_{50} of the R strain, X_2 is the log of the LC_{50} of the F_1 females and X_3 is the log of the LC_{50} of the S strain. This formula will result in a value of –1 if resistance is fully recessive, a value of 0 if there is no dominance and a value of +1 if resistance is fully dominant.

To obtain F_2 females, F_1 females and males were allowed to mate in a similar manner as described above. Because males are haploid and inherit their genes only from the mother, the F_2 progeny obtained from the crosses were genetically equivalent to backcross progeny. The resulting F_2 females were treated with several concentrations (20–30 mites per concentration) in four replicates, covering the range of 0–100% mortality. Analysis of the concentration-mortality data for the F_2 females was done to determine whether the responses fit the model of single major gene inheritance for an arrhenotokous (haplo-diploid) species. The expected responses of the F_2 generation were calculated as described by Georghiou (1969):

$$c = (0.5)\mathrm{W}(\text{parent 1}) + (0.5)\mathrm{W}(\text{parent 2})$$

where *c* is the expected mortality at a given concentration and W is the observed mortality of the parental types at a given concentration. Observed and calculated dose-response lines for F_2 females were compared using a χ^2 goodness-of-fit test.

Enzymatic assays

The *O*-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) by P450 monooxygenases was measured according to the method of DeLuca *et al*. (1988) and Buters *et al*. (1993), but the assay was rescaled and adapted to the 96-well plate format, as previously described (Van Leeuwen *et al*., 2005).

For the determination of esterase activity towards the substrates 4-nitrophenyl-acetate (4-NPA), 4-nitrophenyl propionate (4-NPP), 4-nitrophenylbutyrate (4-NPB), 1-naphthyl acetate (1-NA) and 2-naphthyl-acetate (2-NA), procedures were previously described (Van Leeuwen *et al*., 2005).

Glutathione-*S*-transferase (GST) activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, based on the method of Habig & Jakoby (1981) with slight modifications as recently described (Van Leeuwen *et al*., 2005). GST activity was also determined using the non-fluorescent monochlorobimane (MCB) as substrate, according to Nauen & Stumpf (2002). Thirty fresh adult females were homogenized in 500 µl Tris-HCl buffer (0.05 M, pH 7.5). The homogenate was centrifuged for 10 min at 10,000 g and 4°C. The total reaction volume per well in a 96-well microtiter plate was 300 µl, consisting of 50 µl supernatant, 50 µl buffer, 100 µl MCB (9 mM, dissolved in buffer with a volume fraction of 0.01 ethanol) and 100 µl reduced glutathione (9 mM, dissolved in buffer). The plate was incubated for 20 min at 22°C and fluorescence was measured with a SPECTRAmax^{TM} GEMINI XS dual-scanning microtitre plate spectrofluorometer at 465 nm while exciting at 390 nm. The nonenzymatic reaction of MCB without homogenate served as control.

All protein concentrations were measured with a Coomassie protein assay (Perbio Science, Belgium). All enzymatic assays were repeated independently at least three times.

Results

Toxicity bioassays

The toxicity bioassay data determined on adults of *T. urticae* by foliar spray application indicated high resistance ratios to tebufenpyrad (RR = 184), fenpyroximate (RR≈1500) and pyridaben (RR≈6000) (table 1). Doses of 20,000 mg l^{-1} pyridaben or fenpyroximate did not cause full mortality, but at these concentrations phytotoxic effects were already visible. Resistance to fenazaquin was considerable, but rather low compared to the other METI-acaricides (LC_{50} = 188 mg l^{-1}, RR = 35), and therefore it was not included in further experiments.

Even though the MR-VP strain was under continuous selection pressure of 1000 mg l^{-1} tebufenpyrad during more

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Table 1. Toxicity of tebufenpyrad, fenpyroximate and pyridaben with and without synergists to female adults of strains GSS and MR-VP.

Acaricide Strains		Tebufenpyrad		Fenpyroximate		Pyridaben	
		GSS	MR-VP	GSS	MR-VP	GSS	MR-VP
Acaricide alone	LC_{50} (mg l^{-1})	6.48	1197	6.84	10581	6.19	36959
	(95%CL)	(5.73–7.32)	(1080–1309)	(5.55–8.31)	(8441–13036)	(5.55–6.88)	(26450–59590)
	Slope ± SE	3.6 ± 0.3	3.5 ± 0.3	1.9 ± 0.2	1.5 ± 0.1	4.1 ± 0.4	1.2 ± 0.1
	RR	1	184	1	1547	1	5971
Acaricide + PBO	LC_{50} (mg l^{-1})	5.96	125	0.36	5376	1.01	385
	(95%CL)	(5.27–6.76)	(93–159)	(0.30–0.42)	(4198–6593)	(0.86–1.15)	(290–485)
	Slope ± SE	3.8 ± 0.3	2.1 ± 0.3	2.7 ± 0.2	1.9 ± 0.2	3.4 ± 0.4	1.3 ± 0.1
	SR[a]	1.09	9.57	19.09	1.97	6.13	96.09
	(95%CL)	(0.94–1.25)	(7.27–12.59)	(15.25–23.91)	(1.47–2.63)	(5.19–7.24)	(62.83–146.97)
	RR	1	21	1	14933	1	381
Acaricide + DEF	LC_{50} (mg l^{-1})	5.21	1439	1.71	7922	1.44	560
	(95%CL)	(4.56–5.85)	(785–1682)	(1.43–1.95)	(6083–9859)	(1.21–1.66)	(407–735)
	Slope ± SE	5.03 ± 0.54	4.40 ± 1.55	3.81 ± 0.48	1.79 ± 0.19	3.95 ± 0.39	1.26 ± 0.10
	SR	1.25	0.83	4.00	1.33	4.29	66.01
	(95%CL)	(1.07–1.45)	(0.65–1.06)	(3.19–5.00)	(0.99–1.80)	(3.66–5.03)	(42.07–103.58)
	RR	1	276	1	4633	1	389
Acaricide + DEM	LC_{50} (mg l^{-1})	3.35	953	4.30	11676	3.91	10913
	(95%CL)	(2.53–4.29)	(641–1263)	(3.78–4.86)	(9667–13965)	(2.58–5.64)	(7842–14454)
	Slope ± SE	3.94 ± 0.34	2.87 ± 0.48	2.65 ± 0.20	1.92 ± 0.18	3.34 ± 0.26	1.47 ± 0.19
	SR	1.93	1.26	1.59	0.91	1.59	3.39
	(95%CL)	(1.65–2.26)	(1.00–1.57)	(1.29–1.96)	(0.70–1.17)	(1.34–1.87)	(2.20–5.22)
	RR	1	285	1	2715	1	2791

[a]SR, LC_{50} of acaricide alone/LC_{50} of acaricide + synergist; RR, resistance ratio; SR, synergism ratio; CL, confidence limits.

Table 2. Probit statistics for the crosses (F_1) tested against tebufenpyrad, fenpyroximate and pyridaben.

Acaricide	Strain	*n*	χ^2 (df)	Slope ± SE	LC_{50} (95%CL) (mg l^{-1})	*D*
Tebufenpyrad	GSS♀ × MR-VP♂	564	11.1 (22)	4.5 ± 0.2	407 (363–446)	0.59
	MR-VP♀ × GSS♂	575	22.5 (22)	4.2 ± 0.3	419 (374–462)	0.60
Fenpyroximate	GSS♀ × MR-VP♂	588	29.0 (26)	2.9 ± 0.4	16444 (13588–18886)	1.12
	MR-VP♀ × GSS♂	688	38.4 (26)	2.4 ± 0.2	8086 (6252–9815)	0.93
Pyridaben	GSS♀ × MR-VP♂	635	13.9 (26)	1.9 ± 0.2	10125 (8256–12000)	0.70
	MR-VP♀ × GSS♂	720	24.5 (26)	3.7 ± 0.5	17857 (15667–19782)	0.83

n, number of mites; *D*, degree of dominance.

than half a year, the LC_{50} value (≈1200 mg l^{-1}) was just slightly higher than the selection concentration. In addition, the response of MR-VP to tebufenpyrad appeared to be more homogeneous (slope of probit line = 3.5) than to fenpyroximate, pyridaben or fenazaquin (slope = 1.5, 1.2 and 1.9, respectively).

The GSS strain was fully susceptible, and LC_{50} values were far below the field concentrations of tebufenpyrad (100 mg a.i. l^{-1}), fenpyroximate (50 mg a.i. l^{-1}), pyridaben (180 mg a.i. l^{-1}) and fenazaquin (40 mg a.i. l^{-1}).

Synergism studies

Pre-treatment of the MR-VP strain and the GSS strain with PBO, DEF or DEM revealed different effects on the toxicity of the METI-acaricides (table 1).

PBO caused a 9.6-fold increase in toxicity of tebufenpyrad to MR-VP and reduced the resistance ratio from 184 to 21. DEF and DEM had no synergistic effect on the toxicity of tebufenpyrad.

When the effect of PBO, DEF or DEM was tested on fenpyroximate resistance of MR-VP, the synergism ratios were very low. However, a high synergistic effect could be detected in the GSS strain after pre-treatment with PBO (SR≅19), resulting in a tenfold higher resistance ratio.

PBO and DEF enhanced the toxicity of pyridaben to MR-VP by 96- and 66-fold, respectively, and decreased the resistance ratio to 381 and 389 compared with GSS. Synergism ratios observed after treatment with DEM were rather low.

Inheritance of resistance

In all cases, reciprocal crosses between GSS and MR-VP were successful and yielded female progeny. Results of the crosses are given in table 2.

With all tested METIs, the concentration-mortality data for F_1 females resulting from GSS♀ × MR-VP♂ and MR-VP♀ × GSS♂ reciprocal crosses revealed no maternal inheritance.

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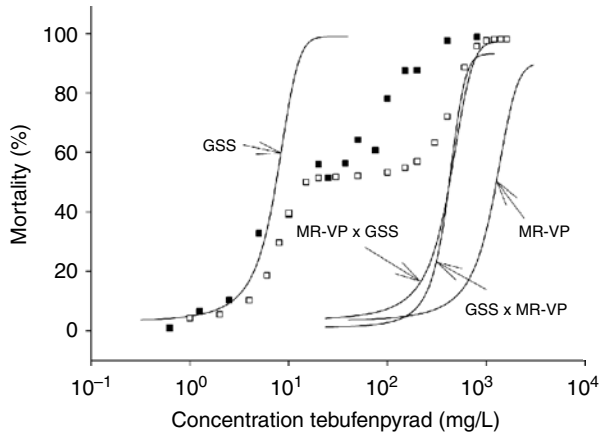

Fig. 1. Concentration-mortality curves for tebufenpyrad in MR-VP, GSS, the reciprocal crosses, the (GSS × MR-VP) F_1♀ × GSS♂ backcross (■) and the theoretical backcross on the basis of monogenic inheritance (□).

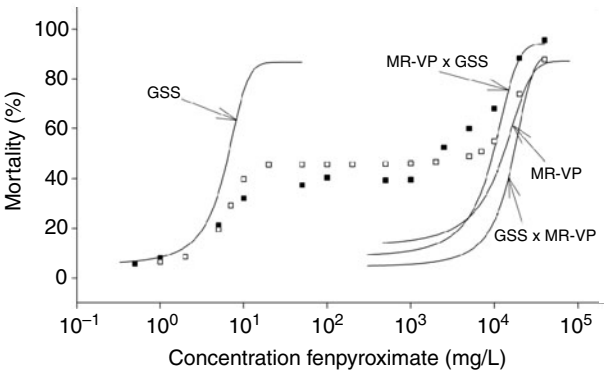

Fig. 2. Concentration-mortality curves for fenpyroximate in MR-VP, GSS, the reciprocal crosses, the (GSS × MR-VP) F_1♀ × GSS♂ backcross (■) and the theoretical backcross on the basis of monogenic inheritance (□).

For tebufenpyrad, the estimated degree of dominance (*D*) for the F_1 females from GSS♀ × MR-VP♂ crosses and from MR-VP♀ × GSS♂ crosses were 0.59 and 0.60, respectively, illustrating an incompletely dominant inheritance of resistance.

Both the concentration-mortality data for fenpyroximate in the reciprocal F_1 females were close to that of the MR-VP strain. The *D* values were 1.12 for cross GSS♀ × MR-VP♂ and 0.93 for cross MR-VP♀ × GSS♂, indicating a completely dominant inheritance of resistance to fenpyroximate.

A dominant inheritance of resistance was also observed for pyridaben ($D = 0.70$ in cross GSS♀ × MR-VP♂ and 0.83 in the reciprocal cross).

Figures 1–3 show the expected (on the basis of monogenic control) and observed concentration-mortality lines of the three acaricides for backcross F_2 females. Since inheritance of tebufenpyrad, fenpyroximate and pyridaben resistance was shown to be (incompletely) dominant, only the offspring of cross (GSS × MR-VP) F_1♀ × GSS♂ was investigated. The Chi-square (χ^2) goodness-of-fit analysis indicated that the

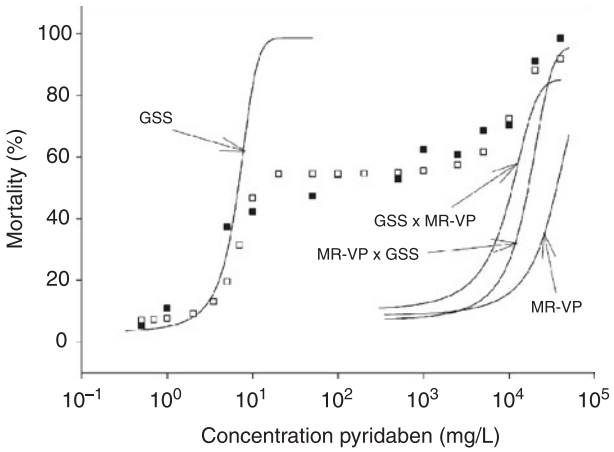

Fig. 3. Concentration-mortality curves for pyridaben in MR-VP, GSS, the reciprocal crosses, the (GSS × MR-VP) F_1♀ × GSS♂ backcross (■) and the theoretical backcross on the basis of monogenic inheritance (□).

observed mortalities of the F_2 females for fenpyroximate and pyridaben were not significantly different ($\chi^2 = 15.88$, df = 13 and $\chi^2 = 7.31$, df = 13, respectively, $p > 0.05$) from the expected values for single gene control. These results suggest that the resistance to fenpyroximate and pyridaben is under monogenic control (figs 2 and 3). The pronounced plateau (at about 50% mortality) in response to the backcross emphasizes that a single factor confers a high level of resistance. Only the observed mortality for tebufenpyrad was significantly different (χ^2-test, $p = 0.05$) from that expected on the basis of monogenic inheritance, leading to the conclusion that more than one gene is involved in resistance.

Detoxification enzymes

A significant difference in monooxygenase-mediated 7-EFC deethylation activity was found between GSS and MR-VP. The activity was increased 23.5-fold in the METI-resistant strain (table 3). Monooxygenase activity in the GSS♀ × MR-VP♂ and MR-VP♀ × GSS♂ reciprocal crosses was 8.8- and 10.5-fold enhanced, respectively, i.e. the activity dropped by 2.7- and 2.2-fold compared with MR-VP (fig. 4). Pre-treatment of the MR-VP strain with PBO could only reduce the monooxygenase activity ex-vivo by 1.6-fold.

Quantitative analysis of general esterase activity measured by the production of the metabolites 4-nitrophenol, 1- and 2-naphthol by various substrates, revealed no significant differences between strains GSS and MR-VP (table 3).

The conjugation of glutathione with CDNB was significantly different between the two strains (*t*-test, $p < 0.05$), though the increase in activity compared to GSS was small (1.2-fold). The GST activity was measured additionally in a fluorometric assay with MCB. Compared to strain GSS, MR-VP exhibited a 2.5-fold increased GST activity.

Discussion

The original field population of *T. urticae*, collected from a greenhouse near Brussels, Belgium, showed a considerable

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Table 3. Detoxifying enzyme activities in the GSS and MR-VP strain

	GSS	MR-VP	Ratio
Monooxygenases			
O-deethylation of 7-EFC[a]	99.2 ± 9.5[A]	2332.7 ± 194.9[B]	23.5
Esterases			
4-NPA[b]	154.9 ± 8.7[A]	153.6 ± 10.7[A]	1.0
4-NPP[b]	331.4 ± 27.0[A]	383.5 ± 16.8[A]	1.2
4-NPB[b]	281.3 ± 32.1[A]	329.0 ± 20.6[A]	1.2
1-NA[c]	304.3 ± 8.0[A]	267.9 ± 18.7[A]	0.9
2-NA[c]	382.2 ± 7.5[A]	349.8 ± 24.1[A]	0.9
Glutathione-*S*-transferases			
CDNB conjugation[d]	1587.1 ± 10.8[A]	1907.1 ± 36.6[B]	1.2
MCB[e]	598.7 ± 30.2[A]	1502.5 ± 48.4[B]	2.5

Means (±SEM) within a row followed by the same capital letter are not significantly different (*t*-test, $P > 0.05$).
[a]pmol 7-hydroxy-4-(trifluoromethyl)-coumarin (30 min)$^{-1}$ mg^{-1} protein (±SEM, $n = 3$).
[b]nmol 4-nitrophenol min^{-1} mg^{-1} protein (±SEM, $n = 3$).
[c]nmol 1- or 2-naphthol min^{-1} mg^{-1} protein (±SEM, $n = 3$).
[d]nmol glutathione conjugated min^{-1} mg^{-1} protein (±SEM, $n = 3$).
[e]relative fluorescence units (RFU) µg^{-1} protein (±SEM, $n = 4$).

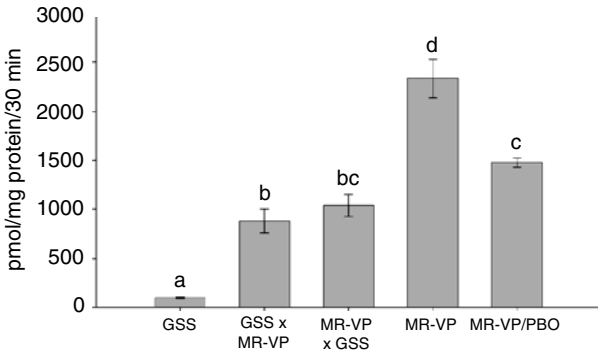

Fig. 4. 7-EFC *O*-deethylation activity of the GSS and MR-VP strain, their reciprocal crosses and MR-VP after 24 h pre-treatment with PBO (5000 mg l^{-1}). Data are mean values ± SEM ($n = 3$). Different letters indicate significant differences (Tukey-test, $p < 0.05$).

cross-resistance between the METI-acaricides tebufenpyrad, fenpyroximate, pyridaben and fenazaquin. None of these METIs were 100% effective at the recommended field dose rate, suggesting structural similarities selecting for similar mechanisms of resistance. After pressurising with tebufenpyrad in the laboratory, the strain proved to be resistant to the tested METIs in the order pyridaben > fenpyroximate > tebufenpyrad > fenazaquin. The extremely high resistance ratios of pyridaben and fenpyroximate are not unusual and were previously reported (Goka, 1998; Nauen *et al.*, 2001; Sato *et al.*, 2004).

METI-acaricides attack a target-site in complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial respiratory pathway (Hollingworth & Ahammadsahib, 1995; Lümmen, 1998). But clearly, a considerable degree of uncertainty exists regarding the exact relationship of inhibitor binding sites on Complex I (Hollingworth & Ahammadsahib, 1995; Schuler & Casida, 2001).

All METIs are compounds containing heterocyclic rings with two nitrogen atoms associated with long hydrophobic tail structures with at least one tertiary butyl group. Despite their similar chemical structure and identical mode of action, a variation in susceptibility of METI-acaricide-resistant *T. urticae* strains to members of this chemical class of acaricides exists. The TUK4-strain, collected in 1999 from hops in England with a short history of tebufenpyrad use, exhibited resistance to tebufenpyrad, pyridaben, fenazaquin and fenpyroximate, with resistance factors of 46, 346, 168 and 77, respectively (Devine *et al.*, 2001). The Japanese METI resistant strain AKITA, collected in 1996, also exhibited 1100-, 870- and 33-fold cross-resistance to pyridaben, fenpyroximate and tebufenpyrad, respectively (Stumpf & Nauen, 2001). In Western Australia, a strain of *T. urticae* collected from an apple orchard, where it had been exposed to five tebufenpyrad applications over four seasons, exhibited 63-, 210- and 25-fold resistance to tebufenpyrad, pyridaben and fenpyroximate, respectively, when tested with adult females (Herron & Rophail, 1998). Cross-resistance between METIs was also found in laboratory selected strains (Kim *et al.*, 2004, 2006).

These observations do illustrate the complexity of mechanisms of resistance to METI acaricides. Based on their chemical structure, hydroxylation is thought to be one common mechanism of oxidative detoxification for all METIs in *T. urticae* (Stumpf & Nauen, 2001). Stumpf & Nauen (2001) reported that the highly METI-resistant strain AKITA showed a 2.4-fold increase in *O*-deethylation activity of the artificial substrate 7-ethoxycoumarin (7-EC). In this study, oxidative detoxification was characterized by the highly sensitive fluorometric microplate assay using 7-ethoxy-4-trifluoromethylcoumarin, a substrate similar to the more common 7-EC. MR-VP showed a highly elevated *O*-deethylation activity (23.5-fold) compared with the susceptible strain GSS. This supports that metabolic detoxification via cytochrome P450 monooxygenases is likely to play a major role in METI resistance in the MR-VP strain, whereas enhanced esterase and GST activity appears to be a minor resistance contributing factor, since only minor differences were found in enzymatic activity between strains.

In the current study, none of the synergists caused METI resistance to drop to full susceptibility. These results suggest either the existence of an additional resistance mechanism toward the METIs, the inability of the synergists to fully suppress the enzymatic detoxification mechanisms or that the amount of synergist may have been too low to fully block detoxification. In case of oxidative METI-degradation, this could also be confirmed with the observation that the *O*-deethylation activity of the MR-VP after 24-h pre-treatment with PBO was only 1.6-fold lower than that of the untreated MR-VP and still 15 times higher than GSS. It has also been reported that the enhanced 7-EC *O*-deethylation activities of the AKITA and UK-99 strains were only partially suppressed by PBO, possibly caused by a restricted binding to the active site of the cytochrome P450-dependent monooxygenases involved (Stumpf & Nauen, 2001).

However, our synergism studies revealed some interesting results in terms of possible differences in resistance mechanisms towards tebufenpyrad, fenpyroximate and pyridaben. Pretreatment with the monooxygenase inhibitor PBO *in vivo* resulted in a significant decrease of tebufenpyrad and pyridaben resistance in the MR-VP strain and

https://doi.org/10.1017/S0007485308006081 Published online by Cambridge University Press

reduced the resistance ratio from 184 to 21 for tebufenpyrad and from 5971 to 381 for pyridaben, which confirms the role of oxidative detoxification for these two compounds. While PBO has also been reported to inhibit resistance-related esterases in some insect species (Gunning *et al*., 1998, 1999; Young *et al*., 2005), there is no evidence that PBO inhibits mite esterases (Van Pottelberge *et al*., unpublished data). Resistance to pyridaben could be significantly reduced by DEF as well, possibly reflecting the role of both monooxygenases and esterases in pyridaben resistance. However, it has been proposed that DEF is not a completely specific inhibitor of esterases and that it can also inhibit microsomal oxidases at high concentration (Scott, 1990; Valles *et al*., 1997). It can be noted that DEM halved the resistance ratio to pyridaben. Taking into consideration the significant, but limited, increase *in vivo* of glutathione-*S*-transferase activities, resistance to pyridaben can be partly caused by these enzymes, although the effect is negligible compared to esterases and monooxygenases.

Surprisingly, the level of resistance to fenpyroximate in MR-VP could not be suppressed by PBO or DEF, suggesting either the involvement of monooxygenases insensitive to PBO inhibition or that the high level of resistance to fenpyroximate is not caused by detoxification. If the lack of synergism on fenpyroximate toxicity finds its origin in an insensitive P450 monooxygenase pathway, this would infer a completely different oxidative detoxification route for fenpyroximate in comparison to tebufenpyrad and pyridaben, since resistance to the latter two could be significantly synergised by PBO. This would also imply that cross-resistance between METIs in this strain is not caused by P450 monooxygenase cross-activity. Up to date, all METI resistance in *T. urticae* could be synergised, at least partially, by PBO (Stumpf & Nauen, 2001; Kim *et al*., 2004). This lack of synergism, together with the clear monogenic inheritance of fenpyroximate resistance, could well point to a target-site-based resistance mechanism. These findings challenge the general thought that METI resistance is solely caused by enhanced oxidative detoxification. This conclusion is mainly based on synergist experiments, which can draw the attention from other possible effects. A detoxification mechanism that is the same in a sensitive and a resistant strain can have a much greater impact on degradation if an altered site of action in the resistant strain retards the intoxication. The effect of a synergist that blocks the detoxification will then be much larger, which could lead to the false conclusion that the detoxification is the major resistance mechanism (Oppenoorth, 1984, 1985).

Regardless of the underlying mechanisms, determination of genetics of the resistance is necessary for resistance risk assessments (McKenzie & Batterham, 1998) since it reveals important information on the susceptibility of heterozygotes and, thus, the stability and the potential to spread on the field. Resistance to all METIs in the MR-VP strain was inherited as a dominant trait as was also found by Devine *et al*. (2001) and Sato *et al*. (2004). However, Goka (1998) examined resistance patterns in a Japanese METI-resistant strain of *T. kanzawai* and reported that the inheritance was intermediately dominant for tebufenpyrad and fenpyroximate, but completely recessive for pyridaben, suggesting that the mechanism conferring pyridaben resistance was almost certainly different from that conferring tebufenpyrad resistance and that the resistance gene loci for the three acaricides are not identical.

Furthermore, the responses of reciprocal crosses between strains revealed no maternal effect for all three METI-acaricides. METI-acaricides attack a target site in complex I of the respiratory pathway. Only a minority of the subunits of complex I are products of mitochondrial genes, while the remainder originate from nuclear DNA (Walker, 1992). Recently, it was suggested that a few subunits of complex I (TYKY, PSST, ND1, ND5 and 49-kDa) form the catalytic core of the enzyme and are possible inhibitor binding sites (Schuler & Casida, 2001). Hence, a mutation in ND1 or ND5, which are encoded by the mitochondrial genome, could result in maternal inherited METI resistance. This was clearly not the case. Autosomally inherited resistance was reported by most authors (Goka, 1998; Devine *et al*., 2001; Sato *et al*., 2004), but Stumpf & Nauen (2001) observed a slight maternal effect in the inheritance of pyridaben and fenpyroximate resistance.

The responses of F_2 females from the reciprocal crosses suggest that the resistance to fenpyroximate and pyridaben was under monogenic control, while resistance to tebufenpyrad was under control of more than one gene (polygenic). It is possible that resistance to tebufenpyrad in MR-VP was originally a monogenic resistance; but, due to selection, additional (minor) resistance mechanisms appeared. Such a hypothesis is supported by earlier findings on *Lucilia cuprina*, the sheep blow fly, where four populations of *L. cuprina*, already nearly fixed for a major allele, were brought into the laboratory for eight further generations of selection, resulting in a polygenic resistance (McKenzie *et al*., 1980, cited in Roush & McKenzie, 1987). Monogenic resistance, which is considered more likely to spread within populations than polygenic resistance, tends to be more stable and is less easily managed (Roush & McKenzie, 1987).

After considering all experimental evidence obtained throughout the study, it can be concluded that a high level of resistance to all METI-acaricides can occur in the field and that different genetically established resistance mechanisms can play a role in resistance to each of these METIs.

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