Metabolic mechanisms of resistance to spirodiclofen and spiromesifen in Iranian populations of *Panonychus ulmi*

Fahimeh Badieinia[a], Jahangir Khajehali[a,*], Ralf Nauen[b], Wannes Dermauw[c], Thomas Van Leeuwen[c,*]

[a] Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan 84156-83111, Iran.

[b] Bayer AG, CropScience Division, R&D, Pest Control, Building 6260, Alfred Nobel Str. 50, D-40789 Monheim, Germany.

[c] Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, 9000, Ghent, Belgium

* Corresponding authors:

Jahangir Khajehali (khajeali@cc.iut.ac.ir) and Thomas Van Leeuwen (thomas.vanleeuwen@ugent.be)

Abstract

The European red mite, *Panonychus ulmi* (Koch), is one of the major pests of apple trees worldwide. Cyclic keto-enol compounds such as spirodiclofen and spiromesifen are frequently used to control phytophagous spider mites in agricultural crops, including *P. ulmi* on apple trees. Spider mites, however, can rapidly develop resistance against acaricides and, in this study, multiple *P. ulmi* populations from apple orchards in Iran were monitored for spirodiclofen and spiromesifen resistance. The Urmia and Shahin Dej population showed the highest spirodiclofen resistance ratio (more than 150-fold) compared to the susceptible Ahar population. Toxicity bioassays also revealed the presence of moderate cross-resistance between spiromesifen and spirodiclofen, but not towards the chitin synthase inhibitor etoxazole. As a first step towards elucidating spirodiclofen resistance mechanisms, the role of detoxification enzymes (cytochrome P450 monooxygenases, carboxyl/choline esterases and glutathione S-transferases) was investigated by *in vivo* synergism and *in vitro* enzyme assays. PBO pretreatment synergized spirodiclofen toxicity in the populations of Urmia and Shahin Dej to a higher extent than in the susceptible Ahar population. Furthermore, enzyme activity measurements showed relatively higher activity of detoxifying enzymes in the resistant populations. In conclusion, increased detoxification is most likely underlying spirodiclofen resistance and results in limited cross-resistance to spiromesifen.

Keywords: Insecticide resistance, Spirodiclofen, Spiromesifen, Cross-resistance, Synergism, Cytochrome P450 monooxygenase.

1. Introduction

Apple is one of the major agricultural productions worldwide (Golding & Jobling, 2012). There are many species of insects and mites that thrive in apple orchards and some can become

destructive to apple trees (Walker et al., 2017). The European red mite, *Panonychus ulmi* (Koch) (Acari: Tetranychidae) is an injurious mite of apple trees (Dekeyser, 2005). The species attacks about 147 host plants and is distributed in 71 countries around the world (Migeon & Dorkeld, 2019). Acaricides play a vital role in the control of *P. ulmi* and closely related mites such as the citrus red mite, *Panonychus citri*, and the two-spotted spider mite, *Tetranychus urticae* (Van Leeuwen, et al., 2015). Resistance development to synthetic pesticides, however, is a major challenge in the chemical control of spider mites. Many factors lead to rapid development of acaricide resistance in spider mites including high fecundity rates, reproduction through arrhenotokous parthenogenesis, a short life cycle and the frequency of acaricide applications (Van Leeuwen & Dermauw, 2016; Van Leeuwen et al., 2015, 2010).

Several chemical classes of acaricides with different modes of action have been discovered and used in control programs of mites (Sparks & Nauen, 2015; Van Leeuwen et al., 2015). Spirodiclofen and spiromesifen, two cyclic keto-enol compounds, have excellent acaricidal activity against phytophagous mites and a few pest insects (Dekeyser, 2005; Nauen et al., 2003). These acaricides inhibit acetyl-CoA carboxylase (ACCase) and interfere with the biosynthesis of lipids in insects and mites (Bretschneider et al., 2012). More specifically, Lümmen et al. (2014) showed that the enol metabolite of the related cyclic keto-enol insecticide, spirotetramat, inhibited *T. urticae* ACCase by interacting with the carboxyltransferase (CT) domain. A number of studies have investigated resistance in *P. ulmi* against acaricides such as clofentezine, dicofol, fenazaquin, tebufenpyrad, hexythiazox, pyridaben, fenpyroximate, abamectin and pyrethroids (Auger et al., 2003; Nauen et al., 2001; Pree et al., 2005; Rameshgar et al., 2019a; 2019b; Thwaite, 1991). Resistance to spirodiclofen has been documented in *T. urticae*, *P. citri* and *P. ulmi* (Demaeght et al., 2013; Kramer & Nauen, 2011; Ouyang et al., 2012; Rauch & Nauen,

2002; Van Pottelberge et al., 2009; Yu et al., 2011) while spiromesifen resistance has also been reported in *Trialeurodes vaporariorum* (Westwood) and *Bemisia tabaci* (Gennadius) (Bielza et al., 2019; Karatolos et al., 2012). Bielza et al. (2019) suggested the involvement of target-site insensitivity in resistance to spiromesifen and spirotetramat in *B. tabaci*, because of lack of a metabolic resistance mechanism. For spider mites, it has been shown that detoxification enzymes such as cytochrome P450 monooxygenases (P450s) are involved in spirodiclofen resistance, while carboxyl/choline esterases (CCEs) are much likely involved in sequestration of spirodiclofen (Demaeght et al., 2013; Kramer & Nauen, 2011; Wei et al., 2019, Bajda et al. 2015). In a recent study, spirodiclofen resistance in *T. urticae* was also mapped to a number of genomic loci (Quantitative Trait Loci (QTL) mapping), with one of the QTLs containing the gene encoding ACCase, the target-site of spirodiclofen (Wybouw et al., 2019). In the ACCase gene of the resistant strain that was used for the mapping experiment, a non-synonymous mutation, A1079T, was also identified. This mutation, however, was located outside the CT-domain of ACCase and awaits functional validation (Khajehali, 2010; Wybouw et al., 2019).

Etoxazole, an acaricide from the oxazoline family, has been registered for spider mite control in Iran (Noorbakhsh et al., 2016). It acts as a mite growth inhibitor and inhibits chitin synthesis in embryonic and immature stages (Demaeght et al., 2014; Van Leeuwen et al., 2012). There are many reports of etoxazole resistance in spider mites (Adesanya, et al., 2018; Demaeght et al., 2014; Herron et al., 2018; Lee et al., 2004; Wu et al., 2019). In *T. urticae*, an amino acid substitution of isoleucine to phenylalanine at position 1017 (I1017F) in the chitin synthase 1 gene (*CHS1*) has been confirmed to confer strong etoxazole resistance (Demaeght et al., 2014; Douris et al., 2016; Herron et al., 2018; Riga et al., 2017; Van Leeuwen et al., 2012; Wu et al., 2019).

Spirodiclofen has been widely used in Iranian apple orchards since 2005, but there is no information on resistance development in Iranian populations of *P. ulmi* to this acaricide. Therefore, the aim of this study was to determine the status of resistance of European red mite populations to spirodiclofen, as an essential step in resistance management and population control. Cross-resistance levels between spirodiclofen, spiromesifen and etoxazole were also assessed. Furthermore, using *in vivo* and *in vitro* assays, we investigated the involvement of detoxification enzymes in the resistance development.

2. Materials and methods

2.1. Field populations of P. ulmi

Eleven populations of *P. ulmi* were collected from East Azarbaijan (Mianeh, Marand, Ahar, and Maraqeh), West Azarbaijan (Urmia, Salmas, Shahin Dej, and Mahabad) and Isfahan (Semirom) during 2016 and 2017 (Table 1). The field-collected populations were kept on leaf discs of *Malus domestic*a var. Fuji, at 25 ±1°C, a photoperiod of 16:8h (light: dark), and 60% relative humidity (RH).

2.2. Acaricides and chemical materials

Bioassays were conducted with the commercial formulation of spirodiclofen (Envidor SC 24%, Bayer CropScience), spiromesifen (Oberon SC 24%, Bayer CropScience) and etoxazole (Baroque SC11%, Sumitomo Chemical, Japan). The synergists including diethylmaleate (DEM) and piperonyl butoxide (PBO) were provided from Sigma-Aldrich (Bornem, Belgium), and triphenyl phosphate (TPP) was provided from Merck (Darmstadt, Germany). Bovine serum albumin was used as a standard for protein determination (Sigma Aldrich, USA). For enzyme assays analytical grades of 3,3',5,5'-tetramethylbenzidine (TMBZ) (Sigma Aldrich, USA),

cytochrome C (Merck, Germany) and 1- chloro-2,4-dinitrobenzene (CDNB) (Sigma Aldrich) were purchased.

2.3. Toxicity assays on P. ulmi larvae

Intoxication symptoms of spirodiclofen on adult spider mite females are less pronounced (Nauen, 2005), so spirodiclofen toxicity bioassays were performed with mite larvae. The bioassay method described by Van Leeuwen et al. (2004) was used, with slight modifications. Briefly, 20–30 adult female mites were transferred to the upper side of apple leaf discs (12.25 cm^2 square-cut) on wet cotton wool, and allowed to lay eggs for 12 hours, after which they were removed. Leaf discs were kept in a climatically controlled room at 25 ± 1°C, 60% RH and 16: 8 h (light: dark) photoperiod. After larval emergence, leaf disks were sprayed with spray fluid (1.05 mL, 1.00 ± 0.03 mg aqueous acaricide deposit per cm^2, at 1 bar pressure) using a Potter spray tower (Burkard Scientific Ltd, Uxbridge, UK). Serial dilutions of spirodiclofen, spiromesifen and etoxazole were used in toxicity tests and a minimum of three replicates per concentration were considered for each assay, and distilled water was sprayed instead of acaricide in the control. Mortality was assessed after 6 days when adults appeared in the control. All control mortalities were lower than 10%. LC_{50} values, slopes and 95% confidence intervals were calculated by probit regression analysis using the software POLO-Plus. Resistance ratios (RRs) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the most susceptible strain (Robertson et al., 2017).

2.4. Synergism assays

Three synergists PBO, DEM and TPP are known as the inhibitors of P450s, glutathione-S-transferases (GSTs) and carboxyl/choline esterases (CCEs), respectively. Resistant and susceptible populations were pre-treated with synergists for 4h before performing spirodiclofen toxicity tests as described above. Larvae were pre-treated with synergists, diluted first in acetone and then mixed with water. In the control, larvae were treated with water + acetone. Based on the preliminary tests, synergist concentrations of PBO 200 mg/L, DEM 400 mg/L and TPP 100 mg/L caused less than 15% mortality on larvae. To estimate synergistic ratios (SRs) LC_{50} values of spirodiclofen were divided by LC_{50} values of spirodiclofen+ synergist (Van Pottelberge et al., 2009). Synergistic ratios and resistance ratios were considered to be significantly different when their 95% confidence intervals did not overlap (Robertson et al., 2017).

2.5. Enzymatic assays

In order to determine *in vitro* enzyme activities of P450s, CCEs and GSTs, about fifty female mites were homogenized. P450 activity was estimated by measuring heme peroxidase activity, using 3,3',5,5'-tetramethylbenzidine (TMBZ) and cytochrome C as substrate and standard, respectively. The assay was conducted according to Brogdon et al. (1997). Briefly, the total reaction volume was 650 µL, consisting of enzyme source (40 µL containing about 5.25µg protein), potassium phosphate buffer (160 µL, 0.625 M, pH 7.2), 50 µL H_2O_2 (3%) and 400 µL of TMBZ solution (0.01 g of TMBZ in 5 mL methanol plus 15 mL of 0.25 M sodium acetate buffer, pH 5.0). The reactions were incubated at room temperature for 2 h. The optical density was measured at 450 nm based on Tiwari et al. (2011) by using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA).

Esterase activity was determined using α-naphthyl acetate (α-NA) (6.4 mM) as the substrate (diluted in phosphate sodium buffer 0.2 M, pH 7) according to the method of Van Leeuwen et al. (2006). Enzyme samples (30 µL) were added to α-NA (200 µL), Fast Blue RR 0.2% (120 µL) and phosphate sodium buffer (0.2 M, pH 7, 200 µL). Finally, absorbance was read at 450 nm every 30 s for 5 min by using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA).

The activity of GSTs was measured using CDNB as substrate, based on the method of Habig et al. (1974). Enzyme samples (25 µl) were added to 200 µl of CDNB and 200 µl of reduced glutathione (GSH; 10 mM). Then, absorbance was read at 340 nm every 30 sec for 5 min using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA).

All protein concentrations were measured with a Coomassie protein assay according to the method of Bradford (1976), and bovine serum albumin (Sigma Aldrich, USA) was used as standard. Thirty µl of enzyme sample was mixed with 470 µl of Bradford reagent then incubated for 5 min at room temperature and absorbance was measured at 590 nm using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA).

Each enzyme test was performed in three replicates. Data represent the mean values of three replicates with the standard error. Analysis of variance (ANOVA) followed by Fisher's protected Least Significant Difference (LSD) mean separation tests was used to determine significant differences in the level of detoxifying enzyme activity between resistant and susceptible populations (GLM Procedure in SAS/STAT version 9.4).

3. Results

3.1. Resistance and cross-resistance in P. ulmi populations

Results of spirodiclofen toxicity against European red mite larvae are shown in Table 2. The Ahar population was found as the most susceptible population to spirodiclofen, with an LC_{50}

value of 2.05 mg active ingredient per liter (mg a.i. L^{-1}). Populations of Urmia and Shahin Dej were most resistant to spirodiclofen with LC_{50} values of 443 and 306 mg a.i. L^{-1}, respectively. Strains Marand, Salmas, Meianeh 2 and Maragheh showed moderate resistance levels with resistance ratios of 20-, 16-, 15- and 11-fold, respectively (Table 2).

Urmia and Shahin Dej populations were found 22.2- and 21.7-fold resistant to spiromesifen, respectively (Table 2). There was a strong correlation between $\log_{10}$ LC_{50} values of spirodiclofen and spiromesifen (r =0.96 and p= 0.0001). The Urmia and Shahin Dej populations were 6.71- and 11.8-fold resistant to etoxazole, respectively (Table 3). There was a relatively weak correlation between $\log_{10}$ LC_{50} values of spirodiclofen and etoxazole (r = 0.79 and p= 0.018). Similarly, the correlation between $\log_{10}$ LC_{50} values of spiromesifen and etoxazole was also week (r = 0.8 and p= 0.017)

3.2. Synergism studies

Results of synergism studies using PBO, TPP, and DEM on selected resistant and susceptible populations are given in Table 4. Pretreatment with PBO significantly synergized spirodiclofen toxicity in resistant Shahin Dej and Urmia populations, but not in the susceptible Ahar population. Pretreatment with DEM and TPP had no significant effect on spirodiclofen toxicity in the tested populations.

3.3. Enzymatic assays

Detoxification enzyme activities in some resistant and susceptible populations are presented in Table 5. The P450 activity in the resistant population of Urmia was significantly higher when compared to strain Ahar (2.01-fold). In addition, significant differences in CCE activity were observed between the susceptible and resistant populations. Esterase activities in Shahin Dej and

Urmia populations were found 1.28 and 1.84 times higher compared to Ahar, respectively. The activities of GSTs in the Shahin Dej and Urmia populations were estimated 3.08 and 3.54 times higher when compared to strain Ahar, respectively.

4. Discussion

European red mite has developed resistance to most classes of acaricides (Mota-Sanchez & Wise, 2019). Tetronic acid acaricides such as spirodiclofen and spiromesifen, and chitin synthase-1 (CHS-1) inhibitors like etoxazole, have high activity on juvenile stages of mites (Demaeght et al., 2014; Sparks & Nauen, 2015; Van Leeuwen et al., 2012, 2015), and have been used extensively to control mites of agricultural importance (Van Leeuwen et al., 2015). Development of resistance to acaricides and insecticides is one of the major problems for sustainable chemical control measures of pests (Hemingway, 2018; Van Leeuwen & Dermauw, 2016; Van Leeuwen et al., 2010). There are a number of reports on resistance to spirodiclofen, spiromesifen, and etoxazole in spider mites, especially in *T. urticae* (Demaeght et al., 2014; Ferreira et al., 2015; Herron et al., 2018; Kramer & Nauen, 2011; Lee et al., 2004; Van Leeuwen & Dermauw, 2016; Wu et al., 2019). However, very few studies have been conducted on monitoring and characterization of *P. ulmi* resistance to spirodiclofen (Bajda et al., 2015; Kramer & Nauen, 2011).

In this study, eleven populations were collected and tested from three important areas of Iranian apple production. The results indicated high spirodiclofen resistance levels in strains collected in Urmia and Shahin Dej, exhibiting resistance ratios of 217- and 149-fold, respectively (Table 2). Such a high resistance level to spirodiclofen has also been reported in some laboratory selected strains of *T. urticae* and *P. ulmi* (Demaeght et al., 2013; Rauch & Nauen, 2002; Van Pottelberge et al., 2009), but up till now, has only been found in Brazilian field populations of *T.*

urticae (Demaeght, 2015; Hu et al., 2010; Kramer & Nauen, 2011; Rauch & Nauen, 2002; Van Pottelberge et al., 2009, Ferreira et al. 2015). Previous studies have reported a 60-fold spirodiclofen resistance ratio in field-collected populations of *P. ulmi*. However, this resistance ratio increased to 7000-fold by laboratory selection, indicating the strong potential of *P. ulmi* to develop resistance levels compromising the field-efficacy of recommended label rates (Kramer & Nauen, 2011).

Cross resistance between acaricides targeting immature stages, such as spirodiclofen, spiromesifen and etoxazole, is another challenge for the implementation of resistance management strategies in the field (Kramer & Nauen, 2011; Van Pottelberge et al., 2009; Wu et al., 2019). Bioassays with spiromesifen, another tetronic acid acaricide, indicated that spirodiclofen resistant populations were moderately cross-resistant to spiromesifen: the populations collected in Urmia and Shahin Dej exhibited a 22- and 21-fold resistance to spiromesifen, respectively (Table 2). A strong correlation was found between spirodiclofen resistance and resistance to spiromesifen in *P. ulmi* populations, suggesting cross resistance between these compounds. However, spiromesifen is not registered for spider mite control in apple orchards in Iran (Noorbakhsh et al., 2016). Resistance ratios against spiromesifen (about 20-fold) were moderate compared to spirodiclofen resistance ratios (more than 150-fold), but are similar to those reported for a spirodiclofen resistant strain of *P. ulmi* (PSR-TK) (Kramer & Nauen, 2011) and a spirodiclofen resistant strain of *T. urticae* (SR-VP) (Van Pottelberge et al., 2009). In addition, cross resistance between spirodiclofen and spirotetramat, another inhibitor of ACCase, has also been reported in *P. citri* (Yu et al., 2011).

The LC_{50} values and RRs of tested *P. ulmi* populations against etoxazole, a mite growth inhibitor acting on chitin synthase-1, showed that spirodiclofen resistant populations of Shahin

Dej and Urmia were 11- and 6-fold resistant to etoxazole, respectively. Correlation analysis of spirodiclofen and etoxazole indicated a relatively weak correlation between spirodiclofen resistance and etoxazole resistance. Because of this weak correlation and the fact that etoxazole is also registered for spider mite control in Iran (Noorbakhsh et al., 2016), it is not clear whether this low etoxazole resistance is caused by elevated levels of detoxification enzymes targeting spirodiclofen, or if it has been selected in the field by frequent etoxazole applications. True cross-resistance is only possible by metabolic mechanisms as both compounds have a different mode of action. Nevertheless, considering the low levels of resistance, etoxazole still seems a viable alternative for spirodiclofen in regions where spirodiclofen resistance has been reported.

Two mechanism have been associated with resistance to cyclic keto-enols in insects and mites (Demaeght et al., 2013; Karatolos et al., 2012), including point mutations in ACCase (Karatolos et al., 2012; Wybouw et al., 2019), and metabolic resistance via enhanced P450 or CCE activity (Demaeght et al., 2013; Wei et al., 2019; Pan et al., 2018). ACCase mutations E645K and A1079T reported by Karatolos et al. (2012) and Wybouw et al. (2019), respectively, are located outside the CT domain where cyclic ketoenols bind and are unlikely to confer the observed levels of resistance. In this study, we investigated metabolic resistance of *P. ulmi* populations to cyclic keto-enols using synergistic assays and *in vitro* measurement of detoxification enzyme activities.The synergist PBO increased spirodiclofen toxicity about 2- and 3-fold in the Urmia and Shahin Dej population, respectively. Similarly, in a highly spirodiclofen resistant strain of *P. ulmi* (RR= 7000-fold), it has been reported that toxicity of spirodiclofen increased by approximately 3-fold after pretreatment with PBO (Kramer & Nauen, 2011). Furthermore, P450 activity in the Urmia population was estimated 2 times higher than that of the Ahar population. This is much less than the 11-fold higher P450 activity found in a spirodiclofen resistant *T.*

urticae strain (Van Pottelberge et al., 2009). In the latter study a different enzyme assay was used (using 7-ethoxy-4-trifluoromethylcoumarin as substrate), so a direct comparison to our data is difficult because the TMBZ assay measures heme content rather than true P450 activity. TPP pretreatment on resistant populations did not significantly enhance toxicity of spirodiclofen in the Urmia and Shahin Dej population. Similarly, another esterase inhibitor, DEF (S,S,S-tributyl phosphorotrithioate), had no effect on spirodiclofen toxicity in a spirodiclofen resistant strain (PSR-TK) of *P. ulmi* (Kramer & Nauen, 2011). In contrast, synergism and enzymatic assays have previously pointed out the involvement of CCEs in spirodiclofen resistance in spider mites (Van Pottelberge et al., 2009), which was subsequently linked to the overexpression of a single esterase isozyme (Wei et al., 2019). Considering the significantly higher activity levels of esterases found in Urmia and Shahin Dej populations compared to the Ahar population (Table 5), the potential involvement of CCEs in spirodiclofen resistance in Iranian *P. ulmi* populations cannot be ruled out. Based on the synergism assays, spirodiclofen toxicity was not affected by DEM pretreatment in the Shahin Dej and Urmia population. Similarly, in other *P. ulmi* and *T. urticae* populations resistant to spirodiclofen, DEM did not synergize spirodiclofen toxicity (Kramer & Nauen, 2011; Van Pottelberge et al., 2009). However, in line with Van Pottelberge et al., 2009, a significantly higher (more than 3-fold) GST activity was found in the Urmia and Shahin Dej population. As the synergist DEM depletes glutathione and thereby, much likely, inhibits all GSTs, the higher GST activity found in spirodiclofen resistant strains might not be related to spirodiclofen resistance, but potentially other compounds.

Given the moderate cross-resistance between spiromesifen and spirodiclofen it seems rather unlikely that target-site resistance is involved. Furthermore, the synergism of spirodiclofen

toxicity with PBO is fairly high, thus rendering target-site resistance unlikely, too. Nevertheless, to exclude any contribution of target-site resistance the ACCase gene should be sequenced.

To conclude, the present study showed that some field populations in Iran are highly resistant to spirodiclofen and showed moderate levels of cross resistance to spiromesifen. Synergistic assays and enzymatic assays indicated that cytochrome P450 monooxygenases and CCEs are likely to be involved in spirodiclofen resistance. Further investigations are necessary to more precisely elucidate which genes are involved in *P. ulmi* resistance to cyclic keto-enols.

Acknowledgements

The authors are grateful for financial support of this work by the Research Council of Isfahan University of Technology. This project was in part funded by the Research Foundation Flanders (FWO, Belgium) (grant G009312N to TVL and grant G053815N to TVL and WD). TVL received support from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program [grant 772026-POLYADAPT and 773902-SuperPests]. During this study WD was a postdoctoral fellow of the Research Foundation Flanders (FWO).

References

Adesanya, A. W., Morales, M. A., Walsh, D. B., Lavine, L. C., Lavine, M. D., & Zhu, F., 2018. Mechanisms of resistance to three mite growth inhibitors of *Tetranychus urticae* in hops. Bull. Entomol. Res. 108, 23-34. https://doi.org/10.1017/S0007485317000414.

Auger, P., Bonafos, R., Guichou, S., & Kreiter, S., 2003. Resistance to fenazaquin and tebufenpyrad in *Panonychus ulmi* Koch (Acari: Tetranychidae) populations from South of France apple orchards. Crop Prot. 22, 1039–1044. https://doi.org/10.1016/S0261-2194(03)00136-4.

Bajda, S., Dermauw, W., Greenhalgh, R., Nauen, R., Tirry, L., Clark, R. M., & Van Leeuwen, T., 2015. Transcriptome profiling of a spirodiclofen susceptible and resistant strain of the European red mite *Panonychus ulmi* using strand-specific RNA-seq. BMC Genomics. 16, 974. https://doi.org/10.1186/s12864-015-2157-1.

Bielza, P., Moreno, I., Belando, A., Grávalos, C., Izquierdo, J., & Nauen, R., 2019. Spiromesifen and spirotetramat resistance in field populations of *Bemisia tabaci* Gennadius in Spain. Pest Manage. Sci. 75, 45–52. https://doi.org/10.1002/ps.5144.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Bretschneider, T., Fischer, R., Nauen, R., & Ag, C., 2012. Tetronic acid insecticides and acaricides inhibiting acetyl-CoA carboxylase, in: Lamberth, C., Dinges, J. (Eds.), Bioactive Heterocyclic Compound Classes: Agrochemicals, Wiley VCH, Weinheim, pp. 265-278.

Brogdon, W. G., McAllister, J. C., & Vulule, J., 1997. Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. J. Am. Mosq. Control Assoc. 13, 233–237.

Dekeyser, M. A., 2005. Acaricide mode of action. Pest Manage. Sci. 61, 103–110. https://doi.org/10.1002/ps.994.

Demaeght, P., 2015. A genomic approach to investigate resistance mechanisms in the two-spotted spider mite *Tetranychus urticae*. Ph.D. Thesis, University of Amsterdam, Amsterdam, The Netherlands.

Demaeght, P., Dermauw, W., Tsakireli, D., Khajehali, J., Nauen, R., Tirry, L., Vontas, J., Lümmen, P., & Van Leeuwen, T., 2013. Molecular analysis of resistance to acaricidal spirocyclic tetronic acids in *Tetranychus urticae*: CYP392E10 metabolizes spirodiclofen, but not its corresponding enol. Insect Biochem. Mol. Biol. 43, 544–554. https://doi.org/10.1016/j.ibmb.2013.03.007.

Demaeght, P., Osborne, E. J., Odman-Naresh, J., Grbić, M., Nauen, R., Merzendorfer, H., Hans C., Richard M., & Van Leeuwen, T., 2014. High resolution genetic mapping uncovers chitin synthase-1 as the target-site ofthe structurally diverse mite growth inhibitors clofentezine, hexythiazox and etoxazole in *Tetranychus urticae*. Insect Biochem. Mol. Biol. 51, 52–61. https://doi.org/10.1016/j.ibmb.2014.05.004.

Douris, V., Steinbach, D., Panteleri, R., Livadaras, I., Pickett, J. A., Van Leeuwen, T., Nauen, R., & Vontas, J., 2016. Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of action on chitin biosynthesis. Proc. Natl. Acad. Sci. 113, 14692–14697. https://doi.org/10.1073/pnas.1618258113.

Ferreira, C. B. S., Andrade, F. H. N., Rodrigues, A. R. S., Siqueira, H. A. A., & Gondim Jr, M. G. C., 2015. Resistance in field populations of *Tetranychus urticae* to acaricides and characterization of the inheritance of abamectin resistance. Crop Protec. 67, 77–83. https://doi.org/10.1016/j.cropro.2014.09.022.

Golding, J., & Jobling, J., 2012. Apples. Crop Post-Harvest: Science and Technology: Perishables, pp. 88–107. https://doi.org/10.1002/9781444354652.ch5.

Habig, W. H., Pabst, M. J., & Jakoby, W. B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hemingway, J., 2018. Resistance: A problem without an easy solution. Pestic. Biochem. Physiol. 151, 73-75. https://doi.org/10.1016/j.pestbp.2018.08.007.

Herron, G. A., Woolley, L. K., Langfield, K. L., & Chen, Y., 2018. First detection of etoxazole resistance in Australian two-spotted mite *Tetranychus urticae* Koch (Acarina: Tetranychidae) via bioassay and DNA methods. Austral Entomol. 57, 365–368. https://doi.org/10.1111/aen.12290.

Hu, J., Wang, C., Wang, J., You, Y., & Chen, F., 2010. Monitoring of resistance to spirodiclofen and five other acaricides in *Panonychus citri* collected from Chinese citrus orchards. Pest Manage. Sci. 66, 1025–1030. https://doi.org/10.1002/ps.1978.

Karatolos, N., Williamson, M. S., Denholm, I., Gorman, K., Ffrench-Constant, R., & Nauen, R., 2012. Resistance to spiromesifen in *Trialeurodes vaporariorum* is associated with a single amino acid replacement in its target enzyme acetyl-coenzyme A carboxylase. Insect Molec. Biol. 21, 327–334. https://doi.org/10.1111/j.1365-2583.2012.01136.x.

Khajehali, J., 2010. Biochemical and molecular mechanisms of acaricide resistance in *Tetranychus urticae*. Ph.D. Thesis, Ghent University, Ghent, Belgium.

Kramer, T., & Nauen, R., 2011. Monitoring of spirodiclofen susceptibility in field populations of European red mites, *Panonychus ulmi* (Koch) (Acari: Tetranychidae), and the cross-resistance pattern of a laboratory-selected strain. Pest Manage. Sci. 67, 1285–1293. https://doi.org/10.1002/ps.2184.

Lee, S.-Y., Kim, G. H., Ahn, K. S., Kim, C. S., & Shin, S. C., 2004. Inheritance and stability of etoxazole resistance in twospotted spider mite, *Tetranychus urticae*, and its cross resistance. Korean J. Appl. Entomol. 43: 43-48.

Lümmen, P., Khajehali, J., Luther, K., & Van Leeuwen, T., 2014. The cyclic keto-enol insecticide spirotetramat inhibits insect and spider mite acetyl-CoA carboxylases by interfering with the carboxyltransferase partial reaction. Insect Biochem. Molec. Biol. 55, 1–8. https://doi.org/10.1016/j.ibmb.2014.09.010.

Migeon, A., & Dorkeld, F., 2019. Spider Mites Web: A comprehensive database for the Tetranychidae. Available from http://www1.montpellier.inra.fr/CBGP/spmweb/. (12/June/2019).

Mota-Sanchez, D., and Wise. J.C., 2019. The Arthropod Pesticide Resistance Database. Michigan State University. on-line at: http:// www.pesticideresistance.org.

Nauen, R., 2005. Spirodiclofen: mode of action and resistance risk assessment in Tetranychid pest mites. J. Pest Sci. 30, 272–274. https://doi.org/10.1584/jpestics.30.272.

Nauen, R., Bretschneider, T., Elbert, A., Fischer, R., & Tieman, R., 2003. Spirodiclofen and

Spiromesifen. Pestic. Outlook. 14, 243–246. https://doi.org/10.1039/b314855f.

Nauen, R., Stumpf, N., Elbert, A., Zebitz, C. P., & Kraus, W., 2001. Acaricide toxicity and resistance in larvae of different strains of *Tetranychus urticae* and *Panonychus ulmi* (Acari: Tetranychidae). Pest Manage. Sci. 57, 253–261. https://doi.org/10.1002/ps.280.

Noorbakhsh, S., Sahraian, H., Soroosh, M. J., Rezaii, V., & Fotoohi, A. R., 2016. List of pests, diseases and weeds of major agricultural products and the recommended methods for their control. Plant Protection Organization.[in Persian]. Iran: Ministry of Jihad-e-Agriculture.

Ouyang, Y., Montez, G. H., Liu, L., & Grafton-Cardwell, E. E., 2012. Spirodiclofen and spirotetramat bioassays for monitoring resistance in citrus red mite, *Panonychus citri* (Acari: Tetranychidae). Pest Manage. Sci. 68, 781–787. https://doi.org/10.1002/ps.2326.

Pan, Y., Chai, P., Zheng, C., Xu, H., Wu, Y., Gao, X., Xi, J., & Shang, Q., 2018. Contribution of cytochrome P450 monooxygenase CYP380C6 to spirotetramat resistance in *Aphis gossypii* Glover. Pestic. Biochem. Physiol. 148, 182–189. https://doi.org/10.1016/j.pestbp.2018.04.015.

Pree, D. J., Whitty, K. J., & Van Driel, L., 2005. Baseline susceptibility and cross resistances of some new acaricides in the European red mite, *Panonychus ulmi*. Exp. Appl. Acarol. 37, 165–171. https://doi.org/10.1007/s10493-005-1423-3.

Rameshgar, F., Khajehali, J., Nauen, R., Bajda, S., Jonckheere, W., Dermauw, W., & Van Leeuwen, T., 2019a. Point mutations in the voltage-gated sodium channel gene associated with pyrethroid resistance in Iranian populations of the European red mite *Panonychus ulmi*. Pestic. Biochem. Physiol. 157, 80–87. https://doi.org/10.1016/j.pestbp.2019.03.008.

Rameshgar, F., Khajehali, J., Nauen, R., Dermauw, W., & Van Leeuwen, T., 2019b. Characterization of abamectin resistance in Iranian populations of European red mite, *Panonychus ulmi* Koch (Acari: Tetranychidae). Crop Protec. 125, 104903. https://doi.org/10.1016/j.cropro.2019.104903.

Rauch, N., & Nauen, R., 2002a. Spirodiclofen resistance risk assessment in Tetranychus urticae (Acari: Tetranychidae): a biochemical approach. Pestic. Biochem. Physiol. 74, 91–101.

Riga, M., Bajda, S., Themistokleous, C., Papadaki, S., Palzewicz, M., Dermauw, W., Vontas, J., & Van Leeuwen, T., 2017. The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in *Tetranychus urticae*. Sci. Rep. 7, 9202. https://doi.org/10.1038/s41598-017-09054-y.

Robertson, J. L., Jones, M. M., Olguin, E., & Alberts, B., 2017. Bioassays with arthropods. CRC press, Boca Raton, Florida, US.

Sparks, T. C., & Nauen, R., 2015. IRAC: Mode of action classification and insecticide resistance management. Pestic. Biochem. Physiol. 121, 122–128. https://doi.org/10.1016/j.pestbp.2014.11.014.

Thwaite, W. G., 1991. Resistance to clofentezine and hexythiazox in *Panonychus ulmi* from apples in Australia. Exp. Appl. Acarol. 11, 73–80. https://doi.org/10.1007/BF01193730.

Tiwari, S., Mann, R. S., Rogers, M. E., & Stelinski, L. L., 2011. Insecticide resistance in field populations of Asian citrus psyllid in Florida. Pest Manage. Sci. 67, 1258–1268. https://doi.org/10.1002/ps.2181.

Van Leeuwen, T., Demaeght, P., Osborne, E. J., Dermauw, W., Gohlke, S., Nauen, R., Grbić,

M., Tirry, L., Merzendorfer, H., & Clark, R. M., 2012. Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc. Natl. Acad. Sci. U.S.A.*109*, 4407–4412. https://doi.org/10.1073/pnas.1200068109.

Van Leeuwen, T., & Dermauw, W., 2016. The Molecular Evolution of Xenobiotic Metabolism and Resistance in Chelicerate Mites. Annu. Rev. Entomol. 61, 475–498. https://doi.org/10.1146/annurev-ento-010715-023907.

Van Leeuwen, T., Stillatus, V., & Tirry, L., 2004. Genetic analysis and cross-resistance spectrum of a laboratory-selected chlorfenapyr resistant strain of two-spotted spider mite (Acari: Tetranychidae). Exp. Appl. Acarol. 32, 249–261.

Van Leeuwen, T., Tirry, L., Yamamoto, A., Nauen, R., & Dermauw, W., 2015. The economic importance of acaricides in the control of phytophagous mites and an update on recent acaricide mode of action research. Pestic. Biochem. Physiol. 121, 12–21. https://doi.org/10.1016/j.pestbp.2014.12.009

Van Leeuwen, T., Van Pottelberge, S., & Tirry, L., 2006. Biochemical analysis of a chlorfenapyr-selected resistant strain of *Tetranychus urticae* Koch. Pest Manage. Sci. 62, 425–433. https://doi.org/10.1002/ps.1183

Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W., & Tirry, L., 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: A review. Insect Biochem. Mol. Biol. 40, 563–572. https://doi.org/10.1016/j.ibmb.2010.05.008.

Van Pottelberge, S., Van Leeuwen, T., Khajehali, J., & Tirry, L., 2009. Genetic and biochemical analysis of a laboratory-selected spirodiclofen-resistant strain of *Tetranychus urticae* Koch (Acari: Tetranychidae). Pest Manage. Sci. 65, 358–366. https://doi.org/10.1002/ps.1698.

Walker, J. T. S., Suckling, D. M., & Wearing, C. H. (2017). Past, Present, and Future of Integrated Control of Apple Pests: The New Zealand Experience. Annu. Rev. Entomol. 62, 231–248. https://doi.org/10.1146/annurev-ento-031616-035626.

Wei, P., Demaeght, P., De Schutter, K., Grigoraki, L., Labropoulou, V., Riga, M., Vontas, J., Nauen, R., Dermauw, W., & Van Leeuwen, T. (2019). Overexpression of an alternative allele of carboxyl/choline esterase 4 (CCE04) of *Tetranychus urticae* is associated with high levels of resistance to the keto-enol acaricide spirodiclofen. Pest Manage. Sci. 76, 1142-1153. https://doi.org/10.1002/ps.5627.

Wu, M., Adesanya, A. W., Morales, M. A., Walsh, D. B., Lavine, L. C., Lavine, M. D., & Zhu, F. (2019). Multiple acaricide resistance and underlying mechanisms in *Tetranychus urticae* on hops. J. Pest Sci. 92, 543–555. https://doi.org/10.1007/s10340-018-1050-5.

Wybouw, N., Kosterlitz, O., Kurlovs, A. H., Bajda, S., Greenhalgh, R., Snoeck, S., Bui, H., Bryon, A., Dermauw, W., Van Leeuwen, T., & Clark, R. M., 2019. Long-Term population studies uncover the genome structure and genetic basis of xenobiotic and host plant adaptation in the herbivore *Tetranychus urticae*. Genetics. 211, 1409–1427. https://doi.org/10.1534/genetics.118.301803.

Yu, D.-Y., Wang, C.-F., Yu, Y., Huang, Y.-Q., Yao, J.-A., & Hu, J.-F., 2011. Laboratory selection for spirodiclofen resistance and cross-resistance in *Panonychus citri*. Afr. J. Biotechnol. 10, 3424–3429. https://doi.org/10.5897/AJB10.2417.

Table 1. Sampling date and geographic origin of *P. ulmi* Populations collected from Iran

Population	Sampling date	Life stage	Latitude and longitude
Ahar	12/7/2016	Adults/nymphs	**38°28′39″N 47°04′12″E**
Semirom1	7/6/2017	Adults	**36°45′47″N 45°43′20″E**
Semirom2	7/6/2017	Adults	**36°45′47″N 45°43′20″E**
Mahabad	13/7/2017	Adults	**36°45′47″N 45°43′20″E**
Mianeh1	12/7/2016	Adults/nymphs	**37°25′16″N 47°42′54″E**
Maraqeh	13/7/2017	Adults	**37°23′21″N 46°14′15″E**
Mianeh2	13/7/2017	Adults	**37°25′16″N 47°42′54″E**
Salmas	3/7/2017	Adults	**38°11′41″N 44°45′53″E**
Marand	13/7/2017	Adults	**38°25′58″N 45°46′30″E**
Shahin dej	12/7/2016	Adults/nymphs	**36°40′45″N 46°34′01″E**
Urmia	3/7/2017	Adults	**37°33′19″N 45°04′21″E**

Table 2. Log-dose probit-mortality data for tetronic acid acaricides tested against larvae of *P. ulmi* field populations.

Acaricide	Population	n*	LC_{50} mg a.i. L^{-1} (Cl 95%)	Slope±SE	χ^2 (df)	RR** (Cl 95%)***
Spirodiclofen	Ahar	356	2.05 (0.87 -3.38)	1.52 ± 0.26	2.90 (4)	
	Shahin Dej	451	306 (185 -449)	1.11 ± 0.14	1.22 (5)	149 (69.6 – 321)
	Mahabad	438	4.76 (2.81 -6.95)	1.44 ± 0.19	1.58 (4)	2.32 (1.08 – 5.01)
	Mianeh1	298	7.06 (4.15 -10.1)	1.49 ± 0.25	0.44 (4)	3.45 (1.62 – 7.34)
	Semirom1	417	2.44 (1.77 -3.16)	1.25 ± 0.13	2.09 (4)	1.19 (0.6 – 2.38)
	Semirom2	387	2.79 (2.31 -3.41)	1.64 ± 0.2	1.15 (3)	1.36 (0.71 – 2.63)
	Urmia	425	443 (243 -737)	0.83 ± 0.10	7.61 (9)	217 (94.1 – 498)
	Salmas	197	33.6 (23.9 -44.3)	1.90 ± 0.27	0.47 (3)	16.4 (8.19 – 33.0)
	Marand	516	42.0 (27.2 -61.3)	1.07 ± 0.15	2.31 (6)	20.6 (9.78 – 43.2)
	Maraqeh	228	22.7 (17.2 -28.5)	1.92 ± 0.25	0.25 (3)	11.1 (5.63 – 21.8)
	Mianeh2	309	32.5 (19.0 -48.2)	1.41 ± 0.24	2.69 (3)	15.9 (7.37 – 34.2)
Spiromesifen	Ahar	194	0.30 (0.12 – 0.56)	1.24 ± 0.34	0.21 (3)	
	Shahin Dej	219	6.60 (5.05 – 8.18)	2.51 ± 0.37	1.20 (3)	21.7 (10.8 – 43.4)
	Mahabad	225	0.40 (0.21 – 0.69)	1.38 ± 0.32	0.51 (3)	1.30 (0.55 – 3.04)
	Mianeh1	466	0.40 (0.30 – 0.50)	1.24 ± 0.34	2.05 (3)	1.32 (0.66 – 2.66)
	Semirom1	356	0.35 (0.28 – 0.42)	2.25 ± 0.30	0.66 (2)	1.15 (0.58 – 2.27)
	Semirom2	202	0.39 (0.22 – 0.62)	1.30 ± 0.26	0.23 (3)	1.28 (0.57 – 2.87)
	Urmia	164	6.77 (4.84 – 9.10)	2.53 ± 0.56	1.79 (2)	22.2 (10.9 – 45.4)
	Salmas	430	0.92 (0.63 – 1.30)	1.20 ± 0.17	1.60 (4)	3.02 (1.43 – 6.36)
	Marand	185	2.19 (1.15 – 3.23)	1.51 ± 0.28	0.31 (3)	7.19 (3.12 – 16.1)
	Maraqeh	189	1.78 (1.14 – 2.65)	1.62 ± 0.38	1.07 (2)	5.85 (2.74 – 12.5)
	Miyaneh2	171	0.89 (0.55 – 1.37)	1.62 ± 0.40	0.39 (2)	2.91 (1.35 – 6.29)

*Number of mites tested.
** Resistance ratio = LC_{50}/LC_{50} Ahar.
***Confidence interval.

Table 3. Log-dose probit-mortality data for a mite growth inhibitor acaricide (etoxazole) tested against the larvae of *P. ulmi* field populations.

Population	n*	LC_{50} mg a.i. l^{-1} (Cl 95%)	Slope±SE	$\chi 2$ (df)	RR** (Cl 95%)***
Ahar	254	0.30 (0.16 – 0.46)	1.15 ± 0.26	0.93 (3)	
Shahin Dej	220	3.51 (2.44 – 4.67)	0.37 ± 1.89	0.62 (2)	11.8 (6.72 – 20.9)
Mahabad	182	1.46 (0.49 – 3.25)	0.33 ± 1.12	0.61 (4)	4.94 (1.98 – 12.3)
Mianeh 1	250	0.72 (0.44 – 1.03)	0.34 ± 1.74	1.04 (3)	2.45 (1.31 – 4.54)
Semirom 1	286	0.30 (0.18 – 0.41)	0.31 ± 1.55	0.61 (2)	1.03 (0.56 – 1.90)
Semirom 2	296	0.77 (0.43 – 1.05)	0.53 ± 2.18	0.99 (3)	2.59 (1.39 – 4.79)
Urmia	220	1.99 (1.20 – 2.77)	0.44 ± 2.46	0.01 (3)	6.71 (3.61 – 12.5)
Marand	217	4.14 (2.38 – 6.36)	0.27 ± 1.32	1.57 (3)	14 (7.21 – 27.1)

*Number of mites tested.
** Resistance ratio = LC_{50}/LC_{50} Ahar.
***Confidence interval.

Table 4. Synergistic effect of PBO (200 mg L^{-1}), TPP (100 mg L^{-1}), and DEM (400 mg L^{-1}) on spirodiclofen resistance in *P. ulmi* populations, compared to the susceptible population of Ahar

	N*	LC_{50} mg a.i. l^{-1} (CI 95%)	Slope±SE	χ^2 (df)	RR** (CI 95%)***	SR**** (CI 95%)
Ahar						
Spirodiclofen	356	2.05 (0.88- 3.38)	1.52±0.26	2.90 (4)		
+PBO	189	1.64 (0.83–2.72)	1.27±0.27	0.53 (4)		1.25 (0.54 – 2.86)
+DEM	213	2.27 (1.11–4.04)	1.24±0.28	0.50 (4)		0.90 (0.38 – 2.13)
+TPP	159	2.07 (1.00–4.51)	1.04±0.29	0.19 (3)		0.99 (0.40 – 2.43)
Shahin Dej						
Spirodiclofen	451	306 (185–449)	1.11±0.14	1.22 (5)	149 (69.6-321)	
+PBO	392	100 (53.3–147)	1.78±0.29	0.12 (3)	61.2 (126-29.8)	3.05 (1.60 – 5.80)
+DEM	176	340 (174–595)	1.06±0.26	0.20 (3)	150 (333-67.2)	0.90 (0.45 – 1.81)
+TPP	139	241.2 (109–393)	1.44±0.34	0.21 (3)	117 (274-49.7)	1.27 (0.62 – 2.58)
Urmia						
Spirodiclofen	425	443 (243–737)	0.83±0.10	7.61 (9)	216 (94.1-498)	
+PBO	288	206 (115–323)	1.10±0.20	0.99 (4)	126 (60.8-260)	2.15 (1.03–4.46)
+DEM	124	460 (226–713)	1.64±0.40	0.33 (3)	202 (93.7-437)	0.96 (0.46–2.03)
+TPP	234	316 (163–504)	1.21±0.29	0.57 (3)	153 (67.9-345)	1.40 (0.67–2.94)

*Number of mites tested.

** Resistance ratio = LC_{50}/LC_{50} Ahar.

***Confidence interval.

****Synergistic ratio.

Table 5. Detoxification enzyme activities in different populations of *P. ulmi* (mean ± SEM).

Population	P450s TMBZ*	Ratio	GSTs CDNB conjugation**	Ratio	Esterases α-Naphthyl Acetate***	Ratio
Ahar	11.6 ± 0.31[b]		67.8 ± 3.63[b]		4614 ± 1.96[c]	
Shahin Dej	7.84 ± 4.42[b]	0.67	209 ± 4.42[a]	3.08	5891 ± 82.1[b]	1.28
Urmia	23.3 ± 1.97[a]	2.01	240 ± 29.9[a]	3.54	8507 ± 297[a]	1.84

* Equivalent units of cytochrome P450 mg^{-1} protein (± SEM).

**nmol glutathione conjugated min^{-1} mg^{-1} protein (± SEM).

***nmol 1-naphthol min^{-1} mg^{-1} protein (± SEM).

Significant differences are indicated by different letters within the columns (one-way ANOVA, $p < 0.05$; LSD test).