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# Molecular analysis of resistance to acaricidal spirocyclic tetronic acids in *Tetranychus urticae*: CYP392E10 metabolizes spirodiclofen, but not its corresponding enol

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ABSTRACT

Spirodiclofen is one of the most recently developed acaricides and belongs to the new family of spirocyclic tetronic acids (ketoenols). This new acaricidal family is an important chemical tool in resistance management strategies providing sustainable control of spider mites such as *Tetranychus urticae*. Spirodiclofen targets lipid biosynthesis mediated by direct inhibition of acetyl coenzyme A carboxylase (ACCase). In this study, we investigated two genetically distant spider mite strains with high resistance to spirodiclofen. Despite the strong resistance levels to spirodiclofen (up to 680-fold), only limited cross-resistance with other members of this group such as spiromesifen and spirotetramat could be detected. Amplification and sequencing of the *ACCase* gene from resistant and susceptible strains did not reveal common non-synonymous mutations, and expression levels of ACCase were similar in both resistant and susceptible strains, indicating the absence of target-site resistance. Furthermore, we collected genome-wide expression data of susceptible and resistant *T. urticae* strains using microarray technology. Analysis of differentially expressed genes revealed a broad response, but within the overlap of two resistant strains, several cytochrome P450s were prominent. Quantitative PCR confirmed the constitutive over-expression of *CYP392E7* and *CYP392E10* in resistant strains, and *CYP392E10* expression was highly induced by spirodiclofen. Furthermore, stage specific expression profiling revealed that expression levels were not significantly different between developing stages, but very low in eggs, matching the age-dependent resistance pattern previously observed. Functional expression of *CYP392E7* and *CYP392E10* confirmed that CYP392E10 (but not CYP392E7) metabolizes spirodiclofen by hydroxylation as identified by LC–MS/MS, and revealed cooperative substrate binding and a $K_m$ of 43 μM spirodiclofen. CYP392E10 also metabolizes spiromesifen, but not spirotetramat. Surprisingly, no metabolism of the hydrolyzed spirodiclofen-enol metabolite could be detected. These findings are discussed in the light of a likely resistance mechanism.



## 1. Introduction

The two-spotted spider mite *Tetranychus urticae* Koch is a highly polyphagous herbivore and an important pest in agriculture worldwide (Jeppson et al., 1975; Migeon and Dorkeld, 2012; Van Leeuwen et al., 2010). Spider mite feeding leads to browning, reduction in photosynthetic capacity and finally abscission of the leaf (Cranham and Helle, 1985). Acaricides are of utmost importance in the control of spider mite populations in many crops. However, the frequent application of these chemicals, combined with the short life cycle and high reproductive potential of spider mites, results in the rapid development of resistance (Van Leeuwen et al., 2008, 2010). Recently, we collected field strains which proved to be resistant to almost all commercially available acaricides (Khajehali et al., 2011).

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Therefore, it is important to implement resistance management strategies based on sequential applications of acaricides with different modes of action in order to prevent resistance development (Nauen et al., 2012).

One of the most recently developed insecticide classes for the control of mites and sucking pests, are the spirocyclic tetronic/tetramic acid (ketoenol) derivatives (spirodiclofen, spiromesifen and spirotetramat) (Fig. 1: 1, 4 and 5) (Bretschneider et al., 2007; Nauen et al., 2003, 2008). The lead compound spirodiclofen was launched in 2002 for the control of economically important mite species belonging to the genera *Tetranychus*, *Panonychus*, *Brevipalpus*, *Phyllocoptruta* and *Aculus* (Wachendorff et al., 2000), and is currently one of the most widely used acaricides. Spiromesifen displays good activity both on mites and whiteflies (Kontseclalov et al., 2009; Nauen et al., 2002). Spirotetramat has systemic properties and can be used to control a broad range of sucking insect and mite pests (Bruck et al., 2009; Cantoni et al., 2008; Nauen et al., 2008). After foliar application, spirotetramat penetrates through the leaf cuticle and is translocated via xylem and phloem as spirotetramat-enol, which is considered the active metabolite (Bretschneider et al., 2007; Bruck et al., 2009; Fisher and Weiss, 2008). Whether hydrolysis to the enol-form is needed for toxicity of spirodiclofen and spiromesifen has not been documented so far. These compounds are mainly effective against eggs and all developmental stages of spider mites, with limited acute toxicity on adults, but with a strong effect on female fecundity and fertility (Bretschneider et al., 2003; Marcic et al., 2010; Van Pottelberge et al., 2009a,b). Although no detailed biochemical nor molecular studies have been reported on the mode of action of spirocyclic tetronic acid derivates, they interfere with lipid biosynthesis and are thought to act as inhibitors of acetyl-coenzyme A carboxylase (ACCase) (Bretschneider et al., 2007; Nauen, 2005; Nauen et al., 2003). Representing a novel mode of action, these compounds show no cross-resistance in spider mite strains resistant to many currently used acaricides (Konanz and Nauen, 2004; Pree et al., 2005; Van Leeuwen et al., 2005; Wachendorff et al., 2002), and recent monitoring programs in Europe have to date not revealed a significant decrease in efficacy (Ilias et al., 2012; Khajehali et al., 2011; Kramer and Nauen, 2011). However, in the light of resistance risk assessment, high levels of resistance have been selected in the laboratory in the mites *T. urticae* and *Panonychus ulmi* (Kramer and Nauen, 2011; Van Pottelberge et al., 2009a,b). Synergism tests and direct measurements of general detoxifying enzymes pointed mainly toward metabolic resistance through the action of P450 mono-oxygenases (P450s) and esterases. In addition, it was shown *in vivo* that a *T. urticae* strain with a 13-fold decrease of spirodiclofen susceptibility, accumulated higher levels of a hydroxylated metabolite, further supporting an important role for P450s (Rauch and Nauen, 2003). However, detailed molecular mechanisms of resistance have not been elucidated so far. Recently, a mutation in the *ACCase* gene of the greenhouse white fly *Trialeurodes vaporariorum* was found and associated with low levels of spiromesifen resistance, suggesting decreased target-site sensitivity in this species, although resistance levels were too low to impair field efficacy at the registered field dose (Karatolos et al., 2012).

In 2011, the genome of *T. urticae* was completely sequenced and became publicly available, leading to new possibilities to study resistance mechanisms (Grbic et al., 2011; Van Leeuwen et al., 2013). Genome-based novel approaches range from the development of powerful bulk segregant mapping protocols to uncover resistance genes (Van Leeuwen et al., 2012), to the construction of genome-wide gene expression microarrays and next generation transcriptome technologies (Dermauw et al., 2013).

In this study, we exploited the genomic information and tools to study the molecular mechanisms of spirodiclofen resistance using two highly resistant *T. urticae* strains with different genetic background. We sequenced and compared the complete *ACCase* genes of resistant and susceptible strains, and collected genome wide expression data to uncover candidate metabolic resistance genes. We finally show in this study that a P450, belonging to the CYP392E subfamily, is induced by spirodiclofen exposure and is constitutively over-expressed in both spirodiclofen resistant strains. Functional expression of CYP392E10 confirmed its potential to metabolize spirodiclofen and spiromesifen, and the main metabolite of spirodiclofen was identified.

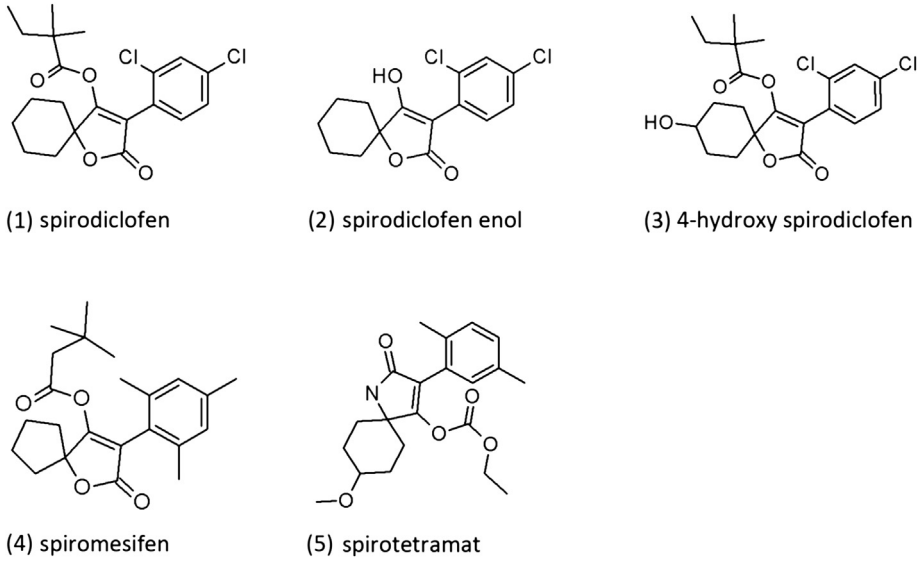


**Fig. 1.** Chemical structures of the tetronic and tetramic acid substrates used in this study. Spirodiclofen (1), spirodiclofen enol (2), 4-hydroxy spirodiclofen (3), spiromesifen (4), and spirotetramat (5).