Molecular mechanisms of resistance to spirodiclofen and spiromesifen in *Tetranychus urticae*

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Abstract

Ketoenol acaricides have been widely used to control *Tetranychus urticae* populations across the world. However, control failure due to resistance development is an increasing concern. To sustain resistance management, it is therefore important to understand the molecular mechanisms underlying resistance, as well as understand the level of cross-resistance they convey between different ketoenol acaricides, such as spirodiclofen or spiromesifen. A *T. urticae* population with moderate levels of resistance to ketoenols was collected from a carnation greenhouse and further selected in the laboratory with spirodiclofen and spiromesifen, separately, until high levels of resistance were achieved. Synergism assays indicated the involvement of P450 monooxygenases and, to a lesser extent, carboxyl/cholinesterases in resistance. Genome-wide gene expression analysis of ketoenol-selected populations compared to the initial field-collected population and a susceptible reference laboratory population further supported the hypothesis of P450-mediated resistance to ketoenols. In addition to metabolic resistance, target-site resistance was also investigated, but no amino acid substitutions in the carboxyl-transferase (CT) domain of the acetyl-CoA carboxylase (ACCase), the target-site of ketoenols, were found in the studied populations. However, increased expression of *ACCase* was found in the spiromesifen-selected, but not in the spirodiclofen-selected population. Finally, changes in resistance levels of some commonly used acaricides were identified after selection with spiromesifen or spirodiclofen.

Keywords: acaricide resistance, spirodiclofen, P450, *ACCase*, spiromesifen

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1. Introduction

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae), is a globally distributed pest of various economically important crops (Van Leeuwen et al., 2010). Chemical pesticides have been the primary method of preventing spider mites from causing high levels of economic impact (Van Leeuwen et al., 2015). However, as a result of intensive, repeated application of pesticides, *T. urticae* has developed resistance to almost all acaricide classes (Van Leeuwen et al., 2010; De Rouck et al., 2023). Considering the challenges of introducing a pesticide with a new mode of action to the market, the available options should be employed carefully to prevent a decrease in their efficacy due to resistance development (Sparks and Lorsbach, 2017). A key to achieving this important goal is to uncover the molecular mechanisms underlying resistance development (Van Leeuwen et al., 2010).

Cyclic ketoenols, including spiromesifen and spirodiclofen, are one of the most commonly used acaricide classes with a considerable worldwide market share (Van Leeuwen et al., 2015). The discovery of cyclic ketoenol acaricides originated from an herbicide group with a similar mode of action, representing a great example of how different branches of the pesticide industry can inspire each other (Bretschneider et al., 2007). They inhibit acetyl-CoA carboxylase (*ACCase*) by interfering with the carboxyltransferase (CT) partial reaction domain which is required for fatty acid biosynthesis (Lümmen et al., 2014). After penetrating the plants, the ketoenols need to be activated to their enol form via a hydrolysis reaction (Brück et al., 2009).

Resistance can arise via multiple mechanisms in phytophagous mites, of which increased activity of detoxification enzymes and alteration of target-sites via point mutations have been most reported (Feyereisen et al., 2015; Van Leeuwen and Dermauw, 2016; De Rouck et al., 2023). Similarly, resistance to ketoenols has been associated with cytochrome P450 monooxygenases (P450s) and carboxyl/cholinesterases (CCEs) in spider mites (Kramer and Nauen, 2011; Demaeght et al., 2013; Badieinia et al., 2020; Wei et al., 2020; İnak et al., 2022). More specifically, a genome-wide gene expression analysis pointed towards the overexpression of two P450 genes (*CYP392E7* and *CYP392E10*) potentially involved in spirodiclofen resistance in *T. urticae* (Demaeght et al., 2013). Functional expression revealed that CYP392E10 could metabolize spiromesifen and spirodiclofen, but not the corresponding enols that are the active form (Demaeght et al., 2013). Supporting this evidence, *CYP392E10* was also overexpressed in a spiromesifen-selected *T. urticae* population (İnak et al., 2022) which might partially explain the moderate cross-resistance between these two acaricides as

previously reported in spider mites (Van Pottelberge et al., 2009; Badieinia et al., 2020). In addition, a quantitative trait loci (QTL) analysis using bulk-segregant analysis was performed to uncover the genetic architecture of spirodiclofen-resistance. QTL mapping revealed three major peaks, pointing towards the polygenic nature of resistance (Wybouw et al., 2019). One of these QTL peaks included several members of CYP392 family, including *CYP392E7*, and the region showed signs of copy number variation. Another peak harbored cytochrome P450 reductase (TuCPR) which is essential for cytochrome P450 catalysis and a D384Y mutation was uncovered (Wybouw et al., 2019). Although this mutation was also uncovered as a QTL in mapping of resistance to mitochondrial electron transport inhibitors acting at complex I (Snoeck et al 2019b), its role in overall P450 metabolism, and ketoenol resistance in particular, awaits further validation. Furthermore, overexpression of an alternative allele of carboxyl/choline esterase 4 (CCE04) in a multi-resistant *T. urticae* strain was reported and associated with a sequestration-based spirodiclofen resistance (Wei et al., 2020). Next to metabolic resistance, several mutations in spider mites have been reported in the target-site gene of ketoenols. However, most of these mutations were located outside the region encoding the CT-domain of ACCase (Wybouw et al., 2019; Papapostolou et al., 2021). Recently, a F1656L mutation in a highly conserved region of the CT-domain was discovered in spiromesifen-resistant field-collected *T. urticae* populations and awaits further validation (İnak et al., 2022). In insects, the target-site mutation A2083V in *ACCase* of both whitefly *Bemisia tabaci* and aphid *Myzus persicae* was reported and validated through CRISPR/Cas9-mediated genome editing in *Drosophila* (Lueke et al., 2020; Singh et al., 2021; Umina et al., 2022). In addition, a comprehensive NCBI search showed the presence of this mutation in predatory mite *Amblyseius swirskii* (Bajda et al., 2022).

In the present study, a field-collected *T. urticae* population was separately selected with both spiromesifen and spirodiclofen. Next, molecular mechanisms of resistance were investigated with a focus on shared and unique mechanisms after selection with both compounds. Finally, changes in resistance levels as a result of ketoenol selection was determined as an important information for resistance management programs.

2. Materials and Methods

2.1. Origin of field collected population and selection

The parental *T. urticae* population (hereafter TP) was originally collected from a carnation greenhouse in Antalya (Türkiye) with an acaricide usage history including spiromesifen, in 2019. The field-collected population was further propagated on kidney bean plants in a climatically controlled room at 26 ± 0.5 °C and 60 ± 2% RH with 16:8 h light:dark photoperiod.

From this population, two independent selection lines were set-up through the use of spiromesifen and spirodiclofen, according to Van Pottelberge et al. (2009). Briefly, these selection lines were created by continuously exposing mites in the population to gradually increasing acaricide concentrations for both acaricides. Both populations (hereafter, SM and SD for spiromesifen- and spirodiclofen-selected, respectively) were selected for more than 20 generations, with final selection concentrations of 1600 and 5000 mg a.i./L for spirodiclofen and spiromesifen, respectively.

2.2. Acaricides

Commercial formulations of all acaricides were used. Adult females were used in toxicity assays for abamectin (Torpedo®; 18 gL^{-1} EC), bifenazate (Floramite®; 240 gL^{-1} SC), pyridaben (Puzzle®; 20% WP), acequinocyl (Kanemite®; 156 gL^{-1} SC), while larval stage of mites were used for spirodiclofen (Smach®; 240 gL^{-1} SC) and spiromesifen (Fibon®; 240 gL^{-1} SC).

2.3. Bioassays and cross-resistance

The concentration-response relationship of five acaricides was assessed both on the initial parental population and the two selected populations. Toxicity assays on both adult females and larval stages of mites were performed as previously described by İnak et al. (2019). Briefly, approximately 20 adult females were transferred onto square-cutted kidney bean leaf discs on wet cotton, after which 2 mL of acaricide solution was sprayed using a Potter spray tower (Burkard Scientific, UK) at 1 bar pressure (1.95 ± 0.05 mg acaricide deposit/cm^{-2}). At least five concentrations and three replicates were used for each experiment. In larval assays, 6-7 adult females were allowed to lay eggs for 24h to obtain synchronized developmental stages. The larval stage of mites was sprayed just after the eggs hatched.

Mortality was assessed after 24h for adult assays and mites that did not move when prodded with a camel-hair brush were considered dead. In the larval bioassays, living and dead mites were counted when the control groups reached the adult stage, as described in İnak et al. (2022).

Probit analysis was used to calculate LC_{50} values using PoloPlus software (Robertson et al., 1980). The LC_{50} values were considered significantly different if the 95% confidence interval did not include the value 1 (Robertson et al., 2017).

2.4. Synergism assays

Synergism assays were performed to reveal a potential contribution of detoxification enzymes in ketoenol resistance. Piperonyl butoxide (PBO), diethyl maleate (DEM) and S,S,S-tributyl phosphorotrithioate (DEF), the inhibitors of main detoxification enzyme groups P450s, glutathione S-transferases (GSTs) and carboxyl/cholinesterases (CCEs), respectively, were sprayed onto larval stage mites as described in İnak et al. (2022). Four hours after synergist treatment, acaricide solutions were applied as described above.

2.5. DNA isolation and amplification of target-site

Genomic DNA was extracted from approximately 150 adult female mites using Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany). Before PCR amplification, the quality and quantity of extracted DNA was checked using a spectrophotometer (Thermo Scientific NanoDrop™ 2000, USA). In the final step, genomic DNAs were eluted by adding 200 µL of elution buffer.

To screen for the presence of point mutations, the target-site of the ketoenol acaricides, *ACCase* (tetur21g02170, accessible at https://bioinformatics.psb.ugent.be/orcae/overview/Tetur), was amplified using the primers and PCR conditions described by Papapostolou et al. (2021).

In addition, the target-site of bifenazate and pyridaben, cytochrome b (cytb) and PSST subunit of mitochondrial complex I, respectively, were screened to check the presence of point mutations in parent and selected populations. The PSST subunit of complex I was amplified using the PSST_exon_new primers according to Bajda et al. (2017), while the amplification of *cytb* gene was performed with PEWYF and WTR primers as previously reported by Van Leeuwen et al. (2008).

PCR reactions were performed in a total volume of 30 µL containing 15 µl of EmeraldAmp® MAX PCR master mix (TaKaRa, Japan), 2 µl of DNA (between 80 and 120 ng μL^{-1}), 1 µl of both forward and reverse primer and 11 µl of PCR-grade water. The alignments and sequencing chromatographs were analyzed using BioEdit 7.0.5 software (Hall, 1999).

2.6. RNA extraction and transcriptome analysis

In order to study genome wide gene expression patterns associated with resistance to the ketoenol compounds spiromesifen and spirodiclofen, RNAseq experiments were performed. For the initial population TP and selected populations SM and SD, RNA extraction was performed from pools of 120 adult female mites, with four replicates, using GeneMATRIX Universal RNA purification kit (EURx, Poland) following the manufacturer's instructions. From the ketoenol susceptible inbred strain SR6i (hereafter ARY), previously described by De Beer et al. (2022) and here used as a general reference strain, RNA was extracted from pools of 100-120 adult female mites using the RNeasy plus mini kit (Qiagen, Belgium). The quality and quantity of extracted RNA were checked using a spectrophotometer (Thermo Scientific NanoDrop™ 2000 (Thermo Scientific, USA) or DeNovix DS-11 (DeNovix, USA)), and visual inspection on a 1% agarose gel after electrophoresis. From the RNA samples of TP, SM and SD, Illumina libraries were constructed using the Illumina TruSeq RNA Library Preparation Kit with polyA selection (Illumina, USA). From the RNA samples of ARY, Illumina libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit. Library preparation and sequencing was performed on an Illumina Novaseq 6000 to generate paired reads of 2x150 base pairs (bp) by Novagene (China) for TP, SM and SD and by Genewiz (Germany) for ARY.Before read-mapping, the quality of the reads was checked using FASTQC (version 0.11.9) (Andrews, 2010). RNA reads were aligned to the three pseudochromosome assembly of *T. urticae* (Grbić et al., 2011; Wybouw et al., 2019) using STAR (version 2.7.9a) (Dobin et al., 2013) with the maximum intron size set to 20 kb. Genes with a normalized read-count lower than ten reads were excluded from further analyses. Aligned reads were sorted and indexed using SAMtools (version 1.11) (Li et al., 2009) and used as an input for HTSeq (version 0.11.2) to perform read-counting on a per-gene basis with the default settings (Anders et al., 2015). To assess the gene expression variation within and between all treatment groups, a principal component analysis (PCA) was performed using the R-package (R version 4.2.1) DESeq2 (version 1.36.0) (Love et al., 2014; R Core Team, 2021). In brief, the raw per gene read-counts were normalized via the regularized-logarithm (rlog) transformation function of the DESeq2 package and the PCA was calculated and plotted for the 5000 most variable genes across all RNA samples using the DESeq2 function PlotPCA. The read count data was also used to perform differential expression (DE) analysis with DESeq2 for all pairwise comparisons between strains (TP, SM, SD and ARY). Genes were considered to be significantly

differentially expressed (DEGs) in the contrast if they have a Log_2 Fold Change (Log_2FC) > 1 and a Benjamini-Hochberg adjusted *p*-value < 0.05).

The raw reads were submitted to the NCBI Short Read Archive (SRA) database under the bioprojects with accession numbers PRJNA983210 and PRJNA930642.

2.7.Determination of relative expression of target-site using qPCR

Relative expression of the target-site *ACCase* of parental and selected populations were determined using real-time PCR (Bio-Rad CFX96 Touch™). Complementary DNAs were synthesized from the extracted RNA via reverse transcription using iScript ™ cDNA synthesis kit (BioRad, USA). The primers for the reference genes (*Actin, rp49, ubiquitin*) and *ACCase* were used according to Demaeght et al. (2013).

The qPCR reactions were performed in a final volume of 20 µL reaction volume containing 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), 1 µL of each primer (10 µM stock), 1 µL of cDNA (100 ng/µL) and 7 µL of sterilized ddH_2O using BioRad CFX96 Touch™ under the following conditions: 98 °C for 30 s, followed by 39 cycles of 95 °C for 10 s, 54 °C for 30 s. The melting curve was analyzed at 60-95 °C after 40 cycles to verify primer specificity. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *P*-values were calculated using an unpaired t-test (CFX Maestro™ Software, Bio-Rad) and values lower than 0.05 were considered significantly different. The analyses were performed using 4 biological replicates.

3. Results

3.1. Toxicity results, selection and cross-resistance pattern

The LC_{50} values of both the parental and selected populations against various acaricides are listed in Table 1. LC_{50} values of all tested compounds are high in the parental line TP, probably indicating that this is a multi-resistant strain. However, spiromesifen selection resulted in more than 30-fold increase in resistance to spiromesifen compared to the parental population, and a two-fold increase of resistance to spirodiclofen. Likewise, spirodiclofen selection resulted in an 11.5- and 6-fold increase in resistance to spirodiclofen and spiromesifen, respectively (Table 1). Selection with the ketoenols also increased resistance ratios to other acaricides, of which

resistance to bifenazate (4.7-fold increase) in SM population and resistance to pyridaben (10.6 fold increase) in the SD population are the most pronounced.

3.2. Effect of synergistic compounds

Pretreatment with PBO, DEM, DEF increased the toxicity of spiromesifen in parent TP population with a synergism ratio (SR) of 2.6-, 1.3- and 1.6-fold, respectively. Although the effect of DEM and DEF could not be calculated due to extremely high resistance levels exceeding 5000 mg a.i./L, PBO synergized the spiromesifen toxicity more than 3.2-fold in SM (Table 2).

PBO and DEF synergized the spirodiclofen toxicity 2.2- and 1.4-fold in TP, respectively, however, pretreatment with DEM did not change the efficacy of spirodiclofen. In addition, 3.1 and 1.7-fold SRs were determined after PBO and DEF treatment, respectively, in SD (Table 2). Similar to parent population, no difference was observed after pretreatment with DEM, the inhibitor of GSTs in SD.

3.3. Comparison of ACCase sequences and screening of other target-site mutations

ACCase sequences of parental and selected populations were compared to both the sequence of the susceptible population ARY and also the reference genome sequence (Grbić et al., 2011). However, no amino acid substitutions were detected in the CT-domain of ACCase. In addition, no mutation was found either in *cytb* or PSST subunit of complex I of both parental and selected *T. urticae* populations.

3.4. RNA mapping and differential expression analysis

The influence of ketoenol selection on the genome-wide expression levels was assessed using RNAseq. Illumina sequencing generated an output of ~25.2 million paired end reads of 150 bp of which an average of 85.1% mapped uniquely against the three chromosome assembly of the *T. urticae* genome (Table S1) (Wybouw et al., 2019). Rlog normalized read-counts of all samples were used to perform a PCA analysis (Figure 1A-B). First, this revealed that all sample replicates per strain cluster closely together, confirming their quality. PCA without ARY showed clear separation between parental TP population and selected populations. After including ARY, parental and selected populations aligned on the same line and SM and TP clustered closely together, which further confirms the results. The first two principal

components (PC1 and PC2) together explain 88% of all variances in the dataset. The largest fraction of the variance is explained by PC1 (79%) which indicates the separation between the susceptible reference ARY and the field strain TP with its selected derivatives (SM and SD). Along with PC2, which explains 9% of the variance, there is a separation between a cluster comprised of TP and SM samples and the SD samples, indicating a larger expression difference with the original field strain due to spirodiclofen selection as compared to spiromesifen selection.

When comparing to the laboratory susceptible population (ARY), 882 and 713 genes over- ($Log_2FC \geq 1$) and underexpressed ($Log_2FC \leq 1$), respectively, in the moderately ketoenol resistant parent population (TP). After further selection, 219 (86 up *vs* 133 down) and 116 (61 up *vs* 55 down) genes were differentially expressed in SD and SM, respectively, compared to parent population (Figure S2, Table S2).

In the parental population, *CYP392A15*, *CYP392A14*, *CYP392E2*, *CYP392A13v1*, *CYP392E6*, *CYP392B3*, *CYP392E8* genes were overexpressed more than 3-fold in TP when compared to susceptible ARY population. Multiple detoxification genes were overexpressed after both selections, of which the P450 genes belonging to the CYP392 family and several UGTs comprise more than half of the most overexpressed gene list (Table 3-4, Figure 1D). In addition, 16 genes were commonly upregulated after selection with both spirodiclofen and spiromesifen. Interestingly, among P450s, *CYP392A14P* (pseudogene in London reference genome), *CYP392A15* and *CYP392A13v1* were upregulated in parental TP and were overexpressed to even higher levels in both selection populations when comparing them to TP, consistently.

In addition to the P450s, four CCEs (namely TuCCE05, TuCCE25, TuCCE65 and CCEincTu06) were overexpressed with $Log_2FC > 2$ in the parental TP population. However, none of them were present among the highest upregulated genes after both ketoenol selections (Table 3-4). Although with low Log_2FC, TuCCE35 was the only CCE that was overexpressed in both parental and selected populations.

The UGTs that were overexpressed with $Log_2FC > 2.5$ were *UGT06*, *UGT79p*, *UGT15* and *UGT18* in parental TP population. Several UGTs were also overexpressed after further selection using ketoenols (Table 3-4). Among them *UGT23* was common in both selected populations.

3.5. Relative expression of ACCase

The relative expression of the target-site, *ACCase*, of parental and selected populations was compared. The *ACCase* expression level was significantly higher in SM population, but not in SD, when compared to the initial parental population (Figure 2).

4. Discussion

Tetranychus urticae has developed resistance to almost all acaricide classes (Van Leeuwen et al., 2015; De Rouck et al., 2023). Cyclic ketoenols, however, present an acaricide class with only a limited number of reported resistance cases. Unfortunately, resistance development is on the move and thus mechanisms underlying resistance and potential cross-resistance between compounds should be uncovered to manage the resistance. In this study, we first determined the differentially expressed genes in a multi-resistant field-collected *T. urticae* population (TP). Then, we further selected this population with spiromesifen and spirodiclofen in order to reveal the shared and unique molecular responses.

In selection studies, the effect of genetic drift on results should be taken into account, unless an adequate number of replications were used. In particular, random selection of a limited number of resistant individuals from a population over time probably also leads to differences in gene expression unrelated to resistance, but merely an effect of drift, which can also influence cross-resistance patterns. However, in this study, the overlap in gene expression responses after selection with both SM and SD were truly independent and led to overexpression of a shared detoxification dataset, potentially indicating a common molecular response rather than genetic drift (see below for detailed discussion).

Considering the contribution of target-site mutations to resistance, we first screened the CT-domain of ACCase in studied populations. However, we could not find any mutation at the CT-domain of either parental or selected populations showing high levels of phenotypic resistance. Besides the alteration of the target-site via point mutations, differences in target-site expression, both higher and lower, have also been associated with resistance to various compounds (Feyereisen et al., 2015). In the case of *ACCase*, significantly higher expression was previously associated with spiromesifen resistance (İnak et al., 2022) and was also observed here. Increased target-site expression has also been reported in *Panonychus citri* following spirodiclofen exposure (Yu et al., 2015). The overexpression of the target-site is most likely to compensate for the proteins inhibited by the acaricide and thus decreased enzymatic activity,

which could result in higher expression via feed-back. Although increases in the amount of target expression has been well documented as a resistance mechanism against certain insecticide groups (i.e. acetylcholinesterase inhibitors) (Bass and Field, 2011), to what extent this mechanism contributes to phenotypic resistance to ketoenols is not known. On the other hand, no difference has been determined between parent and spirodiclofen-selected population, indicating that difference in target-site expression is not a common mechanism for these acaricides with the same mode of action.

P450 monooxygenases are probably the best studied detoxification enzyme family present in metazoans (Dermauw et al., 2020; De Rouck et al., 2023) and the mechanisms of P450-mediated insecticide resistance has been extensively reviewed in Nauen et al. (2022). Elevated toxicity of ketoenols after PBO exposure indicates both increased amount of P450s in parental TP population and their involvement in resistance (Table 2), in line with previous results in spider mites (Rauch and Nauen, 2002; Demaeght et al., 2013; Bajda et al., 2015; Badieinia et al., 2020; İnak et al., 2022). In parent population (TP), among over 1500 differentially expressed genes, overexpression of multiple P450 genes belonging to the CYP392 family (see results for P450 genes overexpressed >3-fold) shows that the usual suspects of detoxification are present, and it might partially explain its multi-resistant nature. After further selection, SRs were slightly increased, indicating elevated activity of P450s and to a lesser extent CCEs. (Table 2). In parallel, presence of several P450s belonging to the CYP392 family are among the most overexpressed genes which further supports their major contribution in ketoenol resistance (Table 3-4). Expression and copy number variation of CYP392 family members were previously associated with xenobiotic metabolism in some studies (Wybouw et al., 2019; Fotoukkiaii et al., 2021). More specifically, overexpression of *CYP392E10* has been reported in a spirodiclofen-resistant *T. urticae* population and the role of this P450 was further validated by functional expression and *in vitro* metabolism experiments (Demaeght et al., 2013). This gene was also highly expressed in TP and two additional selected populations, however, we did not include it in further analysis because the reads were lower than 10 for ARY. In addition, two P450 genes, *CYP392A15* and *CYP392A13v1*, were consistently overexpressed in TP *vs* ARY and in both selected populations when compared to TP (Table 3-4, Figure 1D). Similarly, *CYP392A15* was significantly upregulated in a multi-resistant *T. urticae* population from Greece, with a RR of >20- and >36-fold for spirodiclofen and spirotetramat (Papapostolou et al., 2021) and also in spirodiclofen-resistant SR-VP population reported by Demaeght et al. (2013). Moreover, *CYP392A13v1* was also found to be 2-fold overexpressed in a spirodiclofen-

resistant SR-VP population (Demaeght et al., 2013). Therefore, it might be speculated that these two P450s have a role in cross-resistance between spiromesifen and spirodiclofen, similar to *CYP392E10* that can metabolize both compounds (Demaeght et al., 2013). However, functional validation by *in vitro* metabolization assays should be performed to validate their role in cross-resistance. On the other hand, there are several P450 genes that were significantly overexpressed only in either SM (i.e. *CYP392D6*) or SD (i.e. *CYP392E1*, *CYP392A1*, *CYP392E6*) when compared to the parent population, indicating the presence of individual mechanisms as well. However, the effect of genetic drift caused by random selection on gene expression should be taken into account. Despite being overexpressed in SD *vs* TP, *CYP392E1* was downregulated in SD *vs* ARY and TP *vs* ARY (Table 4), indicating that this gene is most likely not involved in spirodiclofen resistance. On the other hand, *CYP392E6* has been overexpressed in both initial TP population and in SD, that might have a role in spirodiclofen resistance. In support, this gene was significantly overexpressed (Log_2FC of 6.18) in extremely spirodiclofen-resistant *T. urticae* population (Demaeght et al., 2013).

Next to P450s, the involvement of CCEs in ketoetol resistance has also been reported in both laboratory-selected and field-collected *T. urticae* populations (Rauch and Nauen, 2002; Van Pottelberge et al., 2009; İnak et al., 2022; De Rouck et al., 2023). More specifically, overexpression of *TuCCE04* has been associated with spirodiclofen resistance in genetically independent mite populations (Wei et al., 2020). In the present study, pretreatment with DEF partly increased the spiromesifen and spirodiclofen toxicity 1.6 and 1.4-fold, respectively, in the parental population. Spirodiclofen selection resulted in a slight increase in SR of DEF (1.7-fold), however, the effect of CCEs in spiromesifen resistance could not be assessed due to extremely high resistance levels of population SM (LC_{50} value was more than 5000 mg a.i/L even after treatment with DEF). At the gene-level, overexpression of several CCEs including *CCE05*, *CCE25*, *CCE65* with $Log_2FC > 2$ were identified in TP *vs* ARY based on transcriptome results. CCE05 has the closest position with CCE04 in the phylogenetic tree (Wei et al, 2020), however, since this gene is downregulated in two genetically distinct *T. urticae* populations (Wei et al., 2020), it is unlikely to have a role in ketoenol resistance. Further selection with ketoenols caused further overexpression of few CCEs (i.e. *CCE35* in SM; *CCE63* in SD) (Table 3-4). In addition, *CCE35* was the only CCE that was overexpressed in SD and SM, although at a low level. Indeed, it is suggested that sequestration by CCE might delay the activation of spirodiclofen and provide more time for P450 hydroxylation and thus their synergism could confer higher resistance ratios (Wei et al., 2020).

UDP-glycosyltransferases (UGTs) are a relatively overlooked detoxification enzyme group in arthropods. They catalyze the conjugation reaction using a sugar donor (UDP-glucose in case of *T. urticae*) and increase the water-solubility of xenobiotics, allowing for an easier excretion (Bock, 2016; Snoeck et al., 2019a; De Rouck et al., 2023). In *T. urticae*, more than 80 UGT genes were uncovered in the genome, one of the largest sets uncovered in metazoans, with a unique evolutionary history, as the genes were lost in early chelicerate lineages but acquired by spider mites after horizontal gene transfer from bacteria (Ahn et al., 2014). Although many acaricides and plant secondary compounds were shown to be substrates for several UGTs, revealing broad substrate specificity (Snoeck et al., 2019a), further *in vitro* validation experiments were only performed for macrocyclic lactones (abamectin and milbemectin) and pyrethroid bifenthrin (Xue et al., 2020; Wang et al., 2020; De Beer et al., 2022). In the present study, several UGTs were overexpressed in both TP *vs* ARY and TP *vs* selected populations. Among them, *UGT23* is the only UGT overexpressed in both ketoenol-selected populations, in line with Demaeght et al., (2013) who reported the overexpression of same UGT gene (Log_2FC of 2.3). In addition, selection with spiromesifen also showed the overexpression of additional UGTs (*UGT67*, *UGT79p*, *UGT29*, *UGT20* with >1.5 Log_2FC) that might indicate their involvement in resistance. Previously, overexpression of *UGT29* was reported in *T. urticae* populations resistant to macrocyclic lactones and bifenthrin, however, it was only able to glycosylate abamectin (Xue et al., 2020; De Beer et al., 2022). Since *UGT29* was downregulated when compared to susceptible ARY (Table 3), this gene is probably not related to spiromesifen resistance. On the other hand, *UGT23* and *UGT79p* genes were also overexpressed in a Greek *T. urticae* population resistant to multiple acaricides including spirodiclofen and spirotetramat (Papapostolou et al., 2021). Among overexpressed UGT genes in the present study, *UGT20* was also studied, and it was shown that this UGT was able to glycosylate abamectin, but not spirodiclofen (Snoeck et al., 2019a). However, this overexpression did not result in a difference in abamectin resistance after spiromesifen selection (Table 1). The reason for this might be the loss of other putative abamectin-related resistance genes by drift in the initial field-collected population during selection with spiromesifen or downregulation of those genes in the absence of abamectin selection pressure.

Cross-resistance is the term used when resistance in a population to one compound results in resistance to another compound to which the population was not exposed (Tabashnik et al., 2014). However, as discussed above, selection studies have some limitations to detect cross-resistance as it does not allow for the determination of the effect of genetic drift. Previous

studies evaluated the effect of spirodiclofen selection on resistance levels to other acaricides using susceptible laboratory populations of *T. urticae* as a parental population (Rauch and Nauen, 2002; Van Pottelberge et al., 2009). However, no substantially increased resistance levels could be observed. Here, we used a multi-resistant field-collected parental population and assessed the efficacy of five different acaricides before and after selection with either spirodiclofen or spiromesifen. Between ketoenol acaricides, spiromesifen selection (>30-fold RR) resulted in only 2.2-fold spirodiclofen resistance. On the other hand, selection with spirodiclofen (>11-fold RR) increased the spiromesifen resistance 6-fold. Similarly, previous studies showed an increase in spiromesifen resistance level after selection with spirodiclofen in spider mites (Van Pottelberge et al., 2009; Kramer and Nauen, 2011; Badieinia et al., 2020). However, less is known about the mechanisms underlying this cross-resistance. In the present study, a number of potential candidate genes that might contribute to cross-resistance between ketoenols have been uncovered and discussed. Besides ketoenols, spiromesifen selection decreased the susceptibility to bifenazate by more than 4.5-fold. P450-mediated resistance to bifenazate was also reported based on synergism experiments (Sugimoto and Osakabe, 2019), and recently, it was shown that CYP392A11 could hydroxylate bifenazate (Lu et al. 2023). Increased resistance levels in SM, but not in SD, indicates that the underlying mechanism is not shared in selected populations. On the other hand, spirodiclofen selection resulted in 10.6-fold resistance development to pyridaben. These elevated resistance levels can be caused by the shared detoxification route of these acaricides with different modes of action or random selection of resistant phenotypes (i.e. target-site mutations) by genetic drift. Since no mutations at the target-sites of both bifenazate and pyridaben were found, in initial and selected populations, metabolic-based resistance is most likely at play. It is known that extremely high levels of pyridaben resistance (LC_{30} > 10,000 mg/L) can be achieved via mechanisms (in particular P450s) other than target-site mutations (Namin et al., 2020). Other studies showed a synergism between multiple mechanisms in METI-I resistance which is supported by QTL analysis (Snoeck et al., 2019b; Xue et al., 2022; Itoh et al., 2022). Genetic mapping of pyridaben resistance showed involvement of a cluster including PSST and multiple CYPs including *CYP392A1-4* (Bajda et al., 2017). It was later shown that the presence of the PSST resistance mutation is an almost perfect molecular marker for fenpyroximate and tebufenpyrad resistance, at least in a set of 42 European populations (Xue et al., 2022). However, data on pyridaben resistance was not presented in this study. In contrast, overexpression of *CYP392A3* was associated with pyridaben resistance in *T. urticae* from Japan (Itoh et al., 2022). Although we could not detect the up- or downregulation of this P450 gene in either parental or selected

populations, *CYP392A1* showing high similarity to *CYP392A3* was one of the most overexpressed P450 gene in SD. Therefore, the role of *CYP392A1* should be further elucidated in future studies.

In summary, a shared molecular response after spirodiclofen and spiromesifen selection were shown in a field-collected *T. urticae* population. In particular, two consistently overexpressed P450s in both parental and selected populations should be further investigated to reveal cross-resistance risk. Last, decreased susceptibility to some acaricides when exposed to spiromesifen and spirodiclofen can be taken into account in order to efficient use of acaricides.

Acknowledgement

MV was supported by a grant from the Research Council (ERC) under the European Union's Horizon2020 research and innovation program (grant 772026-POLYADAPT) to TVL.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Susceptibility of TP and selected populations to five commonly used acaricides

Population	Acaricide	Slope	LC_{50} (mg a.i./L) (95% CL)	LC_{90} (mg a.i./L) (95% CL)	χ^2	Df	[1]RR_{50}
TP	Spiromesifen	2.07 ± 0.28	165.4 (135.7 - 202.2)	689.7 (485.6 - 1224.7)	9.1	13	-
	Spirodiclofen	0.95 ± 0.09	170.8 (128.5 - 238.3)	3857.8 (1904.8 - 1149.0)	26.0	19	-
	Abamectin	2.69 ± 0.39	27.2 (23.4 -31.6)	81.3 (61.7 - 129.9)	7.6	13	-
	Bifenazate	2.72 ± 0.29	390.9 (335.9 - 452.1)	1155.0 (928.9 - 1570.6)	11.4	13	-
	Acequinocyl	1.65 ± 0.16	113.7 (77.9 - 158.6)	679.03 (427.1 - 1458.1)	33.2	16	-
	Pyridaben	0.97 ± 0.14	619.9 (378.2 - 961.0)	13175.9 (5872.1 - 61007.3)	14.3	13	-
SM	Spiromesifen	-	>5000	-			>30.2
	Spirodiclofen	3.72 ± 0.38	378.3 (334.1 - 419.4)	836.6 (734.5 - 997.9)	14.3	16	2.2 (1.7 - 3.1)
	Abamectin	6.99 ± 0.79	27.3 (25.3 - 29.2)	41.6 (38.1 - 46.9)	8.7	13	1 (0.8 - 1.1)
	Bifenazate	3.56 ± 0.32	1851.2 (1649.4 - 2083.1)	4245.3 (3589.0 - 5303.6)	9.1	16	4.7 (3.9 - 5.7)
	Acequinocyl	0.72 ± 0.09	260.5 (157.0 - 443.7)	15511.8 (5908.3 - 71640.0)	10.0	13	2.2 (1.2 - 3.3)
	Pyridaben	1.42 ± 0.17	1303.6 (999.7 - 1731.8)	10357.7 (6340.0 - 22126.0)	11.9	13	2.1 (1.2 - 3.0)
SD	Spirodiclofen	1.49 ± 0.14	1979.8 (1616.9 - 2536.2)	14220.2 (9024.7 - 27671.3)	12.5	13	11.5 (7.9 - 14.3)
	Spiromesifen	2.43 ± 0.18	1003.1 (896.2 - 1124.3)	3381.6 (2795.7 - 4316.7)	3.6	13	6 (4.3 - 7.3)
	Abamectin	2.90 ± 0.29	22.1 (19.2 - 24.9)	61.0 (51.3 - 77.5)	10.6	16	0.8 (0.6 - 0.9)
	Bifenazate	2.98 ± 0.32	193.8 (165.3 - 222.1)	521.9 (435.4 - 671.5)	7.8	13	0.5 (0.4 - 0.6)
	Acequinocyl	1.71 ± 0.19	241.9 (195.9 - 299.5)	1357.2 (932.4 - 2393.9)	6.4	13	2.1 (1.5 - 2.8)
	Pyridaben	1.03 ± 0.17	6620.7 (4363.7 - 12729.2)	114118.7 (42240.5 - 743321.7)	6.4	13	10.6 (5.5 - 16.9)

[1] RR: Resistance ratio: LC_{50} value of selected population (SM or SD)/ LC_{50} value of parent population (TP)

Table 2. Synergism assays in parent and selected populations

Population	**Treatment**	**Slope**	**LC_{50} (mg a.i./L) (95% CL)**	**[1]SR_{50}**
	Spiromesifen	2.067 ± 0.282	165.4 (135.7 - 202.2)	-
TP	Spiromesifen + PBO	1.220 ± 0.125	62.1 (38.3 - 94.9)	2.6 (1.9 - 3.9)
	Spiromesifen + DEM	1.483 ± 0.136	126.8 (90.7 - 179.1)	1.3 (1.0 - 1.8)
	Spiromesifen + DEF	1.521 ± 0.140	99.0 (67.7 - 140.4)	1.6 (1.2 - 2.3)
SM	Spiromesifen		>5000	-
	Spiromesifen + PBO	1.505+-0.139	1529.2 (1236.2 - 1958.6)	>3.2
	Spiromesifen + DEM		>5000	-
	Spiromesifen + DEF		>5000	-
TP	Spirodiclofen	0.947 ± 0.088	170.8 (128.5 - 238.3)	-
	Spirodiclofen + PBO	1.656 ± 0.123	77.8 (60.7 - 97.3)	2.2 (1.6 - 2.9)
	Spirodiclofen + DEM	0.965 ± 0.105	160.8 (114.8 - 229.3)	1 (0.7 - 1.5)
	Spirodiclofen + DEF	1.570 ± 0.124	117.1 (89.7 - 150.7)	1.4 (1.2 - 2.2)
SD	Spirodiclofen	1.497 ± 0.135	1979.8 (1616.9 - 2536.2)	-
	Spirodiclofen + PBO	1.691 ± 0.132	643.6 (506.7 - 803.8)	3.1 (2.2 - 3.5)
	Spirodiclofen + DEM	1.538 ± 0.147	1749.6 (1385.0 - 2329.0)	1.1 (0.8 - 1.3)
	Spirodiclofen + DEF	1.927 ± 0.154	1178.6 (907.0 - 1553.8)	1.7 (1.2 - 1.9)

[1]SR: Synergism ratio = LC_{50} without synergist/LC_{50} with synergist

Table 3. Overexpressed resistance-related genes in SM *vs* TP and their expression profiles in other comparisons in terms of log2Fold change.

T. urticae gene ID[1]	Gene name	TP *vs* ARY	SM *vs* ARY	SM *vs* TP
tetur08g07930*	CYP392A14P -pse	1.04	4.14	3.09
tetur05g00090*	UGT23	—	1.59	2.54
tetur08g08050*	CYP392A13v1	4.07	6.55	2.48
tetur03g05030	CYP392D6	-1.14	1.02	2.16
tetur06g05080*	SDR	-3.30	-1.15	2.15
tetur03g09941*	CYP392A15	5.41	7.29	1.88
tetur22g00460	UGT67	1.38	3.01	1.63
tetur139g00010	UGT79p	2.99	4.60	1.61
tetur05g05060	UGT29	-2.59	-1.00	1.59
tetur05g00060	UGT20	2.28	3.83	1.56
tetur40g00030	Major facilitator superfamily	-1.69	—	1.54
tetur28g01250	Intradiol ring-cleavage dioxygenase	—	—	1.48
tetur02g03300	UGT09	-1.49	—	1.45
tetur22g00510	UGT69	1.84	3.18	1.35
tetur09g01990*	ABCG-15	—	1.74	1.34
tetur26g01450	GSTd07	—	2.13	1.28
tetur11g05770*	CCE35	1.04	2.26	1.22
tetur12g04600	CCE37	1.03	2.23	1.20
tetur02g14020	CYP392A8	-1.52	—	1.01
tetur35g00180	CCEincTu13	—	—	1.01

[1] accessible at https://bioinformatics.psb.ugent.be/orcae/overview/Tetur

* Genes that are overexpressed in both SM and SD populations

Table 4. Overexpressed resistance-related genes in SD *vs* TP and their expression profiles in other comparisons in terms of log2Fold change.

T. urticae gene ID[1]	Gene name	TP vs ARY	SD vs ARY	SD vs TP
tetur08g07930*	CYP392A14P -pse	1.04	4.06	3.02
tetur08g08050*	CYP392A13v1	4.07	6.56	2.49
tetur05g00090*	UGT23	–	1.31	2.26
tetur06g05080*	SDR	-3.30	-1.07	2.23
tetur03g05540	CYP392E1	-4.43	-2.31	2.13
tetur07g06410	CYP392A1	–	1.79	1.95
tetur21g00500	Major facilitator superfamily	-1.18	–	1.75
tetur27g00330	CYP392E6	3.50	5.19	1.70
tetur03g09941*	CYP392A15	5.41	7.01	1.60
tetur31g00250	CCE63	–	1.98	1.44
tetur04g07780	UGT19	–	1.67	1.42
tetur22g02090	Major facilitator superfamily	–	1.51	1.34
tetur07g06560	Intradiol ring-cleavage dioxygenase, core	1.71	2.95	1.24
tetur21g00420	Major facilitator superfamily;	-1.04	–	1.22
tetur01g10810	CCE07	-1.54	–	1.11
tetur03g04990	CYP392D2	1.06	2.14	1.08
tetur05g05290	GSTm11	–	1.58	1.07
tetur11g05770*	CCE35	1.04	2.08	1.04
tetur07g05940	Intradiol ring-cleavage dioxygenase	3.60	4.63	1.03
tetur09g01990*	ABCG-15	–	1.42	1.02
tetur09g01970	ABCG-13	–	1.65	1.01

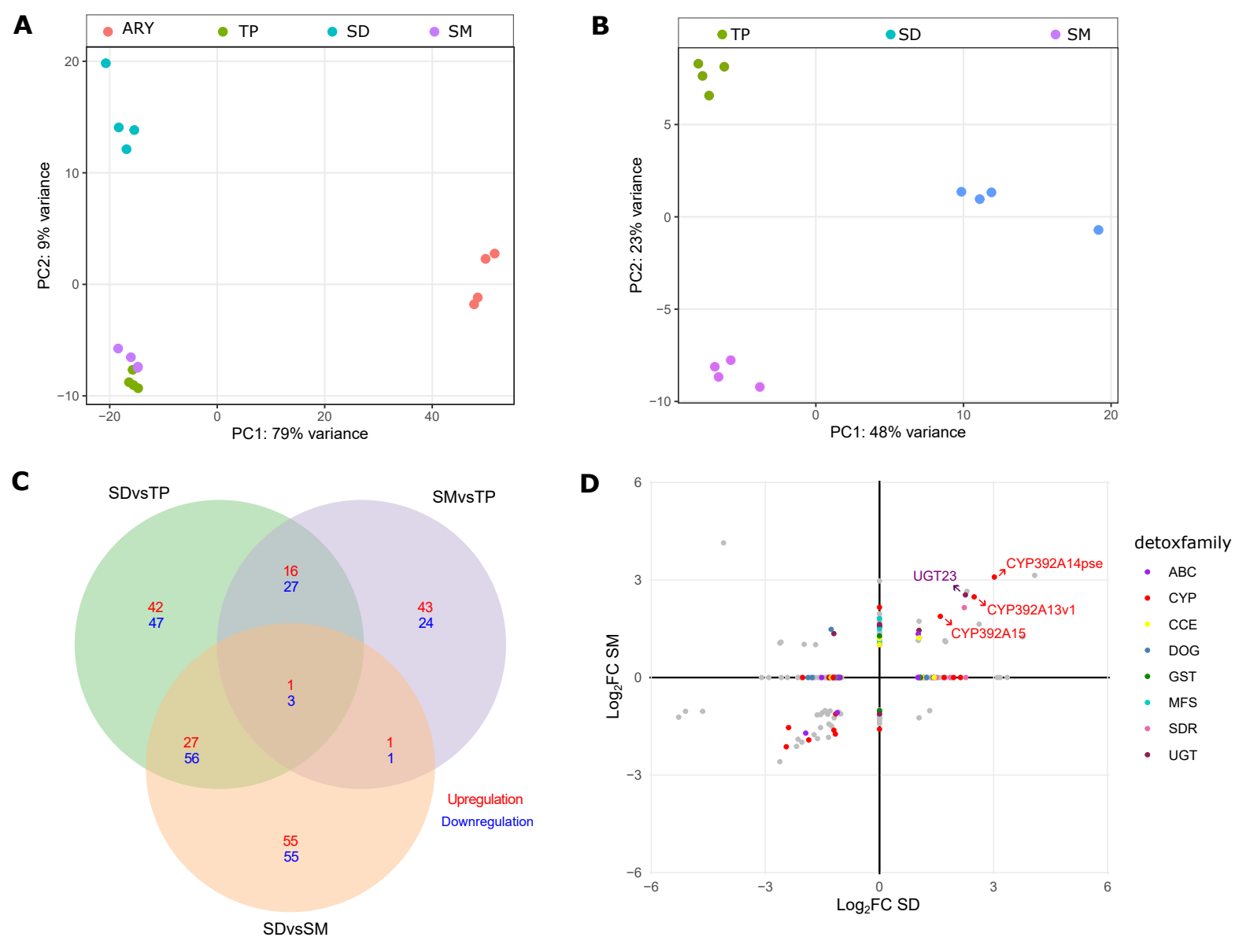
[1] accessible at https://bioinformatics.psb.ugent.be/orcae/overview/Tetur

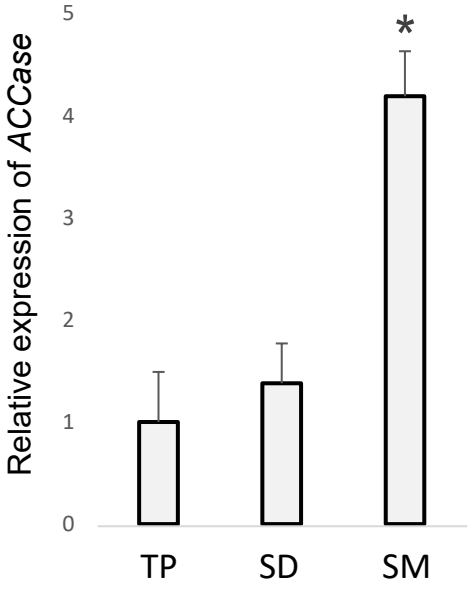
* Genes that are overexpressed in both SM and SD populations

Figure captions

Fig. 1. Transcriptome analysis of the spiromesifen and spirodiclofen selected populations. **A)** Principal component analysis (PCA) of TP, SD, SM and ARY. PC1 and PC2 are represented on the x- and y-axis, respectively, with the percentage of variance explained by each PC. **B)** PCA analysis of TP, SD and SM. **C)** Venn diagram representing the overlap of differentially expressed genes (|Log2FC| > 1, Benjamini Hoghberg adjusted p-value < 0.05) between TP, SM and SD. Indicated in red are the upregulated genes (Log2FC > 1). Indicated in blue are downregulated genes (Log2FC < -1). **D)** Shared and unique differentially expressed genes (|Log2FC| > 1, Benjamini Hoghberg adjusted *p*-value < 0.05) after selection of TP with spirodiclofen and spiromesifen. Differentially expressed resistance-related genes (ABC = ABC transporter; CYP = cytochrome P450 monooxygenases; CCEs = carboxyl/choline esterases; DOG = intradiol ring cleavage dioxygenases; GST = glutathione S-transferase; MFS = Major Facilitator Superfamily; SDR = short-chain dehydrogenases/reductases; UGT = UDP-glycosyl transferases) were highlighted using different colors.

Fig. 2. Relative expression of *ACCase* gene in parental (TP), spirodiclofen selected (SD) and spiromesifen selected (SM) populations. Asterik indicates significant difference between TP and SM based on t-test ($p < 0.05$).


A
ARY
TP
SD
SM
PC2: 9% variance
PC1: 79% variance
B
TP
SD
SM
PC2: 23% variance
PC1: 48% variance
C
SDvsTP
SMvsTP
SDvsSM
42
47
16
27
43
24
1
3
27
56
1
1
55
55
Upregulation
Downregulation
D
UGT23
CYP392A14pse
CYP392A13v1
CYP392A15
Log2FC SM
Log2FC SD
detoxfamily
ABC
CYP
CCE
DOG
GST
MFS
SDR
UGT


Relative expression of ACCase
5
4
3
2
1
0
*
TP
SD
SM