Resistance risk assessment of the novel complex II inhibitor pyflubumide in the polyphagous pest *Tetranychus urticae*

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Abstract

Pyflubumide is a novel selective carboxanilide acaricide that inhibits mitochondrial complex II of mainly spider species such as *Tetranychus urticae*. We explored the baseline toxicity and potential cross-resistance risk of pyflubumide in a reference panel of *T. urticae* strains resistant to various acaricides with different modes of action. A cyenopyrafen resistant strain (JPR) was identified as the only strain with low-to-moderate level of cross-resistance to pyflubumide (LC_{50} = 49.07 mg/L). In a resistance risk assessment approach, JPR was subsequently selected which lead to two highly resistant strains JPR-R1 (LC_{50} = 1437.48 mg/L) and JPR-R2 (LC_{50} = 1893.67 mg/L). Interestingly, compared to adult females, resistance was much less pronounced in adult males and eggs of the two JPR-R strains. In order to elucidate resistance mechanisms, we first sequenced complex II subunits in susceptible and resistant strains, but target-site insensitivity could not be detected. In contrast, synergism/ antagonism

experiments strongly suggested that cytochrome P450 monooxygenases are involved in pyflubumide resistance. We therefore conducted genome-wide gene expression experiments to investigate constitutive and induced expression patterns and documented the overexpression of five cytochrome P450 and four CCE genes in JPR-Rs after pyflubumide exposure. Together, we provide a thorough resistance risk assessment of a novel complex II inhibitor, and provide first evidence for metabolic resistance mediated by cytochrome P450s in *T. urticae*.

Keywords

Pyflubumide, selection, Complex II, Cytochrome P450, Cross-resistance, Cyenopyrafen

Key message

- This study provides a thorough resistance risk assessment of the novel mitochondrial complex II inhibitor pyflubumide in *Tetranychus urticae*.
- We identified a low-to-moderate level of cross-resistance to pyflubumide in the cyenopyrafen resistant strain JPR.
- Under pyflubumide selection pressure, JPR rapidly evolved high level resistance, but only in females.
- Target-site insensitivity could not be detected in the resistant strains.
- Synergism/antagonism experiments followed by genome-wide gene expression experiments strongly suggest that cytochrome P450 monooxygenases are involved in pyflubumide resistance.

Author Contribution

SMF, NW and TVL conceived and designed research. SMF, CM conducted experiments. SMF, TVL and NW analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Arthropoda: Chelicerata: Acariformes) is an important cosmopolitan agricultural pest that causes significant yield losses in the absence of a proper pest and resistance management strategy (Jeppson et al. 1975; Van Leeuwen et al. 2015). The application of acaricides still remains the most frequently used method to keep *T. urticae* populations below economic thresholds for many crops. However, the fast development of resistance in the species, in part due to biological characteristics such as a short life cycle, high reproductive potential, arrhenotokous reproduction, together with a broad host plant range, reduces the efficacy of the application of acaricides (Dermauw et al. 2013; Van Leeuwen et al. 2010; Wybouw et al. 2019). It is therefore important to continue to develop acaricides with new modes of action and limited cross-resistance to other commercially available compounds to secure proper resistance management in *T. urticae* (Nauen et al. 2012; Van Leeuwen, Thomas et al. 2015; Fotoukkiaii et al. 2019). The carboxanilide pyflubumide is a recently developed acaricide by Nihon Nohyaku Co. Ltd with excellent activity against phytophagous mites of the genus *Tetranychus* and *Panonychus* (both belong to the Tetranychidae family) (Nakano et al. 2015). Pyflubumide is structurally similar to the carboxamide fungicides that inhibit succinate-dehydrogenase. Pyflubumide, together with the beta-ketonitriles cyenopyrafen and cyflumetofen, are the first commercially developed acaricides that act as complex II inhibitors in the mitochondrial electron transport chain (Furuya et al. 2017; Van Leeuwen et al. 2015). Similar to the beta-ketonitriles, pyflubumide is a pro-acaricide that is converted to a more potent de-acylated metabolite (Nakano et al. 2015). Although the metabolites of pyflubumide and cyenopyrafen are reported to both strongly inhibit complex II, different binding modes have been suggested with the target-site (Furuya et al. 2015; Nakano et al. 2015). Cross-resistance to conventional acaricides in Japanese field collected resistant populations was not detected for pyflubumide (Furuya et al. 2015). The specificity of pyflubumide and its compatibility with non-target arthropods such as pollinators and natural enemies make it an ideal candidate for integrated pest management (IPM) programs (Furuya et al. 2017; Van Leeuwen et al. 2015).

In this study, the baseline activity and potential cross-resistance risk of pyflubumide was explored by analyzing its toxicity in a reference panel of *T. urticae* strains resistant to various acaricides with

different modes of action. The base line toxicity on adult females, males, and eggs of resistant and susceptible mites was also assessed. Our survey identified a strain resistant to cyenopyrafen (JPR) as the only strain with a low-to-moderate level of cross-resistance to pyflubumide. We subsequently selected JPR for higher levels of pyflubumide resistance and finally obtained two highly resistant strains. To gain further insight into the molecular mechanisms that underpin pyflubumide resistance, we screened for potential target-site resistance mutations, conducted synergism/antagonism experiments, and analyzed transcriptomic changes under various conditions between susceptible and resistant strains.

Materials and methods

Survey of pyflubumide resistance

A reference panel of fifteen acaricide-susceptible and -resistant *T. urticae* strains (Table 1) were screened for resistance to pyflubumide in toxicity bioassays on adult female mites as described below. All strains were maintained on potted bean plants *Phaseolus vulgaris* L. cv. ‘‘Speedy’’ at 25 ± 1°C, 60% RH, and 16:8 h (L:D) photoperiod. Commercial formulations (20% SC, Danikong) of pyflubumide was kindly provided by Ralf Nauen. All other chemicals and synergists were analytical grade and purchased from Sigma-Aldrich.

Selection for pyflubumide resistance

To assess the risk of the development of pyflubumide resistance in *T. urticae*, JPR was reared with pyflubumide selection pressure under two different laboratory regimes, as described below. The two derived strains are referred to as JPR-R1 and JPR-R2.

1) Plate selection regime

Approximately 2000 adult female JPR mites were placed on four detached bean leaves in separate Petri dishes (about 500 females per leaf). Mites were sprayed with 1 ml of 100 mg/L pyflubumide (~ LC90 of JPR) using a Potter spray tower resulting in 2 mg aqueous deposit per cm^2. After 48 h, females that appeared unaffected were transferred to untreated bean plants and allowed to propagate for approximately three generations. The dose-response relationship of pyflubumide toxicity was assessed in this generation, hereafter called SEL1. A second round of selection was undertaken with 1000 mg/L

pyflubumide using the same approach, resulting in generation SEL2. From generation SEL2 onwards, the population was grown on bean plants sprayed until run off with 100 mg/L. The resulting resistant strain was named JPR-R1.

2) On plant selection regime

Approximately 2000 adult female JPR mites were transferred to potted bean plants that were sprayed with a hand-hold spraying device with 100 mg/L pyflubumide until run-off. Before spraying, plant buds were removed and only the primary leaves were kept on the potted bean plants. After seven days, all apparent unaffected mites (from all stages and both sexes) were collected and transferred to untreated bean plants for propagation. The strain was named JPR-R2 and maintained on potted bean plants with a constant selection pressure of 100 mg/L of pyflubumide until analysis.

Toxicity bioassays

Bioassays were performed on eggs, adult females and males. To obtain eggs of a synchronized age for egg bioassays, 50-60 adult females were placed on the upper side of 9 cm^2 bean leaf discs and allowed to oviposit for 4 h. After removing adult mites, the number of eggs were counted per petri dish. After 24 h, the eggs were sprayed with 1 mL of spray fluid at 1 bar pressure in the Potter spray tower, resulting in 2 mg aquous deposit per cm^2. Four replicates of five-eight concentrations of pyflubumide and a control (deionized water) were tested. Mortality was scored after 5 days. Percentage of mortality was calculated by dividing the number of hatched larvae to the number of eggs.

Female and male bioassays were conducted using a standardized method previously described by (Van Leeuwen et al. 2006). Briefly, we tested five-eight concentrations in four replicates. For each replicate 20-30 adults were transferred to 9 cm^2 bean leaf discs on wet cotton wool. Leaf discs were sprayed as outlined above, and mortality was scored after 24 h.

LC_{50}-values and the 95% confidence limits were calculated from probit regressions using the POLO-Plus software (LeOra Software, 2006).

Sequencing of SQR subunits

DNA extraction of the JPR and JPR-R populations was performed as described by Van Leeuwen (2008) (Van Leeuwen et al. 2008). Briefly, approximately 200 adult females were collected and homogenized

in 800 µL SDS buffer (200mM Tris-HCl, 400 mM NaCl, 10 mM EDTA, 2% SDS at pH = 8.3) followed by a phenol-chloroform extraction. The four subunits of JPR-R2 and JPR strains were sequenced using the primer pairs listed in supplemental file 1. PCR reactions were conducted in 50 µL of reaction mixture containing 2 mM of $MgCl_2$, 0.2 µM of each primer, 0.2 mM of deoxynucleotide triphosphate (dNTP) mix (Invitrogen, Merelbeke, Belgium), 5 µL of 10× PCR-buffer (Invitrogen) and 1U of Taq DNA polymerase (Invitrogen), under the following conditions: 2 min at 95 °C, 35 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension of 2 min at 72 °C. After purification using E.Z.N.A. Cycle-Pure kit (Omega Bio-tek, Norcross, GA, USA), PCR products were sequenced at LGC Genomics (Germany). All SQR subunits of JPR-R2 and JPR were screened for nucleotide variants using those of the London strain as reference.

Synergism/antagonism and barbital experiments

Synergism/antagonism experiments were performed according to the methods described by Van Pottelberge et al. (2009). Briefly, 1,000 mg/L of piperonyl butoxide (PBO), 500 mg/L of S,S,S-tributyl phosphorotrithioate (DEF) and 2,000 mg/L of diethylmaleate (DEM) were used as the final concentrations in the experiments. The synergists/antagonists were first dissolved in N, N dimethyl formamide and emulsifier W (3:1), and then diluted to the respective concentrations using deionized water. Mites were sprayed with 1 ml of the synergist/antagonist solution as described above. Exactly 24 h after synergist treatment, mites were used in pyflubumide bioassays. Mortality was recorded after 24 h and synergism ratios (SR) were determined by dividing the LC_{50} of pyflubumide alone by the LC_{50} obtained after synergist pre-treatment.

For barbital assays, the methods described by (Van Pottelberge et al. 2008) were followed. In short, the barbital solution was made by dissolving barbital powder in deionized water. Each replicate (20-30 adult females) was sprayed with 1 ml of 10,000 mg/L barbital solution as described above. After 4 h, mites were sprayed with five-eight concentrations of pyflubumide and a control each with four replicates. The synergism/antagonism ratio was determined by dividing the LC_{50} of treated population by the LC_{50} of non-treated population.

Timing of symptomology

To determine the timing of symptomology, we used 100 mg/L pyflubumide which is a concentration that did not lead to physiological symptoms in JPR-R1 and JPR-R2 strains. About 30 adult female mites from strains JPR, JPR-R1, and JPR-R2 were transferred to the upper side of 9 cm^2 bean leaf discs in ten replicates. All 30 petri dishes were sprayed with 100 mg/L of pyflubumide using the Potter spray tower as described above. Four replicates were sprayed with deionized water as a control. All plates were checked every 1.5 h, starting 1h into the photophase, until the18 h timepoint, with a final observation at 24 h. Dead mites and mites that proved unable to walk the length of their body or displayed uncoordinated behavior (spastic movement) after a gentle touch by a tiny brush were recorded as affected. Significant differences in the percentage of affected mites across time points were determined using a General Linear Model (GLM) followed by Tukey's HSD tests ($\alpha = 0.05$). Statistical analysis was conducted within the R framework [R Core Team (2014), version 3.1.2] (Team 2018).The experiment was conducted at 23-25 °C, 60% RH, and 18/6h (L/D) photoperiod with the photophase started at 6:00 AM.

Experimental set-ups for the transcriptomic analyses of pyflubumide resistance

Three transcriptomic experiments were performed that differed in the way *T. urticae* strains were exposed to pyflubumide. First, four RNA samples were collected from JPO (the ancestral strain of JPR) (Sugimoto and Osakabe 2014), JPR, JPR-R1, and JPR-R2 that were maintained for one generation on non-sprayed plants. Next, four samples were collected from JPR and JPR-R1 that were either exposed to pyflubumide or deionized water for 9 h. Here, approximately 500 adult female mites were placed on detached leaf discs on wet cotton wool and were sprayed with 1 ml of pyflubumide solution (100 mg/L) or deionized water at 1 bar pressure in a Potter spray tower resulting in 2 mg aqueous deposit per cm^2. Last, four RNA samples were collected from JPR-R1 and JPR-R2 that were either sprayed with pyflubumide (100 mg/L) or deionized water 24 h before collection, using the same methodology as for the 9h time point.

RNA extraction and transcriptomic analyses of pyflubumide resistance

Per sample, RNA was extracted from a bulk of 120-150 adult females using a RNeasy minikit (Qiagen), treated with DNase (Turbo, Ambion), and labelled using the Low Input Quick Amplification Kit

(Agilent Technologies). RNA samples were dyed with cyanine-3 or cyanine-5. Cyanine-labeled RNA was hybridized in the respective mixes (cyanine-5 and cyanine-3) to a custom Agilent GE microarray (Gene Expression Omnibus (GEO) Platform GPL16890). After washing using the Gene Expression Wash Buffer kit (Agilent Technologies), microarray slides were scanned with an Agilent Microarray High-Resolution Scanner. The raw intensity values are accessible at the GEO website (GSE138192) and were used for statistical analysis in limma (Smyth Gordon 2004). Background correction was performed using the 'normexp' method (with an offset of 50) (Ritchie et al. 2007). Within- and between-array normalization was applied using the global loess and Aquantile methods, respectively. Data quality was assessed at every step using arrayQualityMetrics (Kauffmann et al. 2008). Using the probe annotation identified in (Snoeck et al. 2018), a linear model was fitted to the processed data, incorporating intraspot correlations (Smyth and Altman 2013). Significant differential gene-expression was identified by empirical Bayesian statistics with cut-offs of Benjamini-Hochberg corrected *p*-values and $\log_2$FC at 0.05 and 1, respectively. A Principal Component Analysis (PCA) was performed on relative transcription levels using the prcomp function within the R environment (Team 2018). An optimal number of clusters for *k*-means clustering was assessed using the gap statistic (global max, seed number set at 54321, cluster number ranging from 2 to 10) (Tibshirani et al. 2001). Using JPO as a common reference, the relative gene-expression levels of genes that were significantly differentially expressed in any comparison were used as input and clustered using the centered Pearson's correlation as distance metric.

Results

Pyflubumide cross-resistance screen and selection

The toxicity of pyflubumide in a number of laboratory and field-collected *T. urticae* strains is shown in Table 1. All strains displayed similar LC_{50} values (2-7 mg/ L), with the exception of JPR, that exhibited an LC_{50} value of 49 (37.62 - 65.63) mg/ L.

To assess the risk of resistance development, JPR was selected for pyflubumide resistance using two different selection regimes. After selection with 100 mg/L on plates, the resulting population SEL1 displayed an LC_{50} value of 247.78 (190.58 - 303.23) mg/L. A second selection with 1,000 mg/L resulted in population SEL2 with LC_{50} of 824.96 (693.47 to 966.43) mg/L. The population was further

maintained on sprayed plants with 100 mg/L and the LC_{50} of the final population, JPR-R1, was 1,437.48 (1,221.26 – 1,666.77) mg/L (Fig. 1).

In an alternative approach, the strain JPR was independently selected for pyflubumide resistance on potted plants, and resulted in the JPR-R2 strain, with an LC_{50} value of 1,893.67 (1,641.63 – 2,156.25) mg/ L.

Toxicity of pyflubumide on females, males, and eggs of susceptible and resistant mites

The LC_{50} values of pyflubumide on females, males and eggs of Wasatch, JPR, JPR-R1 and JPR-R2 strains are reported in Table 2. Pyflubumide was toxic to all tested stages of Wasatch, but males were about 10-fold more susceptible, while eggs were clearly less susceptible (Table 2). While resistance levels were high in adults in both JPR-R strains, eggs showed a marked higher susceptibility (54.10 and 98.19 mg/ L) to pyflubumide, and resistance levels were also lower in males. In contrast, resistance levels in parental JPR were low in adults (49 mg/L) and higher in eggs (318 mg/L), a pattern that was apparently not maintained, nor fortified by selection for pyflubumide resistance.

Sequencing of the succinate: ubiquinone oxidoreductase subunits

Only synonymous SNPs were identified in *tetur01g15710* (*SdhB*), *tetur30g00210* (*SdhC*) and *tetur20g00790* (*SdhD*). In *tetur08g03210* (*SdhA*), we found a substitution V209I between the London sequence (V209) and both JPR (I209) and JPR-R2 (I209). Coding sequences generated in this study are accessible at the NCBI repository (XXXX).

Synergism and antagonism assays

The effect of synergists PBO, DEF and DEM on pyflubumide toxicity are presented in Table 3. Synergism/antagonism assays were performed on susceptible LS-VL, JPR, JPR-R1 and JPR-R2. Pyflubumide toxicity was synergized two-fold by PBO in JPR and much higher in JPR-R1 and JPR-R2 with remarkable SRs of 15- and 20-fold, respectively. DEF antagonized pyflubumide toxicity in all strains, however, the antagonism was about 10-fold higher in JPR. The synergistic effects with DEM was higher in JPR-R1 and JPR-R2 compared with JPR (Table 3). The results of phenobarbital effect on toxicity of pyflubumide are presented in Table 4. Barbital treatment decreased the toxicity of pyflubumide more in JPR-R1 and JPR-R2, as compared with JPR (Table 4).

Constitutive transcriptomic changes associated with selection for pyflubumide resistance

To characterize potential constitutive, or environmentally independent, transcriptomic changes associated with selection for pyflubumide resistance, the transcriptomes were sampled of JPO, JPR, JPR-R1, and JPR-R2, all maintained for one generation without selection pressure on non-sprayed bean plants. A PCA shows that the transcriptomic profile of JPO was highly divergent from JPR and JPR-R1-2 and that a low percentage of the total data variation underlined the differences between JPR and JPR-R1-2 (Fig. 2A). Using JPR as the parental reference, a small number of genes were significantly differentially transcribed in JPR-R1 and JPR-R2 (47 and 37, respectively) (Fig. 2B and Supplemental file 2). Of these, only 14 genes were consistently differentially expressed in both JPR-R1 and JPR-R2. Three genes (*tetur13g01730*, *tetur14g03160*, and *tetur14g01700*) were consistently up-regulated upon pyflubumide selection, but none coded for enzymes that are known to be associated with xenobiotic metabolism (hypothetical proteins and a protein with an 'Immunoglobulin E-set' domain (IPR014756), respectively) (Fig. 2B and Supplemental file 2). One of the genes with a lower transcription level in JPR-R1 and JPR-R2, *tetur03g00830*, coded for the cytochrome P450 CYP392A12 (Fig. 2B). No carboxyl/cholinesterase (CCE) genes were down-regulated in these two comparisons. We hypothesized that the transcriptomic signature of pyflubumide resistance might have been masked in these comparisons because it was already present in the ancestral, cross-resistant JPR strain. Therefore, as a next step, we used the original and susceptible JPO strain (the parent of JPR) as a reference to look at transcriptomic changes. Compared to JPO, 454 genes were differentially expressed in JPR, whereas 362 and 368 differentially expressed genes (DEGs) were detected in JPR-R1 and JPR-R2, respectively (Supplemental Tables). Selecting JPR from JPO was associated with the up-regulation of 16 cytochrome P450 genes, with *CYP392D2*, *CYP392D6*, and *CYP392A12* having a $\log_2$FC higher than four. Two CCE genes (*tetur17g00360* and *tetur30g01290*) were down-regulated in JPR compared to JPO, whereas six CCE genes showed a significant up-regulation. In both JPR-R1 and JPR-R2, 13 cytochrome P450s were up-regulated compared to JPO, of which 11 genes already showed significant up-regulation in JPR (*CYP392D2*, *CYP392D6*, and *CYP392E8* had the highest average transcriptional increase (Supplementary Tables)). JPR-R1 and JPR-R2 each had one unique up-regulated cytochrome P450 (*tetur03g05040* and *tetur06g02620*, respectively (both are not full-length in the reference London

genome (Grbić et al. 2011))). *Tetur03g05010* was the only cytochrome P450 (*CYP392D4*) that was up-regulated in JPR-R1-2, but not in JPR (average log_2FC of 1.02). Only JPR-R1 had a down-regulated CCE gene that was not already down-regulated in JPR; *tetur01g10800* with a log_2FC of -1.08. To gain more insight into the transcriptional evolution of these and other genes, all 560 DEGs were grouped using *k*-means clustering, and three distinct transcriptional patterns became apparent (Fig. 2C and Supplemental Tables). The 513 DEGs of cluster 1 and 2 showed a stable down- and up-regulation, respectively, across JPR, JPR-R1, and JPR-R2. The 47 DEGs of cluster 3 showed a high relative transcription level in JPR-R1-2, but not in JPR. Notably, *CYP392D4* was placed in this group of DEGs.

Symptomology timing

In order to gain insights in the time of toxicity in relation to potential plastic transcriptional changes, mites were exposed to pyflubumide and cumulative percentages of mortality and symptoms were followed during 24 h after spraying with a sub-lethal dose of pyflubumide in JPR and JPR-R1-2 (Fig. 3). The highest level of pyflubumide-induced toxicity symptoms was observed nine hours after spraying in both JPR-R1 (df = 2; F = 7.90; $P = 0.0019$) and JPR-R2 (df = 2; F = 12.63; $P < 0.0001$). The symptom level at this time point was significantly different from that of the previous time points, i.e., 7.5 h (Tukey's post hoc test, $P < 0.001$ for both JPR-R1 and JPR-R2) and the next time point at 10.5 h (Tukey's post hoc test, $P = 0.038$ and $P = 0.037$ for JPR-R1 and JPR-R2, respectively). After 9 h the symptoms declined in JPR-R1-2, whereas JPR showed an increase in the level of symptoms (Fig. 3).

Plastic transcriptomic responses to pyflubumide exposure

As only limited constitutive gene expression differences were observed between JPR and JPR-R1-2, the plastic, environmentally dependent, transcriptomic changes were also characterized to shed more light on the molecular basis of pyflubumide resistance. Based on the symptomology bioassays, we first investigated the transcriptomic responses of JPR and JPR-R1 after nine hours of exposure to a sub-lethal dose of pyflubumide (100 mg/L). Here, no DEGs were detected in JPR-R1 upon pyflubumide exposure, whereas a single DEG (*tetur04g04350*, log_2FC of 1.17) was observed in JPR. Next, we focused on the plastic responses of JPR-R1-2 upon 24 h of exposure to the same sub-lethal dose of pyflubumide, versus water-sprayed controls. As reflected in the PCA (alongside the PC1 axis, Fig. 4A), large transcriptomic

changes were observed here, clearly separating water-sprayed from pyflubumide-sprayed populations. Using water-sprayed populations as respective references, JPR-R1 had 294 DEGs, whereas JPR-R2 exhibited 118 DEGs (Fig. 4B). Over 92% of the transcriptional response of JPR-R2 was also present in the response of JPR-R1, and the shared DEGs (n=109) exhibited a positive relationship in their plastic transcriptional response (Fig. 4B). Eight cytochrome P450s were part of this positively correlated response, of which five showed up-regulation after exposure to pyflubumide (Fig. 4B). Four CCE genes were up-regulated when the resistant strains were exposed to pyflubumide (Supplementary Tables).

Discussion

The development of new acaricides with new modes of action and limited cross-resistance to other conventional compounds remains crucial for efficient resistance management of phytophagous mite pests like *T. urticae* (Nauen et al. 2012). Pyflubumide is a newly developed complex II inhibitor and the first acaricide with a carboxanilide structure (Furuya et al. 2017). In this study, the compound proved to be very active on a collection of strains with varying levels of resistance to commercially available compounds. However, we uncovered decreased susceptibility in a Japanese strain named JPR, which was selected out of JPO with cyenopyrafen (Sugimoto and Osakabe 2014). The decreased pyflubumide susceptibility was only found in JPR, and not its parent JPO, suggesting that selection for cyenopyrafen resistance resulted in decreased susceptibility for pyflubumide (Table 1), a typical case of moderate cross-resistance. As cyenopyrafen and pyflubumide both belong to complex II inhibitors acaricides and their active metabolites act on the same enzyme (Nakano et al. 2015), cross-resistance is not surprising, although pyflubumide and cyenopyrafen belong to different chemical families, and might have slight different binding modes (Nakano et al. 2015). In addition, cross-resistance between cyenopyrafen and cyflumetofen, both beta-ketonitriles, has been demonstrated in JPR (Khalighi et al. 2016).

Before studying the mechanisms of (cross)-resistance, we attempted to further fix pyflubumide resistance in JPR using two different laboratory selection regimes, and obtained two highly resistant strains, JPR-R1 and JPR-R2. Pyflubumide resistance evolved very quickly (Fig. 1), indicating that under field conditions pyflubumide efficacy would be at risk after prior selection with cyenopyrafen.

In the susceptible Wasatch strain, males were much more susceptible to pyflubumide than females, while in contrast, eggs were much less susceptible. Variation in pesticide susceptibility between sexes and developmental stages of pest species can be related to morphological differences (e.g. body size or egg shell thickness) and/or metabolic differences (e.g. differential expression of detoxifying enzymes). A smaller male and egg size in *T. urticae* can provide a higher exposure to acaricides through higher surface-to-volume ratios, although sexual size dimorphism is not always related to variation in pesticide tolerance (Daly and Fitt 1990; Rathman et al. 1992). When assessing stage-specific effects of pyflubumide resistance, we discovered that unlike adult females, males and eggs of the two JPR-R strains were still highly susceptible to pyflubumide, so resistance was strongly female biased. This pattern was however not observed for JPR where eggs retained most of the resistance levels (Table 2). As resistance levels were maintained in eggs versus adults in JPR, but not in JPR-R, this would suggest that at least additional mechanisms were selected. Stage specific effects in resistance has been previously reported in spider mites; spirodiclofen resistance is high in adult *T. urticae* females but absent in eggs (Van Pottelberge, Van Leeuwen, Khajehali et al. 2009). Sex-linked pesticide resistance has been observed in haplodiploid species such as spider mites where unfertilized haploid eggs develop into males and fertilization leads to diploid females (Carrière 2003). One of the underlying mechanisms might be a dosage-effect of the resistance alleles in haploid males (Carrière 2003). This mechanism is similar to sex-linked pesticide resistance in diplodiploid species in which the sex-linked allele is not always present, or expressed in a specific gender (Baker et al. 1994; Marín et al. 2000; Rao and Padmaja 1992). Of practical importance, the effectiveness of resistance management strategies is higher when such sex-linked pesticide resistance patterns are taken into account as the susceptibility of males (and developmental stages such as eggs) might slow down the resistance development process by reducing the overall population growth.

Pyflubumide is structurally similar to the carboxamide fungicides that act as a complex II inhibitors (SDHI), and a similar mode of action has been documented (Furuya et al. 2017). Resistance to SDHI fungicides has been reported to be conferred by specific point mutations in complex II subunits (SDHB, -C, and -D) in many fungal plant pathogens (Avenot and Michailides 2010; Oliver 2014; Sierotzki and

Scalliet 2013). Because of the apparently frequent target-site resistance development in fungi, and the observed cross-resistance between pyflubumide, cyenopyrafen and cyflumetofen (all acting on complex II), we first searched for target-site resistance by sequencing all subunits (SDHA, until -D) that make up complex II. However, we did not find any target-site resistance mutation in subunits SDHB, SDHC and SDHD, previously implicated in QoI resistance in fungi (Avenot and Michailides 2010; Hahn 2014; Sierotzki and Scalliet 2013). The V209I variant in SDHA in JPR and JPR-R2 was not positioned in a conserved region and very likely not associated with resistance, as the ubiquinone-binding pocket where pyflubumide interacts is structurally defined by the interface between the SDHB, -C, and -D subunits (Sierotzki and Scalliet 2013), and is likely the reason why mutations in SDHA have not been uncovered in fungi yet. This is in line with a previous study that did not find target-site resistance in the cyenopyrafen resistant strain JPR (Khalighi et al. 2016) and points toward an alternative mechanism of (cross)-resistance.

Synergism experiments strongly suggested that cytochrome P450 monooxygenases and glutathione S-transferases are involved in pyflubumide detoxification in female resistant mites in the two JPR-R strains (Table 3). To a lesser extent, this is also true for JPR (2-fold PBO synergism). A strong antagonism of toxicity was found after treatment with DEF, which confirms pyflubumide is a pro-acaricide that requires hydrolytic activation (Khajehali et al. 2009; Nakano et al. 2015; Van Leeuwen et al. 2006). Interestingly, antagonism is much higher in JPR compared to both the susceptible LS-VL and resistant JPR-R strains, again pointing towards different mechanisms between parental and selected strains. Pretreatment with barbital dramatically lowered the resistance levels of JPR-R1-2 and JPR, whereas it slightly increased pyflubumide resistance in the susceptible strain LS-VL. This suggests that the detoxifying enzymes, most likely cytochrome P450s, were more suppressed than induced by barbital in the resistant strains. Collectively, it seems likely that the differences in pyflubumide susceptibility of eggs and males of the resistant strains might be due to a different detoxification potential. Higher levels of resistance in the JPR-R strains correlate with higher synergism ratio for PBO and DEM.

Therefore, we performed a genome wide gene-expression analysis between parental (JPR) and selected resistant lines JPR-R. Transcriptome analysis did not show any notable differences between strains in

379 the absence of acaricide pressure (Fig. 2A-B). In contrast, when JPO was used as the reference, over
380 500 genes were differentially transcribed in JPR and JPR-R1-2 with the majority of the DEGs exhibiting
381 stable transcript levels (Fig. 2C). Cluster analysis revealed a small group of DEGs that show an increased
382 expression in JPR-R1-2, compared to JPO (Fig. 2C, cluster 3). The cytochrome P450 CYP392D4 is
383 clustered in this group, and it belongs to the CYP392 family that underwent a spider mite-specific
384 expansion (Grbić et al. 2011) and of which many members have been implicated in resistance in *T.*
385 *urticae* (Demaeght et al. 2013a; Riga, M. et al. 2014; Van Leeuwen and Dermauw 2016). Together,
386 these results suggest that the constitutive transcriptomic signature of pyflubumide resistance was already
387 present in JPR and did not significantly change when selecting for higher resistance levels in JPR-R1-2
388 (Fig. 2A). We therefore tested the hypothesis whether the high pyflubumide resistance of JPR-R1-2
389 could be caused by a heritable increased plasticity (induction by pyflubumide). In our observations of
390 the symptomology timing, the highest level of JPR-R responses to pyflubumide was observed 9 h after
391 exposure to the acaricide. Afterwards, the symptoms declined, which suggests that the detoxifying
392 mechanisms were activated after this time. We indeed found that five cytochrome P450s and four CCE
393 genes were up-regulated when JPP-R1-2 were exposed to pyflubumide for 24 h, compared to water-
394 sprayed JPR-R1-2 mites. Here, the up-regulation of cytochrome P450s further supports our hypothesis
395 that pyflubumide resistance is metabolic.

396 Together, our results show that although many resistant *T. urticae* strains were susceptible to
397 pyflubumide, there could be a risk of rapid development of pyflubumide resistance in the areas that have
398 been exposed to cyenopyrafen and probably other complex II inhibitors. We gathered evidence that
399 indicates that cytochrome P450 enzymes are likely involved in pyflubumide resistance. However, gene-
400 expression patterns have shown to be complex and failed to clearly identify which cytochrome P450(s)
401 might be involved. Future work should focus on trying to get functional data on pyflubumide
402 metabolizing enzymes and characterize resistance with alternative hypothesis-free approaches such as
403 QTL mapping (Kurlovs et al. 2019).

404 **Acknowledgments**

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Tables

Table 1 The toxicity of pyflubumide (LC_{50} and slope) in a acaricide resistance reference panel of *T. urticae*

Strain	Resistant to	Reference	LC_{50} (mg/L) (95% CI)[a]	RR*
LONDON	Susceptible	(Khajehali et al. 2011)	2.55 (2.46 - 2.63)	1
WASATCH	Susceptible	(Riga et al. 2017)	3.08 (2.76 - 3.36)	1.21
JPO	Unknown	(Sugimoto and Osakabe 2014, Khalighi et al. 2016)	3.09 (2.87 - 3.28)	1.21
JPS	unknown	(Asahara et al. 2008, Khalighi et al. 2016)	5.08 (4.88 - 5.28)	1.99
LS-VL	Susceptible	(Van Leeuwen et al. 2005)	3.27 (3.04 - 3.43)	1.28
BR-VL	Bifenazate	(Van Leeuwen et al. 2008)	1.83 (1.63 - 2.02)	0.72
ETOXR	Etoxazole	(Van Leeuwen et al. 2012)	2.70 (2.52 - 2.86)	1.06
TU008R	Cyflumetofen	(Khalighi et al. 2014)	3.18 (2.95 - 3.40)	1.25
AKITA	METIs	(Stumpf and Nauen 2001)	4.20 (3.95 - 4.42)	1.65
SR-VP	Spirodiclofen	(Demaeght et al. 2013b; Van Pottelberge et al. 2009)	4.30 (4.12 - 4.48)	1.69
MAR-AB	Multi	(Dermauw et al. 2013)	5.86 (5.31 - 6.35)	2.30
HOL3	Bifenazate	(Van Nieuwenhuyse et al. 2009)	6.01 (5.58 - 6.43)	2.36
MR-VL	Multi	(Van Leeuwen et al. 2005)	6.39 (5.80 - 6.85)	2.51
MR-VP	Multi	(Dermauw et al. 2013; Van Pottelberge et al. 2009)	7.59 (6.80 - 8.24)	2.98
JPR	Cyenopyrafen	(Khalighi et al. 2016)	49.07 (37.62 - 65.63)	19.24

*Resistance ratio, LC_{50} of resistant strain/LC_{50} of susceptible strain

[a]The lethal concentration required to kill 50% of the population

544 **Table 2** The toxicity of pyflubumide (LC_{50} and slope) in different life stages of susceptible and

545 resistance *T. urticae* mites

		WASATCH	JP-R	JP-RR1	JP-RR2
Female	LC50 (mg/ L)[a]	3.08	49.07	1437.48	1893.67
	(95% CI)[b]	(2.76 - 3.36)	(37.62 - 65.63)	(1221.26 - 1666.77)	(1641.63 - 2156.25)
	Slope ± SE[c]	8.69 ± 0.86	2.15 ± 0.21	2.08 ± 0.20	2.20 ± 0.21
	RR*	-	15.93	466.71	614.83
Male	LC50 (mg/ L)[a]	0.44	2.98	8.13	5.72
	(95% CI)[b]	(0.40 ± 0.48)	(2.73 ± 3.19)	(7.41 ± 8.79)	(4.97 ± 6.34)
	Slope ± SE[c]	4.83 ± 0.5	5.03 ± 0.42	4.55± 0.41	3.59 ± 0.42
	RR*	-	6.77	18.48	13
Egg	LC50 (mg/ L)[a]	23.6	318.20	98.19	54.10
	(95% CI)[b]	(19.93 ± 27.60)	(258.37 ± 381.99)	(78.71 ± 118.97)	(40.07 ± 68.91)
	Slope ± SE[c]	2.03 ± 0.14	1.60 ± 0.15	1.61 ± 0.12	1.42 ± 0.11
	RR*	-	13.48	4.16	2.29

[a]The lethal concentration required to kill 50% of the population

[b]Confdence interval to 95% of the LC estimates

[C]Standard error

*Resistance ratio, LC_{50} of resistant strain/LC_{50} of susceptible strain

546

547 **Table 3** The effect of synergist/ antagonist PBO, DEF, DEM and barbital on pyflubumide toxicity in

548 susceptible and resistant strains of *T. urticae*

		LS-VL	JP-R	JP-RR1	JP-RR2
Acaricide alone	LC_{50} (mg/ L)[a]	3.27	49.07	1437.48	1893.67
	(95%CI)[b]	(3.10 - 3.41)	(37.62 - 65.63)	(1221.26 - 1666.77)	(1641.63 - 2156.25)
	Slope ± SE[c]	11.11 ± 0.92	2.15 ± 0.21	2.08 ± 0.20	2.20 ± 0.21
PBO	LC_{50} (mg/ L)[a]	2.74	22.82	92.79	69.21
	(95%CI)[b]	(2.59 - 2.85)	(19.31 - 26.71)	(81.01 - 104.85)	(55.66 - 84.41)
	Slope ± SE[c]	10.40 ± 1.10	1.99 ± 0.17	2.62 ± 0.19	1.69 ± 0.14
	SR[d]	1.2	2.15	15.49	20.4
DEF	LC_{50} (mg/ L)[a]	7.38	2176.16	3843.95	5028.17
	(95%CI)[b]	(6.86 - 7.79)	(1834.28 - 2498.45)	(3285.81 - 4524.38)	(4245.05 - 6011.25)
	Slope ± SE[c]	7.55 ± 0.86	2.23 ± 0.22	2.08 ± 0.24	1.89 ± 0.24
	SR[d]	0.44	0.02	0.37	0.38
DEM	LC_{50} (mg/ L)[a]	2.36	21.90	152.30	73.73
	(95%CI)[b]	(2.26 - 2.46)	(16.00 - 28.92)	(130.05 - 176.97)	(58.33 - 91.05)
	Slope ± SE[c]	6.95 ± 0.58	1.08 ± 0.15	1.59 ± 0.11	1.56 ± 0.10
	SR[d]	1.38	2.24	9.44	25.68
Barbital	LC_{50} (mg/ L)[a]	5.50	8.93	17.56	32.61
	(95%CI)[b]	(4.90 - 5.99)	(6.88 - 11.26)	(14.39 - 20.94)	(27.98 - 37.56)
	Slope ± SE[c]	4.54 ± 0.57	1.77 ± 0.15	2.14 ± 0.18	2.31 ± 0.17
	BAR Ratio[f]	1.68	0.18	0.01	0.02

[a]The lethal concentration required to kill 50% of the population

[b]Confdence interval to 95% of the LC estimates

[C]Standard error

[d]Synergism ratio, LC_{50} of acaricide alone/LC_{50} obtained after synergist pretreatment

[f] Barbital ratio, LC_{50} of induced population/LC_{50} of non-induced population

549

Fig. Legends

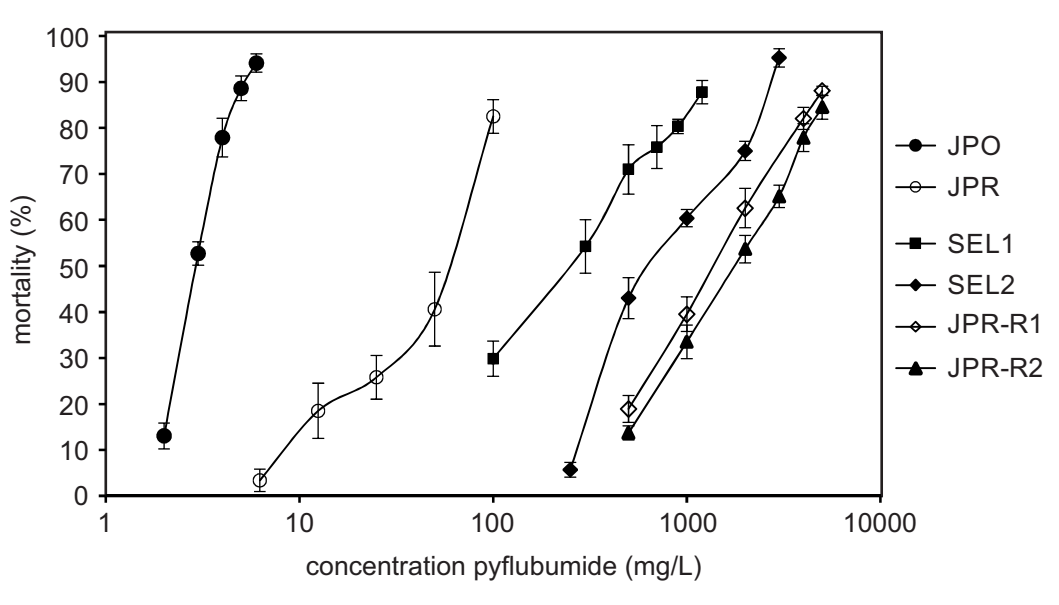
Fig. 1 Dose-response relationships of pyflubumide toxicity on JPO, and the pyflubumide selected strains. JPO is susceptible to complex II acaricides, and was first used in selection experiments. JPO was selected with cyenopyrafen resulting in JPR (Khalighi et al. 2016) and developed low to moderate cross-resistance to pyflubumide. From JPR, two selection lines (JPR-R1 and JPR-R2) were established by pyflubumide selection. SEL1 and SEL2 was the first and second generation of selection that finally generated the JPR-R1 strain. JPRR-R2 as selected out of JPR on plants as described in M&M.

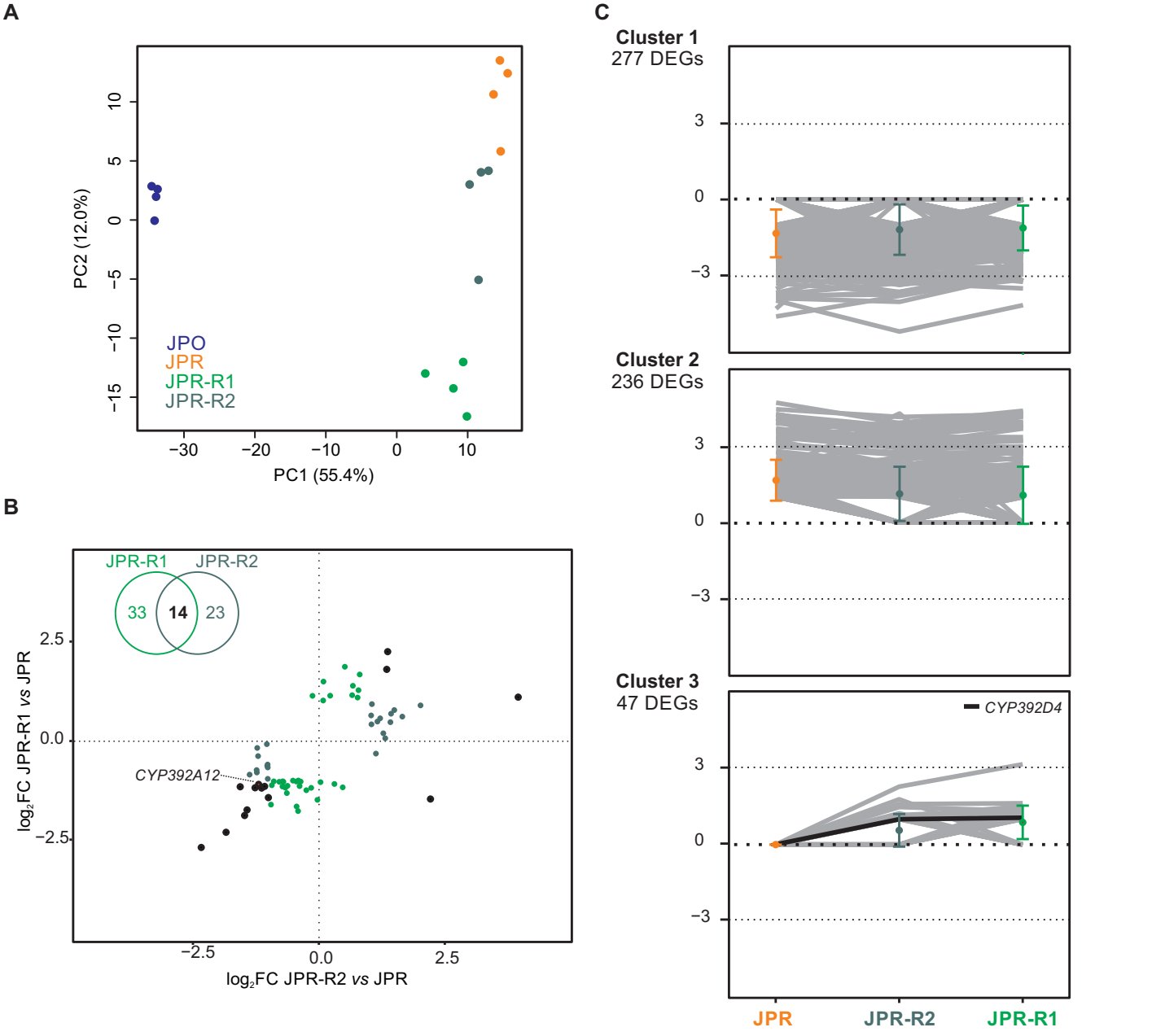
Fig. 2 The constitutive transcriptomic changes between JPO, JPR, JPR-R1, and JPR-R2. Panel A: PCA plot of JPO, JPR, JPR-R1, and JPR-R2. The four samples of each strain are separately plotted. **Panel B**: Scatterplot of the differentially expressed genes in the two pyflubumide-resistant strains JPR-R1 and JPR-R2 versus ancestral JPR. Only the genes with an FDR-corrected *p*-value of < 0.05 and $\log_2 FC \geq 1$ were regarded as differentially expressed. Only one cytochrome P450 was differentially transcribed in both pyflubumide-resistant strains, was down-regulated, and coded for CYP392A12. In panel B: Venn-diagram showing the overlap of differentially expressed genes in JPR-R1 and JPR-R2 versus ancestral JPR. **Panel C**: Using JPO as the common reference, the transcriptional patterns of the three *k*-clustered groups of the 560 DEGs across JPR, JPR-R2, and JPR-R1 are plotted. Circles represent the average (±SD) of gene-expression in each strain (color coded)

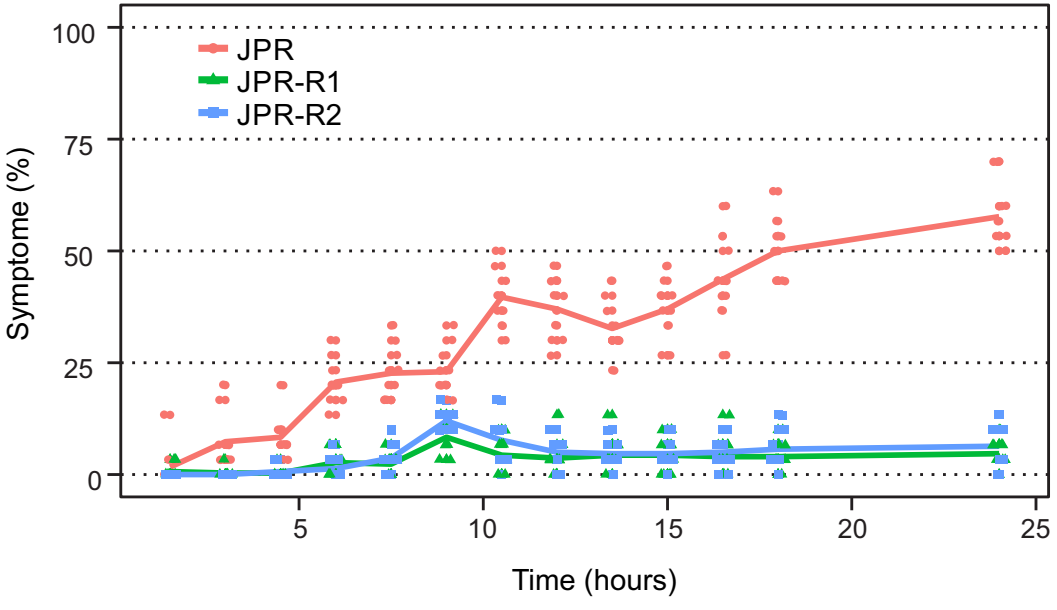
Fig. 3 Timing of symptomology in *T. urticae* strains JPR, JPR-R1, and JPR-R2. Percentage of mites that showed the symptoms of poisoning caused by pyflubumide treatment over 24 hours time course after spraying. Symptoms include death, inability to walk, or uncoordinated behavior (spastic movement) after a gentle touch by a tiny brush

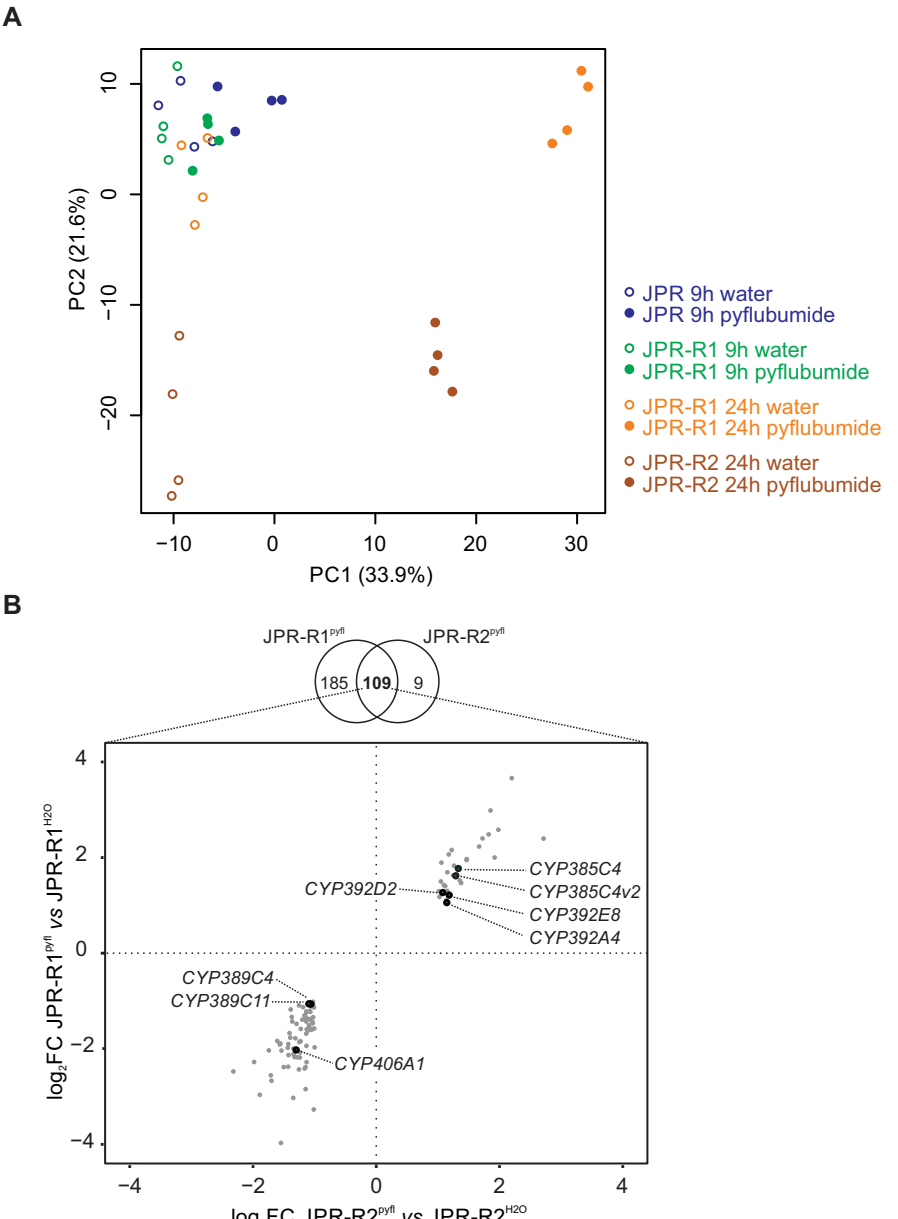
Fig. 4 The plastic transcriptomic changes upon 9 and 24 hours of exposure to a sub-lethal dose of pyflubumide. Panel A: PCA plot of the individual samples alongside PC1 and PC2 (55.5% of total data variation) that were collected to investigate plastic transcriptomic responses. **Panel B:** Above, venn-diagram showing the overlap of differentially expressed genes in JPR-R1 and JPR-R2 after 24 h exposure to 100 mg/L pyflubumide. Below, scatterplot of the 109 shared differentially expressed genes in JPR-R1 and JPR-R2 exposed for 24 h to 100 mg/L pyflubumide. Genes that code for cytochrome

P450s are depicted by black circles and labelled with their CYP identifier. Only the genes with an FDR-corrected *p*-value of < 0.05 and $\log_2 FC \geq 1$ were regarded as differentially expressed


mortality (%)
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
10000
concentration pyflubumide (mg/L)
JPO
JPR
SEL1
SEL2
JPR-R1
JPR-R2


A
PC2 (12.0%)
PC1 (55.4%)
JPO
JPR
JPR-R1
JPR-R2
B
JPR-R1
JPR-R2
33
14
23
CYP392A12
log2FC JPR-R1 vs JPR
log2FC JPR-R2 vs JPR
C
Cluster 1
277 DEGs
Cluster 2
236 DEGs
Cluster 3
47 DEGs
CYP392D4
JPR
JPR-R2
JPR-R1


JPR
JPR-R1
JPR-R2
Symptome (%)
100
75
50
25
0
5
10
15
20
25
Time (hours)


A
PC2 (21.6%)
PC1 (33.9%)
JPR 9h water
JPR 9h pyflubumide
JPR-R1 9h water
JPR-R1 9h pyflubumide
JPR-R1 24h water
JPR-R1 24h pyflubumide
JPR-R2 24h water
JPR-R2 24h pyflubumide
B
JPR-R1pyfl
JPR-R2pyfl
185
109
9
CYP385C4
CYP392D2
CYP385C4v2
CYP392E8
CYP392A4
CYP389C4
CYP389C11
CYP406A1
log2FC JPR-R1pyfl vs JPR-R1H2O
log2FC JPR-R2pyfl vs JPR-R2H2O