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The H92R substitution in PSST is a reliable diagnostic biomarker for predicting resistance to Mitochondrial Electron Transport Inhibitors of complex I in European populations of *Tetranychus urticae*

H92R as METI-I resistance marker in *T. urticae*

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Abstract

BACKGROUND

Mitochondrial Electron Transport Inhibitors of complex I (METI-I), such as tebufenpyrad and fenpyroximate, are acaricides that have been used extensively to control *Tetranychus urticae* Koch (Acari: Tetranychidae) for more than 20 years. Because of the ability of this spider mite to rapidly develop acaricide resistance, field (cross-) resistance monitoring and elucidation of resistance mechanisms are extremely important for resistance management (RM). In the present study, 42 European *T. urticae* field populations were screened for tebufenpyrad and fenpyroximate resistance, and the correlation between resistance and the H92R substitution in PSST was investigated.

RESULTS

According to the calculated lethal concentration values that kill 90% of the population (LC_{90}), tebufenpyrad and fenpyroximate would fail to control many of the collected populations at recommended field rates. Six populations exhibited high to very high resistance levels (200- to over 1950-fold) to both METI-Is. Analysis based on the LC_{50} values displayed a clear correlation between tebufenpyrad and fenpyroximate resistance, further supporting cross-resistance, which is of great operational importance in acaricide RM. The previously uncovered METI-I target-site mutation H92R in the PSST homologue of complex I (NADH:ubiquinone oxidoreductase) was found with high allele frequencies in populations resistant to tebufenpyrad and fenpyroximate. Synergist assays showed this mutation is not the only factor involved in METI-I resistance and additive or synergistic effects of multiple mechanisms most likely determine the phenotypic strength.

CONCLUSIONS

The predictive value of resistance by H92R is very high in European populations and offers great potential to be used as a molecular diagnostic marker for METI-I resistance.

Keywords: METI-I acaricides, resistance management, cross-resistance, *Tetranychus urticae*, molecular marker

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1 Introduction

The two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a major pest with a worldwide distribution [1]. The control of this generalist herbivore is mainly based on synthetic acaricides, several of which inhibit the mitochondrial electron transport (MET) chain, a vitally important pathway that is responsible for ATP synthesis.[2] This chain consists of a series of four protein complexes (I-IV) mediating electron transport from NADH and $FADH_2$ to the final electron acceptor O_2. The proton gradient across the inner mitochondrial membrane that is generated during this process acts as the driving force for complex V, better known as ATP synthase, to generate ATP.[3] The categorization of different MET inhibitors (METIs) is based on the protein complex they target within this chain. Mitochondrial complex I (NADH-ubiquinone oxidoreductase) is the largest complex in the MET chain and plays a key role in cellular energy production by transferring electrons from NADH to ubiquinone.[4–6] Acaricides that inhibit complex I are denominated "METI-I" compounds, and are referred to as group 21 in the Insecticide Resistance Action Committee (IRAC) Mode of Action Classification scheme.[7] These METI-I compounds include the acaricides fenpyroximate, tebufenpyrad and pyridaben, which are highly efficient against all spider mite life stages.[2]

The METI-I acaricides were launched in the early 1990s and quickly gained global importance.[8–12] After a decade of extensive use, the development of resistance increasingly became a problem[13–15], with cross-resistance between different METI's being observed in several cases.[16–20]

This relatively fast resistance development to METI-Is is not surprising, as *T. urticae* is known as the "resistance champion", as it developed resistance to all major pesticide classes used for its control, often in only a few years.[2,21] The factors that contribute to this spider mite's ability to rapidly develop resistance include its very short life cycle and its high fecundity, demanding frequent acaricide application for successful control.[14] Aggravating the problem, the mite has an arrhenotokous reproduction system, in which fertilised eggs develop into females, while unfertilised eggs give rise to haploid males. Therefore, recessive resistance mutations in the hemizygous males are directly exposed to selection, allowing for rapid resistance development.[1,13] With more than 1,100 recorded host plant species [22], the mite's extreme polyphagous nature is also proposed to contribute to the fast resistance development. Indeed, mechanisms that evolved for detoxifying a variety of phytotoxins appear to be of service when confronted with man-made pesticides as well.[23,24] This so called "pre-adaptation syndrome", together with some remarkable cases of horizontal gene transfer[23], has largely expanded the metabolic tool kit of spider mites. Nevertheless, other ecological and operational factors might be more predictive for the incidence of resistance development across pests with different host range.[24]

The adaptations that underlie resistance development to acaricides can be classified as either "pharmacokinetic" or "pharmacodynamic", which respectively involve mechanisms of decreased exposure (e.g. increased metabolism) and decreased sensitivity (e.g. target-site insensitivity).[21,25] Unravelling the molecular mechanisms underlying resistance is of major importance to control the development and spread of resistant populations.[2]

Synergism and enzyme activity tests first linked *T. urticae* METI-I resistance to increased metabolism, mainly involving elevated cytochrome P450 monooxygenase activity [19,20,26,27]. Recently however, the involvement of target-site insensitivity was reported as a potential main resistance factor by Bajda et al. (2017).[28] Although inhibition of complex I by fenpyroximate in *T. urticae* was already demonstrated by Motoba et al. in 1992, the fact that complex I consists of more than 40 subunits [29] has hampered the identification of the exact binding-site for METI-I compounds, and consequently also the discovery of point mutations that could lead to target-site resistance. Complex I is composed of nuclear- as well as mitochondrially-encoded subunits.[29] Sugimoto and Osakabe observed a maternal effect in pyridaben resistance that was only present in eggs and disappeared in adult females and a follow-up study revealed that no mutations in mitochondrially encoded subunits were present.[17,30] Indeed, complete maternal inheritance for METI-I resistance has not yet been reported and thus there are no indications for the presence of mutations in mitochondrially encoded subunits. In contrast, photoaffinity labelling experiments with a pyridaben and a fenpyroximate derivative pointed towards the nuclear encoded PSST subunit, and the interface between the PSST and 49 kDa subunits (both nuclearly encoded) of complex I as the binding site for the respective inhibitors.[31,32] With this information at hand, and exploiting the available genome sequence of *T. urticae* [1], Bajda et al. (2017) sequenced the PSST and 49 kDA subunits in METI-I resistant and susceptible *T. urticae* strains, leading to the discovery of the H92R substitution in the PSST protein (*Yarrowia lipolytica* numbering[33] as a reference, corresponding to H110R in *T. urticae* PSST (ORCAE gene ID *tetur07g05240*)). The H92R substitution however only partially explained the observed resistance phenotype in *T. urticae*, as introgression of the mutation in a susceptible genetic background did not result in mites that were as strongly resistant as the resistant parental strain.[28] To further investigate resistance mechanisms and their interplay, Snoeck et al. (2019) used an unbiased bulked segregant analysis to map resistance loci in the genome (QTL mapping). Based on independent mapping experiments for both fenpyroximate, tebufenpyrad and pyridaben, the H92R target-site mutation was identified as a significant factor in resistance to all three compounds.[34]

The occurrence of resistance in populations of *T. urticae* could result in field control failure and associated crop losses. Furthermore, sustained selection pressure would lead to increasing resistance genotype frequencies and increased geographical spread of resistance genes. To sustain the effectiveness of the approved and registered compounds, it is of utmost

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importance to keep resistance at bay.[35] Early detection of resistant populations, even before the application of the compound to which resistance is present in the field, is crucial to resistance management (IRM). In addition, the presence of resistance genes, even at low frequency, can inform resistance management programs. While toxicity bioassays are a highly relevant resistance screening method, faster and more cost-efficient monitoring approaches do exist, if molecular diagnostic markers are available. For example, target-site resistance mutations can easily be detected using methods such as PCR-sequencing, TaqMan qPCR and droplet digital PCR.[35] However, the predictive value of a molecular marker needs to be validated in a certain geographical region across multiple crops (for a review on the value of molecular markers we refer to Van Leeuwen et al. (2020).[35]

The main aim of present association study was to investigate the incidence of METI-I resistance and cross-resistance, and to validate the predictive value of the PSST H92R substitution in tebufenpyrad and fenpyroximate resistance in *T. urticae*. To that purpose, populations collected throughout Europe between 2018 and 2020 were subjected to full dose-response bioassays and genotyped for the PSST mutation. In addition, synergist assays were performed to better interpret the observed phenotypic strength of resistance.

2 Material and methods

2.1 Mite populations

During 2018, 2019 and 2020, 42 *T. urticae* populations were collected from various locations in Europe (**Figure 1**). These field populations (FP) were named according to the collection year (FP18-xx/19-xx/20-xx) and numbered in order of arrival in the lab. Additional information on the populations is provided in **Table S1**. All *T. urticae* FPs were maintained on untreated bean leaves (*Phaseolus vulgaris* L. cv. “Prelude”) under laboratory conditions (25 ± 1°C, 60% relative humidity and 16:8 h light:dark photoperiod).

2.2 Acaricides and toxicity bioassays

Adulticidal bioassays were carried out with commercially formulated tebufenpyrad (Masai, 20% WP) and fenpyroximate (Kiron SC, 51.2 g L^{-1}). At least five concentrations, causing mortality ranging from < 20% to > 80%, were tested in four replicates, while controls were sprayed with deionised water. For each replicate, 20 to 30 young adult female mites were transferred to 9-cm^2 square-cut bean leaf disks and sprayed with 0.8 mL of serial acaricide dilutions at 1 bar pressure (1.5 ± 0.05 mg aqueous acaricide deposit cm^{-2}) using a Cornelis spray tower.[36,37] The mites were kept at laboratory conditions and mortality was evaluated after 24 h. Mites were scored “dead” if they did not move after prodding with a fine brush. If 5000 mg a.i. L^{-1} did not cause ≥ 50% mortality, no further attempts were made to determine the LC_{50}. The lethal concentrations (LC_{50} and LC_{90} values), resistance ratios (RR), and the

corresponding 95% confidence interval (CI) were determined using PoloPlus (LeOra Software, Berkeley, CA, USA, 2006).

2.3 Synergist assays

Mites from FP18-4, FP20-19, FP20-21 and FP19-9 were subjected to a synergism study, as described by Van Pottelberge et al. (2009).[19] In short, adult females were sprayed with the synergist PBO (piperonyl butoxide, 1000 mg L^{-1}) 24 h before being used in fenpyroximate or tebufenpyrad bioassays as mentioned above. Survival was scored 24 h after acaricide treatment. LC_{50} values, synergism ratios (SR) and the corresponding 95% CI were determined using PoloPlus. If the 95% CI of the SR did not include 1, synergism was considered to be significant.

2.4 DNA isolation

Genomic DNA for populations collected during 2018 and 2020 was extracted from approximately 200 *T. urticae* female adults per population, as described by Van Leeuwen et al. (2008).[38] Briefly, mites were homogenized in a mix of 800 µL of SDS buffer (2% SDS, 200 mM Tris-HCl, 400 mM NaCl, 10 mM EDTA, pH 8.33), RNase A and proteinase K, followed by DNA extraction using a phenol-chloroform-based protocol.[39] For populations collected during 2019, the DNeasy Blood & Tissue Kit (QIAGEN) was used, according to the manufacturer's instructions. The concentration and integrity of the DNA samples was assessed using a DeNovix DS-11 spectrophotometer (DeNovix, Willmington, DE, USA) and by agarose gel electrophoresis. The concentration of the DNA samples was additionally determined with the Qubit™ dsDNA BR Assay (Invitrogen, Carlsbad, CA) on a Qubit fluorometer 2.0 (Invitrogen, Carlsbad, CA), in order to precisely calculate the input amount of double stranded DNA (dsDNA) for ddPCR reactions.

2.5 Survey of genotypes in the PSST subunit gene fragment

A quantity of 1 µL DNA was used as a template for PCR amplification. The PSST subunit gene (Orcae gene ID: tetur07g05240) fragment was amplified using forward primer 5'-ACAGGTCAGCCAATCGAATC-3' and reverse primer 5'-ATACCAAGCCTGAGCAGTGG-3', according to Bajda et al. (2017).[28] PCR-products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek) and were sequenced (LGC genomics, Germany). All sequencing data were analysed using BioEdit version 7.0.5.2..[40] Mutation frequencies were estimated by comparing the height of the wild type and mutant peaks in the sequencing chromatogram (proportional sequencing), as described by Van Leeuwen et al. (2008).[38]

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2.6 H92R frequency determination using ddPCR

In order to accurately determine the proportion of mutant H92R sequences in the DNA sample of each population, droplet digital PCR (ddPCR) was optimized and performed according to Mavridis et al. (2021).[41] The 20.0 µL reactions included 1x ddPCR Supermix for probes, 5U restriction enzyme EcoRI-HF® (New England Biolabs), 10 ng of dsDNA, 1200 nM of primers (forward primer: 5'-TTTGACTTTTGGATTAGCCTGTTG-3', reverse primer: 5'-TGCTCTGAATAACATACCAAATCTTTC-3'), 375nM of the wild type (wt) probe (5'-HEX-CGTTGAAATGATGCACA -MGB-3'), 500 nM of the mutant (mut) probe 5'- FAM-TTGAAATGATGCGCATAG -MGB-3'), and were adjusted to the final volume with DEPC-treated water. Samples were mixed with 70.0 µL of droplet generator oil for probes (Bio-Rad), inserted in the QX200 droplet generator (Bio-Rad) and transferred on 96-well plates (Bio-Rad), where PCR was performed on a C1000 Touch thermal cycler (Bio-Rad) with the following thermal protocol: 95 °C for 10 min, and 50 cycles of 94 °C for 30 s, 54 °C for 1 min, and 98 °C for 10 min. Endpoint fluorescence was measured in the FAM and HEX channels in the QX200 droplet reader (BioRad), and raw data processed with the QuantaSoft Analysis Pro Software (v.1.0.596). Synthetic double stranded DNA sequences (gBlocks™ gene fragments) of known copy numbers that contained the wild type or mutant sequences were used as standards in order to calculate the limit of detection (LoD) for the H92R assay. The mutant gBlocks™ were diluted in a stable background of wild-type DNA to produce different mutant allelic frequency (MAF) standards (50.0%, 1.0%, 0.50%, 0.20%, and 0.10%). LoD, was defined as the lowest MAF that can reliably be detected and is distinguishable from the wild type background, and determined to be 0.2% (**Figure S1**).

The number of positive and negative droplets were used to provide an absolute quantification of target DNA molecules as target copies/µL of reaction for wild type (HEX-positive) and mutant H92R (FAM-positive) alleles. Mutant and wild type allele copies (MUTcp and WTcp) per reaction were used to calculate the percentage mutant allelic frequency (%MAF) = (MUTcp/ (MUTcp + WTcp)) × 100 for each sample. The MAF for H92R can be found in **Table S1**.

The correlation of H92R allelic frequency, as determined by ddPCR versus proportional sequencing (see 2.5), was analysed (R studio, method= 'lm').[42]

3 Results

3.1 Resistance to tebufenpyrad and fenpyroximate in *T. urticae* field populations

The concentration-mortality relationship for both tebufenpyrad and fenpyroximate toxicity was assessed for 27 European field populations (**Table 1**). Probit analysis revealed that toxicity varied significantly across populations, with LC_{50} values ranging from below 5 to over 5000 mg L^{-1}. FP19-13 was considered as the most susceptible population to tebufenpyrad (LC_{50} of 2.56

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mg L^{-1}) and population FP20-17 the most susceptible population to fenpyroximate (LC_{50} of 5.07 mg L^{-1}). Compared to these reference populations, six (FP18-4, FP18-3, FP20-19, FP18-5, FP20-21, and FP18-7) out of these 27 populations exhibited high levels of resistance against both acaricides, with resistance ratios (RRs) ranging from 200- to over 1950-fold (**Table 1**). For FP18-4 and FP18-3, LC_{50} values were not determined in all cases as the upper limit of the toxicity assay, i.e. 5000 mg a.i. L^{-1}, did not result in 50% mortality. These six resistant populations originated from Italy, Belgium, the United Kingdom and Romania (**Table 1** and **Table S1**).

A correlation analysis was performed between the tebufenpyrad and fenpyroximate $\log_{10}(LC_{50})$ values available for 25 populations, which resulted in a strong correlation coefficient (R^2) of 0.92 (p= 2.6 10^{-14}) (**Figure 2**). The two most resistant populations (FP18-3 and FP18-4) were not included in this analysis as not all LC_{50} values could be calculated (exceeding the assay threshold).

A different set of 15 populations collected during 2019 was screened for tebufenpyrad toxicity only, populations FP19-8, FP19-15 and FP19-22, originating from Italy, Belgium and Spain, respectively, were found to be 1000-fold resistant compared to FP19-13 (**Table 2**).

The LC_{90} values for both acaricides for all field populations are shown in **Table S2**. Comparison with the advised field doses of tebufenpyrad (100 mg a.i. L^{-1} for Masai) and fenpyroximate (102.4 mg a.i. L^{-1} for Kiron) makes clear that these products would fail to control several of the screened populations.

3.2 Synergism assays

Synergism assays revealed a significant synergistic effect for the monooxygenase inhibitor PBO in three out of four tested populations (resistant FP20-19 and FP20-21, susceptible FP19-9) (**Table 3, Figure S2**). For the fourth and most resistant population (FP18-4), synergism could be detected but the synergism ratios could not be calculated as the LC_{50} values for the assays without PBO were higher than the assay threshold of 5000 mg L^{-1}.

Tebufenpyrad toxicity was synergized about two-fold in susceptible FP19-9 and resistant FP20-19, and more than threefold and as high as 21-fold in FP18-4 and FP20-21 respectively. The synergistic effects for fenpyroximate were higher in FP20-19, FP20-21 and FP19-9 compared to tebufenpyrad. PBO strongly synergized fenpyroximate toxicity in the susceptible FP19-9 (9-fold), yet to an even greater extent in the resistant FP20-19 (17-fold) and FP20-21 (39-fold). In contrast, SR was relatively low for FP18-4, with an LC_{50} value that remained relatively high even with PBO pretreatment.

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3.3 Presence and frequency of PSST mutations by proportional sequencing and ddPCR

METI-I resistance has been linked to the H92R target-site mutation in the PSST subunit of complex I[28], while Alavijeh et al. (2020) additionally suggested the involvement of A94V in the related mite *Panonychus citri*. All field populations were screened for these target-site mutations via sequencing of DNA from pooled mites per population.[43] Four different non-synonymous mutations were observed in the PSST gene: the H92R (CAC -> CGC) mutation, which appeared to be fixed in five populations (FP18-4, FP18-3, FP18-5, FP19-8 and FP19-22) while it was segregating in five additional populations (FP20-19, FP20-21, FP18-7, FP19-15 and FP19-5) with frequencies over 45% (**Figure 3** and **Table 4**). In FP19-23, the mutation frequency was about 17%. None of the screened populations was found to possess the A94V substitution. Next to H92R, three additional mutations were discovered. M117L (*Y. lipolytica* numbering; M135L in *T. urticae*) was segregating in FP18-4 and FP20-21 with frequencies of 15% and 74%, respectively, while 14 populations were detected with fixed or segregating mutation V36I (V65I in *T. urticae* numbering) (**Table 4**). Substitution A55T (*T. urticae* numbering[44]) was segregating (50% allelic frequency) in FP19-4 only.

As the H92R substitution is of main interest to present study, its MAF was additionally determined using ddPCR. The H92R mutation was found to be present in 24 out of the 42 populations, ranging in allele frequency from completely fixed to below 1%. More precisely, ddPCR was able to detect frequencies in field populations as low as 0.25%, i.e., 1 heterozygote pooled with about 200 wild type individuals.

The correlation between the H92R allelic frequency as determined by ddPCR versus proportional sequencing was determined. The lowest frequency obtained using latter detection method accounted to 16.7%. Using this value as an arbitrary detection limit for proportional sequencing, a strong correlation was detected (R= 0.95, p= 5.7 10^{-6}) (**Figure 3)**.

4 Discussion

Although chemical treatment should only be considered as a last resort in Integrated Pest Management (IPM)[45], the application of synthetic acaricides remains the cornerstone of *T. urticae* control.[2] Amongst these acaricides are fenpyroximate and tebufenpyrad, which are mitochondrial electron transport inhibitors of complex I (METI-Is). As with most other acaricide groups, *T. urticae* has developed resistance against these compounds.[2,21] In order to assure the sustainable use of the acaricides in a decreasing portfolio of approved products, monitoring of the resistance status of field populations is of utmost importance. Molecular markers of resistance could soon prove to be crucial for rational guidance of resistance management programs and for attaining IPM goals, yet are still not well developed or validated for *T. urticae*.[35]

In the present study, 42 *T. urticae* field populations (**Figure 1**) were collected throughout Europe between 2018 and 2020, and subjected to adulticidal concentration-response toxicity assays using tebufenpyrad (populations from 2018, 2019 and 2020) and fenpyroximate (populations from 2018 and 2020). Based on the observed toxicity values, it could be concluded that the advised field concentration for both tebufenpyrad (100 mg L^{-1}) and fenpyroximate (102.4 mg L^{-1}) would fail to efficiently control many of the screened populations. Indeed, nine populations were found to be highly resistant to tebufenpyrad, with RR values ranging from 333- to over 1950-fold. Six of these populations were included in the fenpyroximate toxicity assay, and exactly these six populations were most resistant to fenpyroximate as well (RR values ranging from 200- to over 1000-fold) (**Table 1**). A comparison of the available LC_{50} values for both acaricides revealed a strong correlation, further supporting cross-resistance between both METI-I acaricides (**Figure 2**).

Two groups of populations could be distinguished based on the tebufenpyrad and fenpyroximate toxicity values: those with LC_{50} values lower than 100 mg L^{-1}, and those with values higher than 1000 mg L^{-1} (**Figure 4**). Populations with an LC_{50} in the "gap" between both thresholds appeared to be virtually non-existent in present screening. The observed acaricide resistance can be the result of a number of mechanisms, among which increased detoxification and target-site insensitivity are the most common.[21,25] Although METI-I resistance and cross-resistance was first attributed to cytochrome P450 mediated detoxification[19,27], Bajda et al. (2017) were the first to uncover a target-site resistance mechanism. Several lines of evidence suggested that an amino acid substitution (histidine to arginine at position 92 (H92R); *Y. lipolytica* numbering) in the PSST subunit of complex I caused METI-I resistance.[28] Reassuringly, the same mutation was also independently identified by Snoeck et al. (2019), via unbiased QTL mapping, as a major factor involved in fenpyroximate, tebufenpyrad and pyridaben resistance.[34] In present study, a relevant fragment of the PSST gene was sequenced for all *T. urticae* field populations. This allowed for the identification of potential new PSST target-site mutations and for an estimation of their allelic frequency via proportional sequencing. Highly sensitive frequency data were additionally obtained for the H92R allele using droplet digital PCR (ddPCR), proposed to reliably detect one mutant spider mite in a pool of 1000 specimens.[41] By comparing H92R proportional sequencing frequencies with the corresponding ddPCR data (**Figure 3**), proportional sequencing appeared to be an adequate estimator of the H92R allele frequency in populations, at least when this frequency was higher than 10-20%. Likewise, proportional sequencing could give a good estimation of the frequency of other substitutions observed in PSST. Substitution V36I was detected in 13 out of the 42 field populations (**Table 4**). This mutation is unlikely to be involved in resistance as most of these populations exhibited RRs lower than 4-fold for both acaricides. In addition, V36I is not in proximity of a conserved domain. Yet another substitution, M117L, was found to be

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segregating in two highly resistant populations, with frequencies of 15.4% and 74.0% (**Table 4**). This M117 is separated by 32 amino acid residues from conserved cysteines that could serve as ligands for iron-sulfur cluster N2. Even more, the adjacent mutation V119M in the PSST homologue of *Y. lipolytica* was predicted to have influence on electron transport and results in a catalytically impaired complex I.[33] In Bajda et al. (2017), substitution M117L, together with H92R, was found to be fixed in resistant strain MR-VP.[28] Due to their proximity to one another, this M117L mutation was also transferred to the susceptible background when the MR-VP strain was used in the back-crossing experiment to determine the role of H92R. Also in the genetic mapping experiments from Snoeck et al. (2019), M117L was on QTL1, together with H92R.[34] Whether the M117L mutation contributes to METI-I resistance still remains unclear. Substitution A55T (*T. urticae* numbering), which was uncovered in a multi-resistant strain by Papapostolou et al. (2021), was detected in population FP19-4 only. This population was however susceptible to tebufenpyrad (LC_{50} of 48.5 mg L^{-1}). As the substitution is also not located in the proximity of the METI-I binding site nor in a conserved region [44], it is most likely not involved in resistance. Lastly, the A94V substitution in PSST of the citrus red mite *P. citri* was of interest to Alavijeh et al. (2020) as it is located only two amino acids away from H92R, while it was present with higher allele frequencies in a moderately resistant Iranian population.[43] In present extensive screening of *T. urticae* populations, A94V was not detected.

As H92R is the focus of present study, ddPCR was used to precisely determine its frequency in each population. The high sensitivity of ddPCR allowed the detection of very low frequencies of the mutation, down to 0.25%. As such, this technology would make it possible to detect incipient resistance, which is critical for resistance management.[35] The H92R mutation was found to be present in more than half of the screened populations, in five of which it was fixed or virtually fixed (allelic frequency >95%). Exactly these five populations appeared to be the most resistant ones (**Figure 4**). A regression analysis between H92R frequency and fenpyroximate or tebufenpyrad toxicity indeed revealed strong correlations (**Figure S3**). This appears to be in contrast with earlier findings by Mavridis et al. (2021), which did not detect a strong correlation between the H92R mutant allelic frequency and the mortality caused by the METI-I pyridaben at field recommended dose.[41] However, these authors stated that the underestimation of the predictive strength of H92R could be due to the relatively small number of analysed populations[41], while we additionally remark that the percent mortality at field dose, which was in all but one population below 30%, may not have been the ideal proxy for resistance.

Next to sequencing and ddPCR analysis, screening for the H92R mutation in *T. urticae* would also be possible using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays. Three isoschizomers (CviRI, HpyCH4V and HpyF44III) cut at restriction site TG^CA which is present at position M91-H92 in wild type mites, yet not in

H92R mites (TGCG). Based on the 42 sequenced populations, this restriction site appears to be unique within 273 nucleotides bordering the H92 locus, allowing opportunity for amplicon optimization.

The involvement of the PSST H92R target site mutation in METI-I resistance appears to be quite well established based on genotype-phenotype correlation data, but also in unbiased genetic mapping experiments (present study, Bajda et al. (2017), Snoeck et al. (2019)).[28,34] Reassuringly, a tetranychid from the same subfamily as *T. urticae*, the citrus red mite *P. citri*, was found to possess an identical mutation in Iranian populations resistant to METI-I's Alavijeh et al. (2020).[43] The fact the same substitution independently evolved in a related species is a striking example of parallel evolution, and further supports a causal role of the H92R substitution in METI-I resistance.

Surprisingly however, introgression of the H92R mutation into a susceptible background revealed only limited phenotypic strength.[28] Indeed, only moderate resistance levels up to 30- to 60-fold were recorded for tebufenpyrad and pyridaben, while for fenpyroximate, lower and less consistent resistance levels were reported.[28] Unfortunately, attempts to genetically modify *Drosophila* flies with this mutation failed as it was found to be lethal in this species.[28] This indicates the importance of the residue in functioning of the protein, but does not allow to validate its role in resistance. However, resistance in *T. urticae* may often have a polygenic basis, as was further supported by recent studies, including genetic mapping experiments.[34,46,47] The involvement of both mechanisms of decreased exposure and decreased sensitivity may result in synergism.[48] This has also been documented for METI-I resistance, where the role of P450-mediated detoxification is indeed very well supported.[26,27,34] With regard to this cross-resistance, fenpyroximate and tebufenpyrad both contain heterocyclic rings with two nitrogen atoms, associated with long hydrophobic tails with at least one tertiary butyl group.[27] This structural relatedness is at the basis of their shared mode of action, while it would also explain common mechanisms of detoxification.[27] Nevertheless, structural differences in METI-I acaricides may also result in resistance mechanisms that are compound-specific.[34]

Because of the previously documented low phenotypic strength of H92R and the suggested involvement of P450s, we also investigated in the present study the involvement of P450s in resistance with synergist assays. Four populations were included in these assays, ranging from highly resistant with fixed H92R, to highly susceptible with complete absence of H92R. In all of these populations, the P450 inhibitor PBO severely increased the susceptibility to both tebufenpyrad and fenpyroximate (**Table 3**, **Figure S2**). In the most resistant population, with H92R in a fixed state (FP18-4), LC_{50} values after synergist treatment were reduced, yet remained exceptionally high. In this population, PBO appeared unable to sufficiently break

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resistance. The apparent involvement of P450-mediated detoxification does not exclude a role for the H92R target-site substitution in resistance. Already in 2009 Van Pottelberge et al. (2009) state that "a detoxification mechanism that is the same in a sensitive and a resistant strain can have a much greater impact on degradation if an altered site of action in the resistant strain retards the intoxication.[19] The effect of a synergist that blocks the detoxification will then be much larger, which could lead to the false conclusion that the detoxification is the major resistance mechanism. [19,49,50] This statement appears to align with the observations in present study, and this synergism between mechanisms was at least partially experimentally validated in *Drosophila*.[48]

A P450 known to be involved is described in Riga et al. (2015), which showed that the CYP392A11 was capable of catalysing the hydroxylation of fenpyroximate into a non-toxic metabolite *in vitro*, while transgenic expression in *Drosophila* resulted in significant levels of fenpyroximate resistance. Although this P450 could additionally metabolize the METI-II cyenopyrafen, tebufenpyrad appeared not to be a suitable substrate.[26] The CYP392A11 enzyme was found to be upregulated in multi-resistant *T. urticae* strains, including the METI-I resistant strain MR-VP[23], which also possesses the PSST H92R substitution.[28] Besides, Snoeck et al. (2019) also identified a D384Y substitution in the electron transfer flavoprotein CPR as a potential factor in tebufenpyrad and pyridaben resistance, yet not in fenpyroximate resistance.[34] CPR serves as an electron donor for all microsomal P450s and several other enzymes found in the endoplasmic reticulum of most cells.[34] Again, a link with CYP-mediated detoxification is clear, and it is highly likely that both CYPs and (factors associated with) H92R are involved. Recently, Itoh et al. (2021) studied the effect of the H92R substitution in combination with candidate cytochrome P450s on pyridaben resistance.[30] A candidate causal factor for high resistance levels was found to be CYP392A3. This CYP alone only marginally contributed to resistance, yet when combined with H92R, the CYP appeared to have a synergistic or cumulative effect on pyridaben resistance. CYP392A3 is member of the CYP cluster that was located within the same QTL region in which PSST H92R was identified.[28,30]

It is tempting to speculate that the different, potentially synergistic, mechanisms of resistance are responsible for the pattern seen in **Figure 4**. The wide range of LC_{50} values within the well-separated "susceptible" and "(highly) resistant" group (i.e. LC_{50} <100 mg L^{-1} or >1000 mg L^{-1}, respectively) is proposed to be the result of variability in factors involved in detoxification, while high allelic H92R frequencies appear to be a requirement to bridge the gap from the "susceptible" group to the "(highly) resistant" group. Indeed, in our screening, only one population does not follow this pattern. FP19-5 has a high H92R allelic frequency (64%), yet clusters closely to the "susceptible" group.

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It is important to realise that a good diagnostic marker needs to correlate with resistance, but is not necessarily the only causal factor in resistance. This is clear for this study, as the presence of the H92R mutation, especially at high frequency, predicts resistance very accurately, even if it is also clear that additional (synergistic) mechanisms are needed to attain very high resistance levels. We consider this marker as highly valuable in European populations of *T. urticae*. The present study supports the geographical relevance of the H92R substitution as a biomarker for *T. urticae* METI-I resistance for several European countries, including screened populations from Belgium, Bulgaria, Germany, Hungary, Italy, The Netherlands, Poland, Romania, Spain and the UK (**Figure 1**). Resistant populations, with high H92R allelic frequencies, were found to be present throughout mainland Europe, yet also on the islands Sicily and Great Britain. In addition, a literature search revealed that also in Greece, the H92R mutation was detected in a multi resistant strain.[44] Furthermore, METI-I resistant populations from Korea[16], Japan[28,30] and Ethiopia[51] were shown to harbour this mutation. The H92R mutation does however not appear to be universally present in METI-I resistant strains. In a Canadian *T. urticae* strain with high resistance to pyridaben (LC_{30} >10,000 mg L^{-1}), for example, the mutation appeared to be absent.[52]

In summary, *T. urticae* populations collected throughout Europe were subjected to tebufenpyrad and fenpyroximate toxicity screens. Many of the tested populations proved to be (cross-)resistant to the extent that field control using METI-I compounds would be impaired. All of the resistant populations harboured the H92R substitution in the PSST gene, the proposed METI-I target site. And *vice versa*, populations in which the H92R variant was the predominant allele, were virtually always highly resistant to either of the tested acaricides. Even though it is clear that, next to the H92R substitution, also other mechanisms are at play in METI-I resistance, requires further elucidation, the present study strongly supports the use of H92R as a reliable molecular biomarker in resistance management.

5 Acknowledgements

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6 Conflict of Interest Declaration

The authors declare that they have no conflicts of interest.

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8 Figure legends

Figure 1. Distribution of *T. urticae* field populations sampled in Europe. The green to red color gradient represents the PSST H92R allelic frequency in each population, as determined by ddPCR analysis. Slight random jitter has been added to separate overlapping data points. (Plot generated using ggplot2)

Figure 2. Correlation between tebufenpyrad and fenpyroximate toxicity (log_{10} of LC_{50}) in the collected European field populations supports cross-resistance (R^2= 0.92, p= 2.6 10^{-14}). The green to red color scale represents the H92R mutation frequency as determined by ddPCR. Two populations with LC_{50} higher than the assay threshold (5000 mg L^{-1}) are not included in the analysis. (Plot generated using ggplot2)

Figure 3. Correlation analysis between the H92R allelic frequency (%) in the population sample as determined by ddPCR versus proportional sequencing. The lowest detected non-zero frequency for proportional sequencing (i.e. 16.7%) was used as a threshold to divide the populations in below (green triangles) and above (red circles) the detection limit. Darker shading indicates overlaying data points. A strong correlation was found for samples above the proportional sequencing detection limit (R= 0.95, p= 5.7e^{-6}). The dashed line represents x=y. (Plot generated using ggplot2)

Figure 4. Toxicity (LC_{50}, in mg L^{-1}) of tebufenpyrad and fenpyroximate to the collected *T. urticae* field populations. The red dashed lines represent the recommended field doses. If LC_{50} values for both acaricides were determined for the same population, the corresponding dots are connected. The green to red color gradient represents the PSST H92R allelic frequency (%) in each population, as determined by ddPCR. The values for two populations above the assay threshold (5000 mg L^{-1}) are indicative and are represented by a triangle. (Plot generated using ggplot2)

Figure S1 Determination of the limit of detection for the H92R ddPCR assay using a variable number of mutant sequence copies in a stable wild type background. The Y-axis is in logarithmic scale.

Figure S2. Toxicity (LC_{50}) of tebufenpyrad and fenpyroximate to four *T. urticae* field populations, without and with pretreatment of the synergist PBO. For FP18-4, the LC_{50} values without PBO were higher than the assay threshold of 5000 mg L^{-1}. (Plot generated using ggplot2)

Figure S3. LC_{50} of fenpyroximate and tebufenpyrad in function of H92R frequencies as determined by ddPCR or proportional sequencing. (Plot generated using ggplot2)

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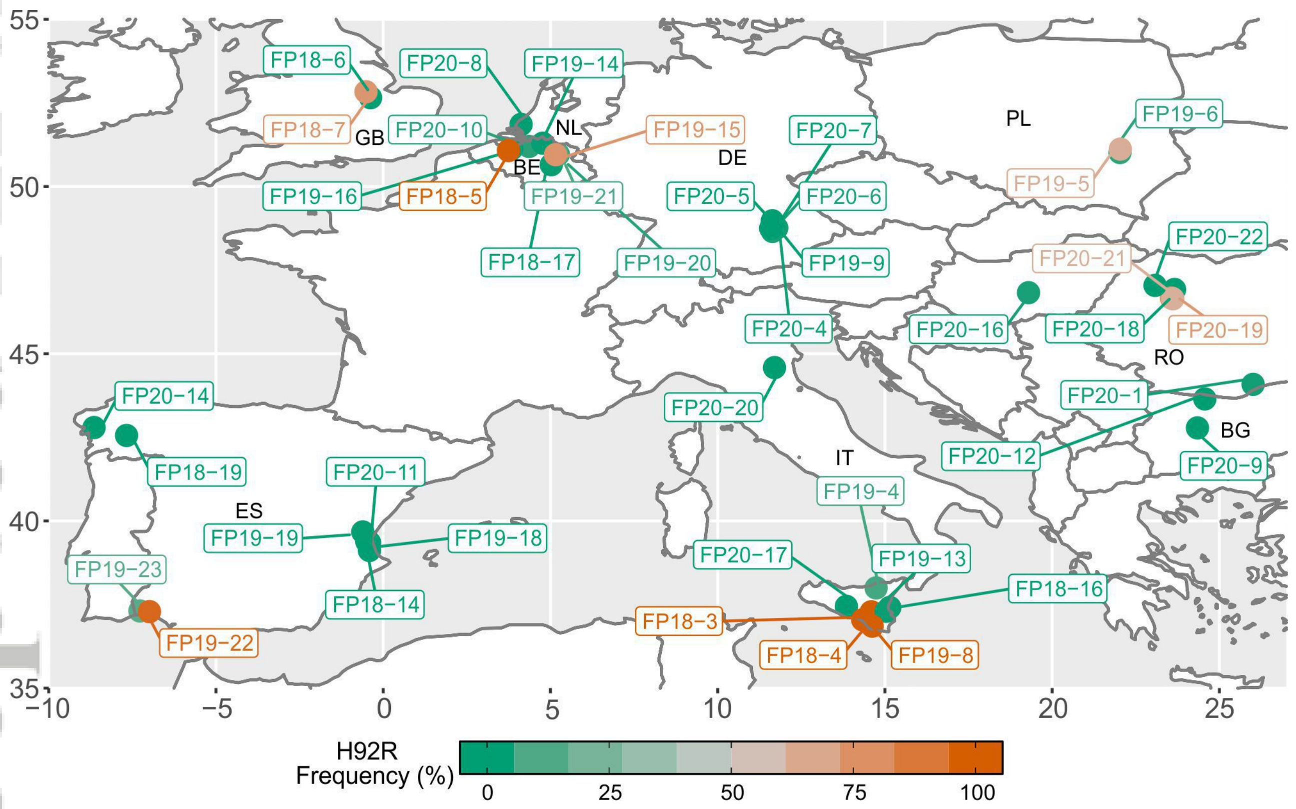

Fig1.jpg

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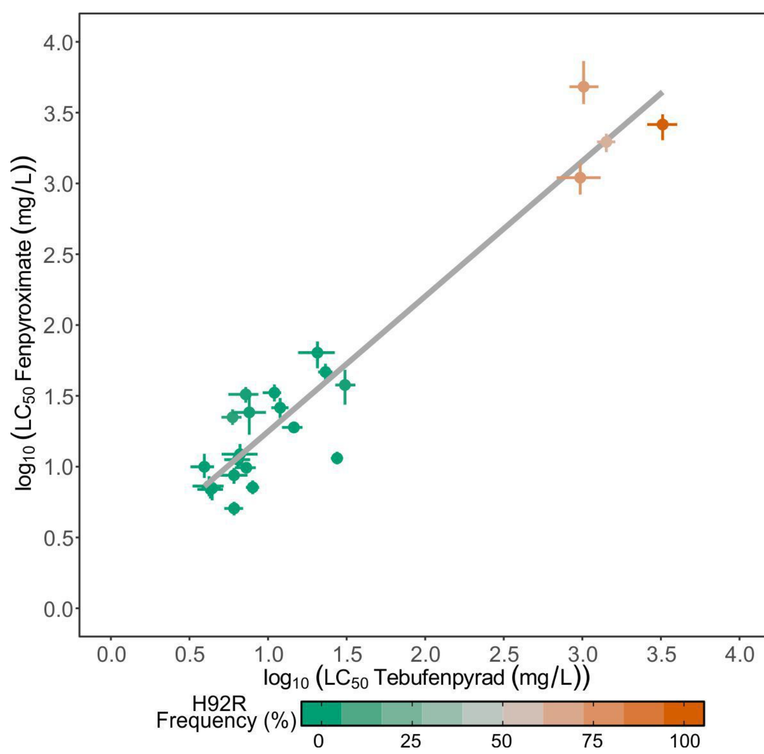

Fig2.jpg

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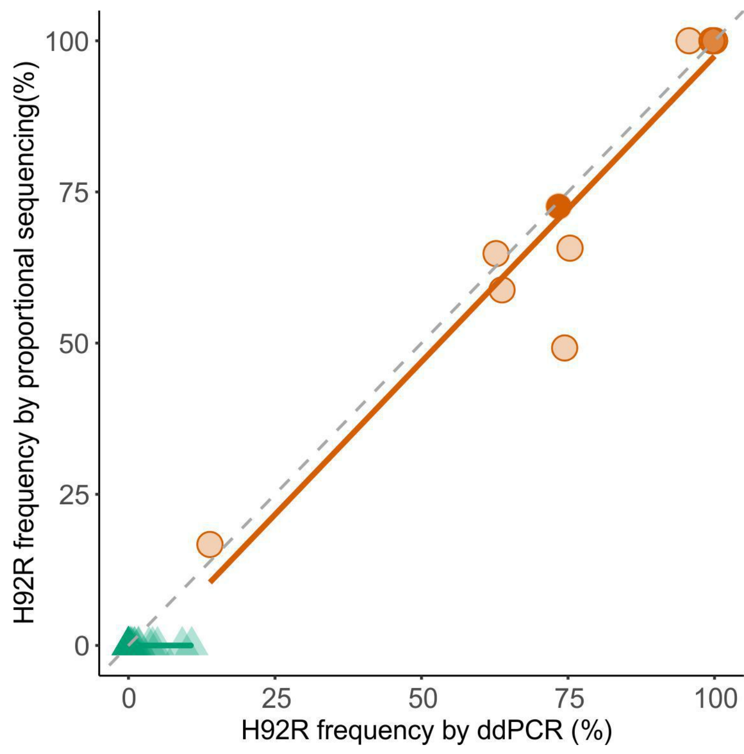

Fig3.jpg

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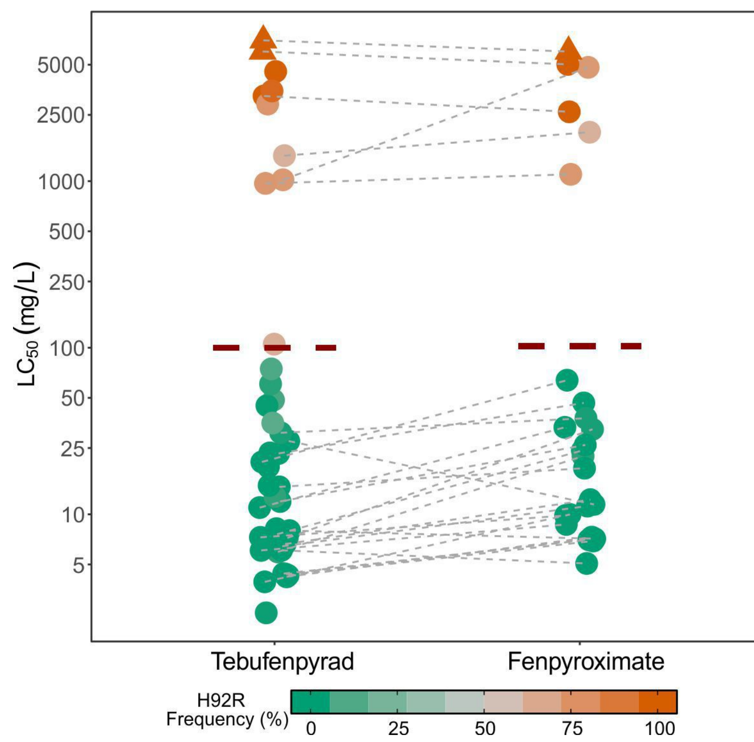

Fig4.jpg

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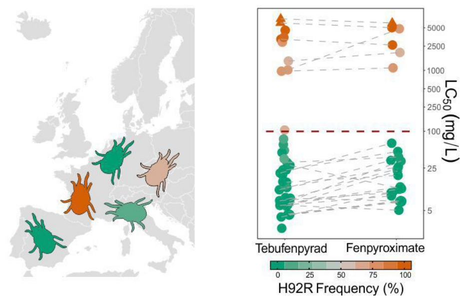
PSST H92R target-site mutation
as METI-I resistance marker in *T. urticae*



Graphical Abstract Image.jpg

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The H92R substitution in PSST is a reliable diagnostic biomarker for predicting resistance to Mitochondrial Electron Transport Inhibitors of complex I in European populations of *Tetranychus urticae*

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The H92R substitution in the METI-I target site PSST is a reliable marker for tebufenpyrad and fenpyroximate resistance in European populations of *Tetranychus urticae*.

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Table 1. LC_{50} values of tebufenpyrad and fenpyroximate for 27 *T. urticae* field populations collected in Europe during 2018 and 2020.

Population	Tebufenpyrad			Fenpyroximate		
	Slope (±SE)	LC_{50} (95% CI)[a]	RR_{50} (95% CI)[b]	Slope (±SE)	LC_{50} (95% CI)	RR_{50} (95% CI)
FP20-1	5.43 (±0.46)	7.59 (5.95-9.70)	2.97 (2.42-3.64)	3.06 (±0.48)	24.2 (16.8-32.0)	4.76 (4.00-5.68)
FP20-4	5.19 (±0.51)	4.37 (3.77-5.00)	1.71 (1.38-2.11)	6.23 (±0.71)	7.12 (6.50-7.75)	1.40 (1.23-1.61)
FP20-5	5.00 (±0.42)	4.22 (3.31-5.22)	1.64 (1.34-2.03)	3.90 (±0.29)	7.29 (6.18-8.56)	1.44 (1.25-1.66)
FP20-6	5.93 (±0.75)	7.21 (5.59-8.69)	2.82 (2.27-3.48)	3.24 (±0.37)	32.4 (28.3-36.5)	6.41 (5.49-7.41)
FP20-7	3.24 (±0.23)	4.30 (3.55-5.17)	1.68 (1.35-2.08)	4.05 (±0.31)	6.92 (5.95-8.08)	1.36 (1.19-1.57)
FP20-8	4.11 (±0.33)	27.5 (25.1-29.9)	10.8 (8.77-13.2)	3.72 (±0.27)	11.5 (10.4-12.7)	2.26 (1.96-2.60)
FP20-9	4.23 (±0.33)	7.99 (7.23-8.78)	3.12 (2.54-3.83)	3.02 (±0.25)	7.14 (6.39-7.99)	1.41 (1.21-1.64)
FP20-10	3.93 (±0.35)	5.95 (5.05-6.78)	2.32 (1.88-2.87)	3.22 (±0.22)	22.3 (19.7-25.4)	4.41 (3.80-5.10)
FP20-11	3.44 (±0.31)	23.2 (20.8-25.7)	9.09 (7.30-11.24)	2.61 (±0.30)	46.6 (39.3-53.3)	9.17 (7.81-10.9)
FP20-12	3.93 (±0.51)	14.6 (12.3-16.6)	5.71 (4.57-7.14)	4.08 (±0.28)	18.9 (17.3-20.8)	3.73 (3.25-4.29)
FP20-14	2.67 (±0.21)	11.9 (10.5-13.5)	4.65 (3.72-5.85)	2.04 (±0.18)	26.1 (22.0-30.5)	5.15 (4.24-6.25)
FP20-16	3.39 (±0.27)	30.9 (26.8-35.9)	12.0 (9.71-14.9)	2.10 (±0.19)	37.7 (27.4-48.3)	7.46 (5.99-9.26)
FP20-17	2.65 (±0.21)	6.08 (5.27-6.94)	2.38 (1.88-3.00)	3.74 (±0.30)	5.07 (4.53-5.65)	1.00
FP20-18	2.28 (±0.18)	6.43 (5.27-7.69)	2.51 (1.92-3.28)	2.86 (±0.25)	11.2 (9.77-12.7)	2.21 (1.87-2.61)
FP20-19	1.55 (±0.13)	1020 (828-1270)	333 (333-500)	1.41 (±0.17)	4820 (3640-7330)	1000
FP20-20	4.19 (±0.42)	4.41 (3.93-4.86)	1.72 (1.39-2.14)	2.27 (±0.28)	7.04 (5.79-8.27)	1.39 (1.13-1.70)
FP20-21	1.96 (±0.20)	1420 (1240-1620)	500	3.14 (±0.27)	1970 (1660-2250)	333 (333-500)
FP20-22	1.93 (±0.15)	6.63 (5.07-8.57)	2.59 (2.02-3.31)	3.25 (±0.36)	12.2 (10.1-14.5)	2.42 (2.04-2.85)
FP18-3	2.76 (±0.95)	>5000	>1950	3.28 (±0.64)	5020 (4070-8530)	1000
FP18-4	0.74 (±0.26)	>5000	>1950	3.01 (±1.22)	>5000	1000
FP18-5	2.33 (±0.34)	3240 (2590-4020)	1000	4.09 (±0.49)	2610 (2020-3080)	500
FP18-6	6.02 (±0.85)	3.93 (3.21-4.53)	1.54 (1.23-1.92)	4.30 (±0.65)	9.98 (8.33-12.3)	1.97 (1.67-2.32)
FP18-7	1.49 (±0.12)	970 (686-1300)	333 (333-500)	1.64 (±0.14)	1101 (834-1390)	200 (167-250)
FP18-14	8.26 (±0.91)	11.0 (9.25-12.1)	4.29 (3.52-5.21)	2.23 (±0.18)	33.3 (28.9-38.0)	6.58 (5.52-7.81)
FP18-16	3.54 (±0.34)	7.27 (6.15-8.36)	2.84 (2.27-3.55)	4.16 (±0.36)	9.83 (8.92-10.8)	1.94 (1.68-2.23)
FP18-17	2.61 (±0.21)	6.07 (4.80-7.41)	2.37 (1.89-2.98)	2.91 (±0.22)	8.70 (7.57-9.79)	1.72 (1.48-1.99)
FP18-19	1.46 (±0.13)	20.6 (15.6-26.6)	8.06 (6.02-10.75)	2.85 (±0.40)	63.8 (49.5-76.6)	12.7 (10.4-15.2)

[a]Median lethal concentration (expressed as mg L^{-1}), with 95% confidence interval

[b]Resistance Ratio: LC_{50} relative to the LC_{50} of the most susceptible strain (FP19-13 for tebufenpyrad, FP20-17 for fenpyroximate)

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Table 2. LC_{50} values of tebufenpyrad for 15 *T. urticae* field populations collected in Europe during 2019.

Population	Tebufenpyrad		
	Slope (±SE)	LC_{50} (95% CI)[a]	RR_{50} (95% CI)[b]
FP19-4	2.27 (±0.27)	48.5 (34.8-62.4)	18.9 (14.1-25.6)
FP19-5	1.76 (±0.17)	105 (76.7-135)	41.7 (31.3-52.6)
FP19-6	1.46 (±0.15)	12.8 (7.56-18.6)	5.00 (3.55-7.04)
FP19-8	2.36 (±0.40)	4550 (3340-9130)	1000
FP19-9	2.06 (±0.20)	8.20 (5.97-12.72)	3.21 (2.44-4.20)
FP19-13	3.09 (±0.37)	2.56 (1.32-3.55)	1.00
FP19-14	1.65 (±0.19)	44.9 (24.3-65.0)	17.5 (12.5-24.4)
FP19-15	2.35 (±0.31)	2900 (2160-3730)	1000
FP19-16	2.63 (±0.52)	19.3 (15.0-26.3)	8.85 (5.21-15.15)
FP19-18	3.69 (±0.34)	14.9 (12.5-17.6)	4.98 (3.86-6.45)
FP19-19	5.49 (±0.70)	23.4 (19.3-27.1)	5.99 (4.74-7.63)
FP19-20	3.42 (±0.29)	60.6 (53.3-68.8)	21.7 (16.4-28.6)
FP19-21	2.18 (±0.20)	74.6 (56.4-93.1)	43.5 (32.3-58.8)
FP19-22	2.60 (±0.45)	3480 (2650-4730)	1000
FP19-23	1.73 (±0.17)	35.3 (24.8-48.8)	29.4 (19.2-43.5)

[a]Median lethal concentration (expressed as mg L^{-1}), with 95% confidence interval
[b]Resistance Ratio: LC_{50} relative to the LC_{50} of the most susceptible strain (FP19-13)

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Table 3. Toxicity of tebufenpyrad and fenpyroximate, without and with the synergist PBO pre-treatment, to female adults of three METI-I resistant (FP18-4, FP20-19 and FP20-21) and one susceptible (FP19-9) *T. urticae* field population.

	Tebufenpyrad				**Fenpyroximate**			
	FP18-4	**FP20-19**	**FP20-21**	**FP19-9**	**FP18-4**	**FP20-19**	**FP20-21**	**FP19-9**
Acaricide								
LC_{50} (mg L^{-1})[a]	>5000	1240	942	8.56	>5000	1870	2170	27.2
95% CI[b]	/	1040–1480	533–1310	7.32–10.29	/	1500–2230	1600–2770	24.3–30.4
Slope ± SE	/	1.83±0.17	1.12±0.20	3.37±0.35	/	3.30±0.27	1.80±0.25	3.90±0.36
RR[c]	>584	144	110	1	>184	68	80	1
PBO + Acaricide								
LC_{50} (mg L^{-1})	1390	501	44.3	3.60	3550	107	56.3	2.99
95% CI	1260–1520	424–582	33.8–57.0	3.00–4.28	3070–3950	87.8–134	41.4–75.6	2.86–3.13
Slope ± SE	3.32±0.21	2.13±0.19	0.98±0.08	3.53±0.51	4.36±0.56	1.39±0.14	0.85±0.08	11.9±1.49
SR[d]	**>3.61**	**2.47***	**21.3***	**2.38***	**>1.41**	**17.4***	**38.5***	**9.10***
95% CI	/	1.97–3.09	14.0–32.5	2.02–2.81	/	13.7–22.1	27.2–54.6	8.22–10.1

[a] Median lethal concentration

[b] Confidence interval of LC_{50}

[c] Resistance ratio: LC_{50} relative to LC_{50} of FP19-9

[d] Synergist ratio: LC_{50} of acaricide alone relative to LC_{50} of PBO + acaricide

* PBO significantly synergized toxicity

Table 4. Populations with non-synonymous mutations in PSST (*Y. lipolytica* numbering) and mutation frequency, as estimated by proportional sequencing (%).

Population	Substitution		
	H92R	M117L	V36I
FP18-4	100	15.4	0
FP18-3	100	0	40.9
FP20-19	72.6	0	0
FP18-5	100	0	0
FP20-21	64.8	74.0	0
FP18-7	49.2	0	0
FP20-6	0	0	19.2
FP18-16	0	0	100
FP18-17	0	0	24.9
FP20-4	0	0	13.4
FP20-7	0	0	34.8
FP20-20	0	0	100
FP20-17	0	0	24.5
FP19-8	100	0	0
FP19-22	100	0	0
FP19-15	65.7	0	0
FP19-5	58.8	0	0
FP19-20	0	0	28.4
FP19-14	0	0	18.1
FP19-23	16.7	0	0
FP19-16	0	0	55.2
FP19-6	0	0	29.5
FP19-9	0	0	29.1

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