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Mitochondria COI-Based Molecular Characterization and Genetic Analysis of the Fenazaquin Selected Resistant Strain of Two-Spotted Spider Mite, *Tetranychus urticae* Koch

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A B S T R A C T

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) has emerged as an important agricultural pest in a wide range of outdoor and protected crops worldwide. Fenazaquin is METI-acaricide which is used extensively and frequently for the management of this mite has resulted in the development of resistance. So, present studies were conducted to investigate molecular characterization and genetic analysis based on mtCOI sequence between fenazaquin resistant and susceptible population of *T. urticae* as very limited information is available regarding mutation/variability in genes involved in imparting resistance. Fenzaquin resistance population was developed in the laboratory by giving selection pressure with fenazaquin for 15 generations leading to 166.49 fold resistance when compared with susceptible population. Molecular characterization of resistant and susceptible population revealed no changes in genes structure of *mtCOI* in the resistant compared to the susceptible population. In our studies high level of resistance to fenazaquin didn't show any change in the amino acid sequence of COI region of resistant and susceptible populations Thus results revealed mtCOI as a stable gene which is least influenced by acaricide resistance.

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is an important agricultural pest with a global distribution and is one of the economically most important pests in a wide range of outdoor and protected crops worldwide (Belay *et al.,* 2018). This mite has a great potential to produce high population which depends particularly on temperature, humidity and host plant and these in turn make it one of the most important pests of greenhouses, farms and orchards in different regions of the world (Jeppson *et al.,* 1975 and Zhang 2003. It has been found that *T. urticae* has the potential to quickly develop resistance to almost all kinds of acaricides because of their

high prolific rate, short life-cycle, high reproductive potential, arrhenotokous reproduction, polyphagous feeding habit, coupled with their extremely dispersal behavior (Stumpf *et al.,* 2001; Croft *et al.,* 1988; Knowles *et al.,* 1997 and Ramasubramanian *et al.,* 2005).

Mitochondrial electron transport inhibitors (METI's) belong to a class of acaricides, which are known to effectively control *T. urticae* and other tetranychid mite species for many years, including populations resistant to other chemical classes of insecticides/acaricides. There are many reports of acaricides becoming ineffective against *T. urticae* after short period of their use. TSSM or *T. urticae* has developed resistance to many categories of acaricides like organotins compounds, carbamates, bifenthrin, organophosphates, dicofol, abamectin, METI compounds (like fenazaquin, fenpyroximate, pyridaben etc.) hexythiaziox, clofentezine and chlorfenapyr. As a consequence, *T. urticae* has attained the dubious reputation to be “the most resistant species” in terms of the total number of pesticides to which it has become resistant (Van Leeuwan *et al.,* 2010)

Fenazaquin attack a target-site in complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial respiratory pathway (Hollingworth *et al.,* 1995). Resistance to METIs has been reported in a number of regions and crops (Sharma and Bhullar, 2018). The resistance of two-spotted spider mite to METI-acaricides has already been reported from many countries all over the world, including Korea, England, Australia and Belgium (Van Pottelberge *et al.,* 2009, Cho *et al.,* 1995 and Herron *et al.,* 1998)

Molecular approaches based on marker DNA sequence comparison have been introduced as tools for the identification of these species (Vogler and Monaghan, 2007). Two molecular markers, mitochondrial cytochrome oxidase subunit I (mtCOI) and ribosomal RNA internal transcribed spacer 2 (ITS2), have been used extensively in the classification of Tetranychidae mites (Navajas *et al.,* 1992). The mitochondrial gene coding for the subunit I of the cytochrome oxidase (mt COI) are commonly employed as molecular markers and they have already proved to be useful for separating distant groups of individuals within an insect species and resolving population genetic structures (Behura, 2006).

Navajas *et al* (1998) reported that 5’ end of the mitochondrial COI gene is extensively used as a barcode to identify *Tetranychus* species and to analyze their phylogenetic evolution. The control of *T. urticae i*n Punjab has been and still is largely based on the use of acaricides. Fenazaquin is widely used for control of *T. urticae* and other pests such as *P. ulmi* on apple and citrus. The extensive and frequent use of this acaricide facilitates resistance development in some populations of *T. urticae* in Punjab (Anonymous, 2018). Molecular basis helps in better understanding the development of resistance including strategies to avoid resistance and to manage spider mites when resistance is present. The objectives of this research were molecular analysis of resistant and susceptible population of *T. urticae* for genetic differences based on mtCOI.

Materials and Methods

Rearing of susceptible *T. urticae* population

The susceptible population of *T. urticae* was maintained on brinjal without exposure to any acaricide in the screen house and isolated from possible contaminants (i.e. pesticides and other arthropods for generations. Fenazaquin (Magister) was used for resistance

studies that act as Mitochondrial complex I electron transport inhibitor.

Development of fenazaquin resistant population

The adult populations of *T. urticae* population that was collected from Patiala exhibited maximum resistance (24.65) against fenazaquin. Further resistance population was developed as per protocol by Sharma *et al.,* (2018). The acaricide resistant and susceptible populations of *T. urticae* were used to assess the genetic diversity in mtCOI.

Molecular characterization and genetic analysis of resistant and susceptible *T. urticae* populations

DNA was isolated using NucleoSpin® Tissue XS (Macherey-Nagel-MN) kitas per manufacturer's protocol, which Isolated was analyzed by agarose gel electrophoresis for quality and by UV spectrophotometry for concentrations. The resistant and susceptible populations were investigated for molecular differences using mitochondrial cytochrome oxidase I (mtCOI) gene region, which has been universally accepted as taxonomically important' DNA barcode' region. Set of primers specific to mtCOI region of *T. urticae* were designed from the genome database specific to mitochondrion (http:// bioinformatics.psb.ugent.be/orcae/overview/ Tetur) of this organism and custom synthesized through Integrated DNA Technologies, Inc, Coralville, IA, USA.. PAU_Acro 8204: TATCAACAAATCAT AAAAATATTGG; PAU_Acro 8912: TATACTTCTGG ATGA CCAAAAAATCA

PCR amplification of mtCOI DNA

PCR amplification of mtCOI from *T. urticae*total DNA was carried out in a reaction volume of 20 µL, which contained:

Component	Stock concentration	Volume (µL)
Insect DNA	~20.0 ng/µl	2.0
F primer	10.0 µM	1.0
R primer	10.0 µM	1.0
dNTPs mix	1.0 mM	5.0
Taq polymerase	5.0 units/µL	0.6
Taq buffer (with 1.5mM $MgCl_2$)	10X	2.0
Sterile Milli-Q H_2O to make 20 µl	-	8.4

PCR amplified products were resolved by horizontal agarose gel electrophoresis using 1.0 per cent (w/v) agarose gel (supplemented with ethidium bromide @ 1.0 mg/l) in 1X TAE buffer. The agarose blocks containing the specific amplified DNA band were cut from the agarose gel with a clean, sharp scalpel blade and transferred to a 1.5 µL microcentrifuge tube and purified using 'QIAquick Gel Extraction Kit' (Qiagen) as per manufacturer's protocol. The purified DNA fragments were cloned into a 'PCR cloning vector' pGEM®-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* JM109 host cells for mass multiplication of plasmid. One hour grown culture was spread (80-100µl) for selective growth of transformants on LB-Amp-X-GAL-IPTG agar (LB agar supplemented with ampicillin @ 100 µg. mL^{-1}in Petri plates. The Petri plates were incubated overnight at 37°C for selection of the white recombinant clones from individual bacterial isolates.

Three individual recombinant (white) clones from individual plates were picked up with a sterile tooth pick, inoculated into culture tubes containing 3 mL of LB-Ampicillin broth and the tubes were incubated overnight at 37°C under shaking conditions (180 rpm). Using this broth culture, miniprep plasmids were isolated using 'alkaline lysis method'. The size of insert DNA, in different recombinant plasmids was determined by PCR amplification using insert specific primer sets (CO I) and universal M_{13} primers. The recombinant plasmid was also double restricted with restriction enzymes EcoR1 and Pst1 (Fermentas Life Sciences) for further confirmation of the insert. The sequencing grade plasmid DNA was purified from the respective recombinant clone using 'Gene Elute ^{TM}Miniprep Plasmid Kit' of 'Sigma' as per manufacturer's protocol. The clones were sequencedthrough Custom Sequencing Services of 'M/S Eurofin Genomics, Bangalore, India. The obtained sequences were analysed using Megalin, SeqMan, editSeq and Seqbuilder suits of lasergene-DNA star for nucleotide alignment amongst individuals of resistant and susceptible population. Any change in nucleotide sequence in resistant population was recorded.A

Results and Discussion

Development of fenazaquin resistant population of *T. urticae*

T. urticae population that was collected from Patiala (exhibited maximum resistance of 24.65 fold against fenazaquin) was exposed to serial concentrations, mortality was recorded after 24 hrs and LC_{50} was determined and selection pressure were applied unless there was no further change in LC_{50} value. Finally, the LC_{50} values were calculated for the F_{15} generation as there was not much change in LC_{50} value and this population with 166.49 fold resistant was designated as fenazaquin selected resistant population. The acaricide resistant and susceptible populations of *T. urticae* were used to assess molecular mechanism of resistance.

Molecular Characterization of resistant and susceptible populations of *T.urticae*

The *T. urticae* populations from Patiala-Punjab have been compared with laboratory maintained susceptible population from based on mtCOI region.

Extraction and quantification of total DNA from *T. urticae* adults from resistant and susceptible population

The DNA was isolated from both resistant and susceptible populations of *T. urticae* and was run on 1.0 per cent agarose gel in TBE buffer. A single condensed high molecular weight band free from degradation was obtained in from whole body tissues of both susceptible and resistant populations (Fig. 1). The DNA concentration as determined by spectrophotometer ranged between 0.76µg/ µl and 1.23µg/ µl. The quality of DNA was determined by A_{260}/A_{280} ratio which ranged between 1.78 and 1.92 for two sample tissues each of resistant and susceptible populations. The gel electrophoresis, quantity and quality revealed the good quality of DNA obtained for subsequent molecular analysis of *T. urticae* adult samples from resistant and susceptible population.

Molecular characterization of resistant and susceptible *T. urticae* populations- mtCOI region

Mitocondrial COI region has gained global importance and is being universally accepted as taxonomically conserved region for insect species and biotype/ strain identification. Studies have suggested that high level of acaricides/insecticide resistance may bring about slight changes in nucleotide sequences

of this mitochondrial DNA region (Maitra *et al.,* 2000, Catania *et al.,* 2004 and Feyereisen, 2005). The specific primers were used to amplify ~709bp DNA fragments of COI gene from the mitochondrial DNA of both susceptible and resistant population. Size of these fragments was similar to the expected size available in the NCBI database (NCBI, 1988) (www.ncbi.nlm.nih.gov). The single clean amplified band supported the specificity of desired region from both resistant and susceptible populations (Fig. 2).

Custom sequencing of cloned COI DNA fragments

The sequencing grade recombinant plasmid (Fig. 3) from three different clones of COI fragments from resistant and susceptible variants were purified and sequenced bidirectional through Eurofin Genomics Ltd, Bangalore using M_{13} reverse and forward primer. The raw sequence data was processed using seqMan module of Lasergene DNA star software for removing the vector sequences and making a single sequence contig from the respective sequences of resistant or susceptible populations. The individual clones as well as both the complimentary DNA strands were proof read for any misread bases by comparison with chromatograms of original sequence (Fig. 4). The whole sequence of each individual strand was completed by aligning the sequence of one strand with that of the reverse complimentary sequence to yield a single sequence contig.

The final contigs from the respective population were aligned to form a single sequence in seqMan which was translated using seqBuilder module of Lasergene- DNA star. The mtCOI region sequence from susceptible and resistant populations was submitted to "GenBank Database" using Banklt. The GenBank accession number assigned to COI sequence from both the submitted sequences are MF152824 and MF152825.

Multiple alignment of COI nucleotide sequence and derived amino acid sequence

Multiple alignment of COI nucleotide sequence for both the resistant and susceptible population of *T. urticae* established existence of codon substitution at nucleotide position at 177, 444, 580 and 687 represented by substitution with G,T, A and T, respectively in resistant population compared to susceptible population (Fig. 5).

The codon substitution however didn't result in any change in the predicted amino acid sequence of both resistant and susceptible population consequently no change in the protein has been observed (Fig. 6). The mtCOI region of mitochondrial DNA is highly conserved and has been globally accepted as the gene of taxonomic importance. This fragment has been widely used for the identification of *T. urticae* species as well. The usefulness of the COI region for delineating tetranychid species has been investigated in several studies (Hinomoto *et al.,* 2001, Hinomoto and Takafuji., 2001, Navajas *et al.,* 1994, 1996a, 1996b, 1998, Toda *et al.,* 2000 and Xie *et al.,* 2006a). Recently, a DNA barcoding approach was used to identify tetranychid species (Hinomoto *et al.,* 2007). Partial COI sequence of 1257 nucleotides amplified and sequenced in ten *T. urticae* strains identified all strains as *T. urticae* when compared with available COI sequences in public databases (NCBI). The 709 bp sequences of mtCOI region of susceptible and resistant population showed no significant differences in nucleotides.

Fig.1 Total DNA isolated from fifty *T. urticae* female adults. 2µl of each DNA sample was loaded in 1 % Agarose ETBR Gel (Susceptible-S1, S2, S3 and resistant- R1, R2, R3 population)

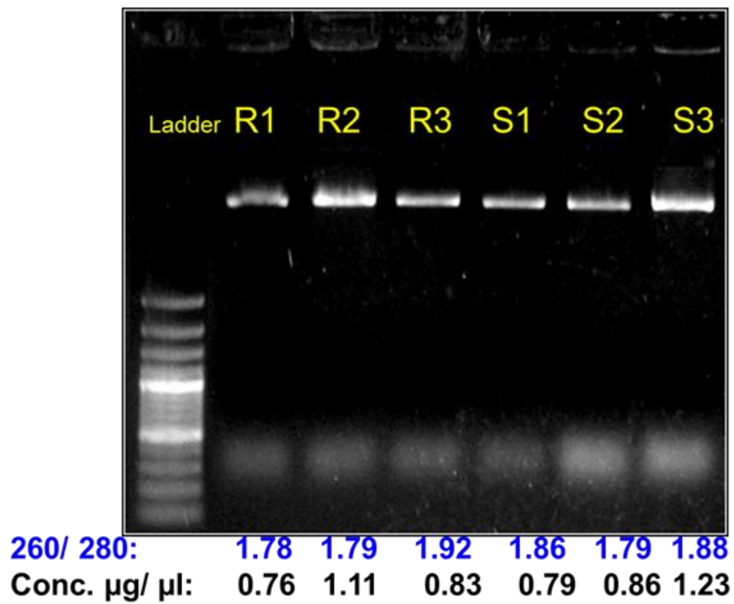

Fig.2 e PCR amplification of mt COI region (709bp) with mt COI specific primer set Custom sequencing of cloned mtCOI DNA fragments

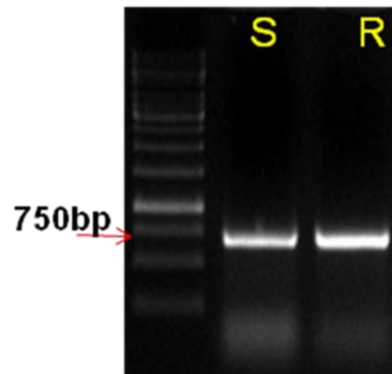

Fig.3 Sequencing grade plasmid on 1 % agrose gel for quality check prior to sequencing

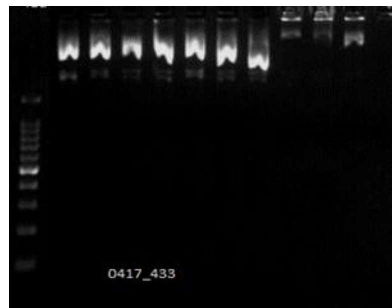

Fig.4 Comparative analysis of the two similar sequences from different clones using chromatogram

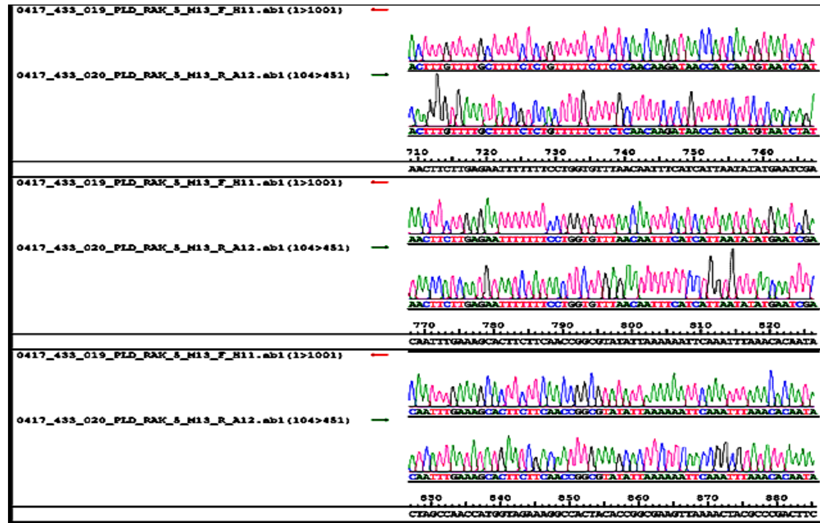

Fig.5 Multiple alignment of COI nucleotide sequence of resistant and susceptible populations of *T. urticae*

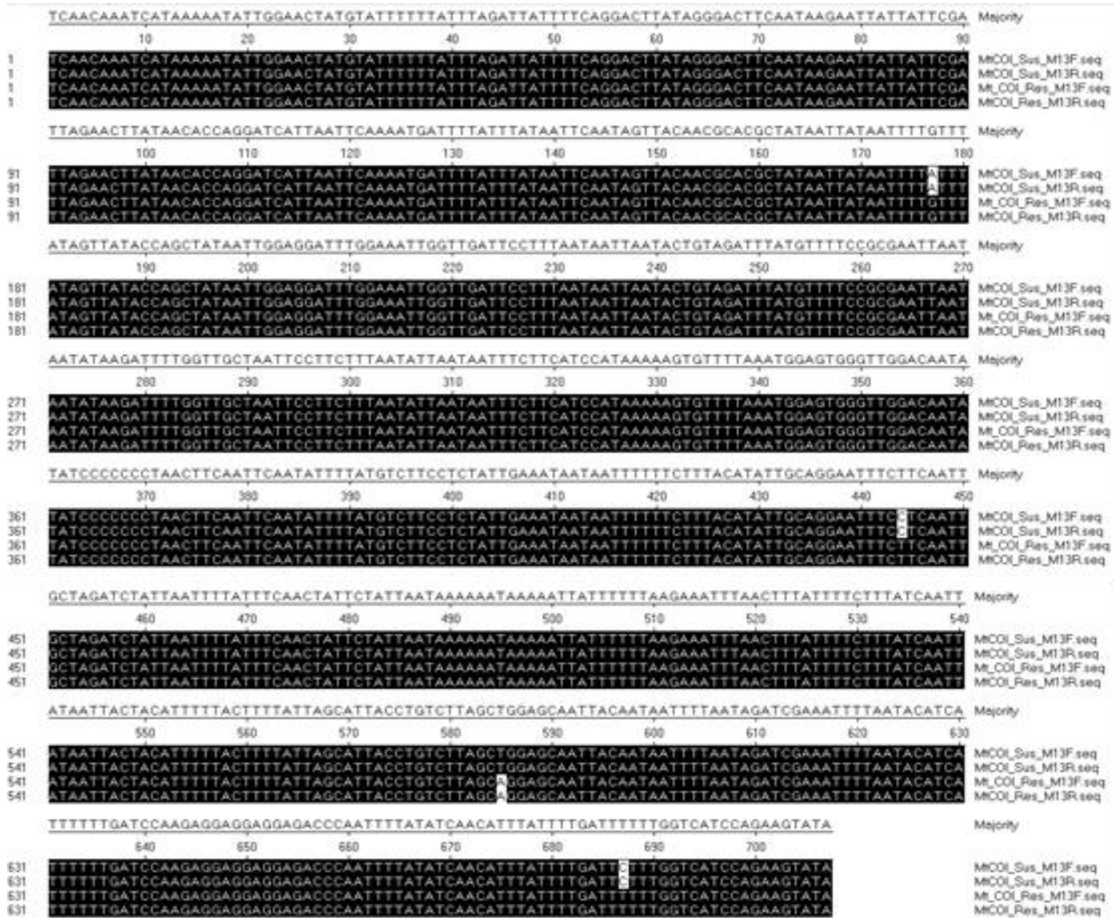

Fig.6 Derived amino acid sequences of COI region variants of *T. urticae*

So the consensus sequence of their alignment was blasted in NCBI database and this showed high level of similarity with the existing COI sequences. The low to moderate level of resistance in the selected population has not shown any change in the amino acid sequences and thus no change in the protein structure. Navajas *et al.,* (1998) and Xie *et al.,* (2006b) while characterizing ten different strains based on COI region, detected a total of six haplotypes which showed no insertions or deletions in the sequenced region. The COI region has been almost widely accepted for barcoding animals because of its generally conserved priming sites. Moreover the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species (Cox and Herbert, 2001, Wares and Cunningham, 2001). When looking at the genetic distance between strains expressed as nucleotide divergence of COI sequence, there was no correlation between COI polymorphism, geographical location of sampling and resistance status. Thus most of the studies have reported COI as a stable gene which is least influenced by insecticide/ acaricide resistance. In our studies high level of resistance to fenazaquin didn't show any change in the amino acid sequence of COI region of resistant and susceptible populations.

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