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Molecular mechanisms of resistance to some pesticides in the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae) from Egypt

Mohamed S. Salman[1], Akram M. Abouzied[2*], Ayat K. El-Gammal[1], Ahmed H. Abo-Ghalia[2]
[1]Plant Protection Research Institute, Agriculture Research Center, Egypt
[2]Department of Zoology, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt

ABSTRACT

The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), is the major pest affecting crops in Egypt. To date, synthetic acaricides are used extensively to control it. These include two specific acaricides: i.e. abamectin and chlorfenapyr compounds that are toxic to *T. urticae* and used widespread in Egypt. Nevertheless, progressive insensitivities to these acaricides are problematic in many areas under field conditions currently used in Ismailia province, Egypt, to control mites. The overall aim of the present study was to study the molecular mechanisms of resistance to the pesticides abamectin and chlorfenapyr in the two-spotted spider mite collected from Ismailia governorate in Egypt. In this paper, we investigated *T. urticae* collected from sprayed vegetables, fruits and citrus by compounds abamectin and chlorfenapyr where control failures were reported, together with a laboratory strain collected from unsprayed districts. Oligonucleotide specific primers of *T. urticae* diagnostics were used to screen the genomic DNA as templates based on the end-point PCR analysis. In the present work, searching of *T. urticae* DNA by PCR for resistance mutations revealed that the amplified DNA fragments had the expected resistant common kinds of mutations. The resulting PCR products were sequenced and compared with the laboratory strain collected from unsprayed districts. Neither mutation was detected in DNA samples of the laboratory strains examined in this study. So, it could be that the toxicological and mutation mechanisms data of abamectin and chlorfenapyr resistance determined in this study will be essential in devising the resistant management strategies for the future use of these compounds in the cultivation in Egypt.

Keywords: Abamectin, Chlorfenapyr, *Tetranychus urticae*, PCR, Resistance, Toxicity, Spider mite

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) is a major agricultural mite pest of a broad range of vegetables, fruits, citrus and ornamental crops (Pinese and Piper, 1994; Bolland *et al*., 1998; Nyoni *et al*., 2011).

T. urticae is widely distributed (Van Leeuwen *et al*., 2007; Nyoni *et al*., 2011), for which biological control has not yet been achieved. Currently, the most effective and widely used method for controlling *T. urticae* is through the use of chemical control by pesticides such as abamectin and chlorfenapyr (Montasser *et al*., 2011). Nevertheless, resistance and pesticide residue are problematic (El Gammal *et al*., 2012). Abamectin acts on the insect nervous system by modifying the normal operation of voltage-gated sodium channels, leading to paralysis and death (Dermauw *et al*., 2012). Chlorfenapyr works by disrupting the production of ATP leading to cellular death, and ultimately organism mortality (Van Leeuwen *et al*., 2006).

The development of resistance of *T. urticae* to abamectin and chlorfenapyr compounds have been widely reported (Ismail *et al*., 2007). This resistance has caused ongoing problems for agricultural production nationwide in Egypt including Ismailia governorate (Anazawa *et al*., 2003; Ghadamyari and Sendi, 2008). Ismailia governorate has encouraged this study through an initiative to supply subsidized pesticides to subsistence farmers. A primary threat imposed by intensive use of these compounds in both agriculture and as part of vector control strategies, is the potential selection for resistance (Ito and Fukuda, 2009).

One important mechanism of resistance to abamectin, termed knockdown resistance, has been shown to arise through alterations (point mutations) in the *para*-type sodium channel protein, leading to reduced sensitivity of the insect nervous system to this compound (Dermauw *et al*., 2012). In the present study, reports of abamectin and chlorfenapyr resistance in *T. urticae* populations in Ismailia governorate have been confirmed using acaricide bioassays (Ochiai *et al*., 2007; Ghadamyari and Sendi, 2008; Ito and Fukuda, 2009; El Gammal *et al*., 2012), the cloning and sequencing of regions (Abdollahi *et al*., 2006; Kwon *et al*., 2010), encoding domains II to IV of the *T. urticae para*-type sodium channel (Ito and Fukuda, 2009; Dermauw *et al*., 2012) in two acaricides resistant from Ismailia province and in two acaricides susceptible from laboratory strains (Van Leeuwen *et al*., 2004) in Ismailia Agricultural Research Station are described.

It has been suggested that the frequency of mutations could be an effective indicator of stress from acaricides in *T. urticae* DNA structure. Studies that involve exposing *T. urticae* to acaricides, isolating of DNA and determining the change in DNA by PCR to show mutations that formed by exposing to acaricides are discussed (Ito and Fukuda, 2009; Dermauw *et al*., 2012).

The objective of the present study was to report laboratory and field studies of resistance mechanisms against two pesticides of major economic importance (abamectin and chlorfenapyr) broadly used in Ismailia

* *Corresponding author:* akramabouzied@science.suez.edu.eg

province, Egypt, in field-collected populations and one susceptible strain of the two-spotted spider mite *T. urticae*. We further elucidate through a series of biological and molecular experiments the accumulation of mutations associated with high levels of abamectin and chlorfenapyr resistance in *T. urticae*, in an attempt to use this knowledge to control the development and spread of resistant populations.

MATERIAL AND METHODS

Extraction of genomic DNA

T. urticae mites were collected from major horticultural centres in Ismailia governorate according to El Gammal *et al*., 2012. Genomic DNA (g DNA) samples were isolated from adult female mites using a DNA extraction method by standard procedures as previously described. Briefly, genomic DNA was extracted from *T. urticae* females (20-30 individuals) following directions provided by the DNA Preparation Kit, Jena Bioscience according to the manufacturer's instructions. Females were washed in 0.01% bleach, rinsed in sterile water and tissues were digested in 900 *µl* of *Lysis Solution* for 2 hours in 1.5-ml microcentrifuge tubes and vortexed and centrifuged at 15,000 rpm for 1 min. The supernatant was removed by suction up to about 20 *µl*; 1 ml of water was added, after centrifugation for 1 min as above, the supernatant was removed again up to about 20 *µl* and vortexed. 300 *µl Cell Lysis Solution* was added and vortexed. 100 *µl Protein Precipitation Solution* was added to the suspension and vortexed vigorously for 30 seconds to mix well and centrifuged at 15,000 rpm for 1 min. The supernatant was transferred into a cleaned 1.5-ml microcentrifuge tubes containing 300 *µl isopropanol >99%* and then vortexed and centrifuged again at 15,000 rpm for 1 min. The DNA precipitate was recovered by centrifugation at 14,000 rpm for 20 min, washed with 70% ethanol, and resuspended in 20 µl of sterile distilled water. The DNA white pellets were taken and kept at 4°C until they were used as templates in PCR. DNA concentration and purity was measured using a quantitative NanoDrop 1000 spectrophotometer according to the manufacturer's protocol. The extracted genomic DNA was subjected to 1 % agarose gel containing 0.5 µg/ml of ethidium bromide; the DNA bands were detected by UV light and documented using a Biorad gel doc 1000 system. All products obtained were sized using a standard 100-bp molecular weight ladder.

Primers used and PCR protocol

Primers used are forward primer GluCl1_F1 (5′-TTGGATTGACCCTAACTCAGCA-3′) and reverse primer GluCl1_R1 (5'-TTGCACCAACAATTCCTTGA-3') which were obtained from published sequences (Dermauw *et al.*, 2012). These primers were designed to specifically anneal to conserved regions within the gene from ion channels gated by acetylcholine, GABA, glutamate in *T. urticae* (Tsagkarakou *et al.,* 2002; Ilias *et al.,* 2012). These specific primers were 20 sequences GluCl3_ F1 (5'-CCGGGTCAGTCTTGGTGTTA-3') and reverse primer GluCl3_ R1 (5′-CACCACCAAGAACCTGTTGA-3′).

Primers synthesis were performed and ordered from Thermo Hybrid Premier Biosoft International (www.Premier.Biosoft.com). The DNA templates for PCR assay were the purified genomic DNA sequence extracted from the adult *T. urticae* females. All PCR amplifications were made in final volumes of 50 µL in a nuclease-free Eppendorf tube with 40 µL of PCR Universal TaqMan Master Mix (Applied Biosystems) containing 0.5 µL each of the two amplification primers at a final concentration of about 30 picomoles for each primer and 4 µL of DNA templates (0.078µg/1µL) and kept on ice prior to amplification. The PCR tubes were placed and carried out in a DNA Thermal Cycler apparatus (Perkin-Elmer). Then the temperature in the block reached 95°C and kept at that temperature for 4 min for strand separation. After that, 35 cycles of amplification were performed. The cycle of denaturation at 95°C for 0.5 min, annealing at 58°C for 1 min and nucleotide addition (extension) at 72°C for 1 min. When the last cycle was completed, the reactions were kept for another 8 min at 72°C for a final extension step followed by cooling at 4°C until the tubes were removed from the machine. The amplified PCR products were mixed with a 6X gel DNA loading dye (Fermentas, Cat. No. R0611) and examined by electrophoresis on 2% agarose TBE gels stained with 0.5 µg/ml of ethidium bromide (EB) for visualization under UV light. The DNA bands were documented using a Biorad Gel Doc 1000 system and quantified using Quantity One version 4.2.3 software (Bio-Rad) according to the manufacturer's descriptions. All products obtained were sized using a standard 100-bp DNA Ladder molecular weight (Jena Bioscience GmbH, Cat. No. M214). Positive control PCR reactions were also performed to evaluate the efficiency of the PCR reactions with the same guidelines as mentioned above. Negative controls were included in all PCR-runs to prevent misjudging following the risk of contaminations of the samples.

Direct sequencing of amplified DNA:

With the advent of DNA methodologies, like the introduction of PCR and nucleotide sequencing, it has become common practice to sequence the PCR products commercially. Briefly, the amplified PCR products were extracted once with ethanol precipitation after the phenolchloroform treatment (Sambrook *et al*., 1989). Direct sequencing of the PCR products was performed with the automatic sequencing assay using the Taq Dye Deoxy Terminator Cycle Sequencing Kit with the ABI 3700 automated DNA sequence (Applied Biosystems) following the manufacturer's protocol for the sequencing reaction. One strand of the DNA was sequenced. The sequencing results have been further grouped and analyzed. The nucleotide sequences from this study were multiple aligned and edited manually by visual inspection. The sequences from this study were compared alongside with those of *T. urticae* reference

susceptible laboratory strains which are available from the deposited sequences in DDBJ/EMBL/GenBank database with accession numbers of AB076369-72 (Vontas *et al.*, 2001).

RESULTS

DNA techniques and PCR amplification:

The results of the genomic DNA extracted from *T. urticae* adult females strains (as the males were too small to produce enough DNA to obtain reliable results) are shown in Fig. 1 lanes three to seven. Sample number five was low DNA concentration (Fig. 1 lane seven). The five DNA samples were amplified by using the primers GluCl1_F (5′-TTGGATTGACCCTAACTCAGCA-3′) and reverse primer GluCl1_R1 (5′-TTGCACCAACAATTCCTTGA-3′). Analysis of the agarose gel electrophoresis revealed several interesting findings. The high fidelity PCR yielded successfully the expected ~ 0.7 kb long band size including the PCR primers. Only a single common 700 bp band of PCR products was obtained by using the primers set (Fig. 2 lanes three to seven). The amount of amplified PCR products detected by agarose gel (Fig. 2 lanes three to seven) was almost thick and the extent of amplification nearly reached a peak level after 35 cycles of amplification, and was performed for the five different samples of *T. urticae* strains, suggesting that the primers set were specific. This size was equivalent to the previously reported sequence data of *T. urticae*, with 700 bp, of Hoy and Jeyaprakash (2005).

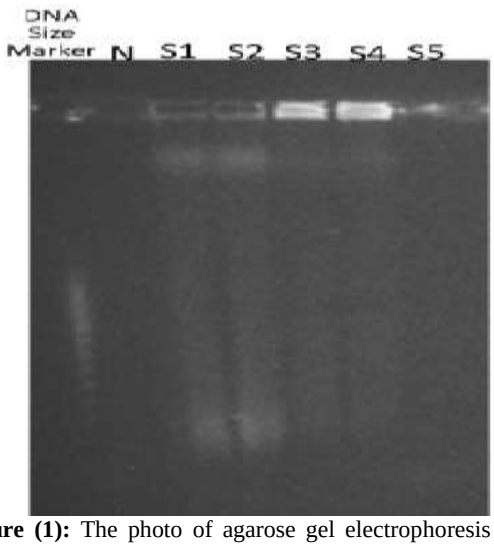

Figure (1): The photo of agarose gel electrophoresis shows genomic DNA extracted from *T. urticae* adult females. Negative control is present on lane number 2. S sample.

Additionally, the banding patterns of the fragments that detected by electrophoresis (see Fig. 2) were also expected by the fragment sizes calculated from the sequencing data and the recognition sites (in this work). The optimal concentration of the PCR DNA products for the samples was quantified using a NanoDrop ND 1000 spectrophotometer instrument (Thermo Scientific) is applicable for all five different samples of *T. urticae* without modification. All experiments were repeated at least two times with the same conditions, and the mean value was used for further analysis. No differences between the amplified DNA concentrations in each of the repeated PCR products were observed as judged by the NanoDrop and the agarose-stained gel results. These results revealed that PCR amplifications induced by the primers set were specific and can be seen in Fig. 2. Moreover, the DNA concentrations of the five tested samples of *T. urticae* identify homologous stretches of DNA assuming identical mobilities and hence represent the same sequences (Fig. 2 lanes three to seven). A negative control experiment was done without mite DNA in order to check for contamination (See Fig. 1 and Fig. 2).

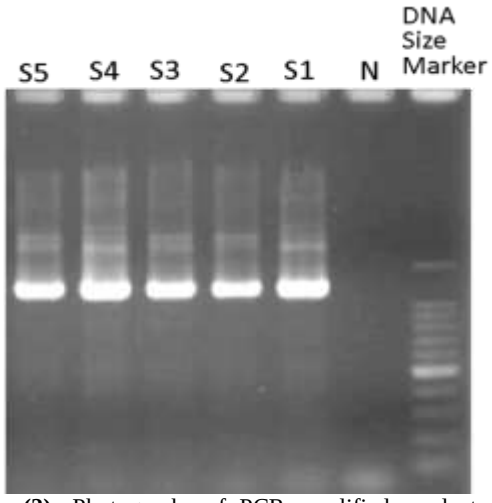

Figure (2): Photographs of PCR amplified products from adult mites (Females) separated by the agarose gel electrophoresis. The primers set used were GluC11_F1 and GluC11_R1. S sample. N negative control.

Direct sequencing of PCR amplified products:

Direct sequencing of the PCR amplified genomic DNA was performed to confirm the PCR products. Only one PCR amplified product was successfully sequenced using a dilution of the previously described original PCR primers with the sequence strF GluCl1_F1 (5′TTGGATTGACCCTAACTCAGCA-3′) and the sequence strR GluCl1_R1 (5′TTGCACCAACAATTCCTTGA-3′). The obtained sequencing data was identified and were compared alongside with those of *T. urticae* reference susceptible laboratory strains.

When the sequences of the700 bp long PCR product were compared to these sequences provided by Ilias *et al.* (2012), our sequences data displayed some differences. Differences in the positions or number of resistance target sites among samples were found in resistance *T. urticae* compared to the reference susceptible laboratory strains (Tsagkarakou *et al.*, 2002), and displayed the expected higher levels for resistance to at least one of the two current tested acaricides, abamectin and chlorfenapyr, that are well established due to the heavy use of insecticides treatment history in Ismailia province, Egypt. The results revealed sequence highest similarity for the two of the selected sequences and to other resistant

sequences due to the heavy use of insecticides treatment history in different countries and crops (Van Leeuwen *et al.*, 2010).

Two different mutations were identified after the 700 bp long PCR products were sequenced; one of them was closely related to the target site resistance to abamectin across a range of insect and mite species (Ben-David *et al.*, 2007). The two mutations were found only in both the 138 base position and the 172 base position of the resistant strains but not in the susceptible *T. urticae* strains, suggesting that they are likely associated with the resistance phenotype due to the abamectin. Very similar mutation has been found in other arthropod species that are resistant to acaricides (Bernardi *et al.*, 2013).

At least one recognition site for the mutations at the 138 base position was found on each sequence that caused resistance in *T. urticae* is a worldwide phenomenon and it has been reported from a number of different countries and crops (Van Leeuwen *et al.*, 2010). This could be the result of selection or genetic drift if resistance was involved it is likely to be the result of the heavy use of insecticides treatment history of the two current tested abamectin and chlorfenapyr (Van Leeuwen *et al.*, 2010; Clotuche *et al*., 2013). This indicating that susceptible genotypes had been eliminated for the tested strain with the heavy use of abamectin and chlorfenapyr. These 700 bp long product sequences are also related sequences obtained from the mites *T. urticae* from Greece, *T. kanzawai*, *T. puerariсola* and *T. ludeni* using PCR-RFLP (Demaeght *et al*., 2013).

DISCUSSION

T. urticae is one of the most important agricultural pests worldwide. It can feed on more than 1000 host plant species, including crops of major economic importance (Dermauw *et al*., 2012). The extreme short life cycle, combined with frequent acaricide applications, facilitates resistance build-up (Chiasson *et al*., 2004; Van Leeuwen *et al*., 2005). Moreover, *T. urticae* is known to have a particularly high tendency to develop resistance to acaricides among other mite species (Schoknecht and Otto, 1992).

The *T. urticae* collected from Ismailia governorate in Egypt, and sprayed with abamectin and chlorfenapyr pesticides when compared to the susceptible strain showed a wide range of cross resistance. In order to verify this hypothesis, it would be necessary to compare the laboratory susceptible and resistant strains from parts of Ismailia province.

In our study, *T. urticae* showed molecular mutations level of resistance to abamectin and chlorfenapyr. Our laboratory results revealed that PCR amplification showed induced point mutations by abamectin in *T. urticae*. All expected banding patterns were represented by electrophoresis, and the primers set used were convenient for this study. These observations, and the fact that resistance in *T. urticae* appeared rapidly in different regions of Ismailia governorate suggest that mutations conferring resistance may have appeared independently in the different localities. The efficacy of abamectin and chlorfenapyr against *T. urticae* and sensitive strains was compared by DNA analysis. Additionally, the results of PCR and followed by sequencing using the primers are in agreement with those obtained by Osakabe *et al*. (2002, 2008), and the molecular analysis have shown that the sensitivity to at least the insecticide abamectin increased and resulted in a single point mutation or a combination of point mutations. This has been reported in strains from the Greece (Vontas *et al.*, 2001) and Egypt (this study).

In this work, evidence for *T. urticae* to develop resistance to acaricides was confirmed by PCR method. However, these findings need to be verified in a larger prospective study. Therefore, we do recommend gaining greater understanding of possible side effects of abamectin and chlorfenapyr resistance when *T. urticae* populations are being controlled in Egypt by chemical means only.

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ميكانيكيات المقاومة الجزيئية لبعض المبيدات الاكاروسية لأكاروس العنكبوت ذو البقعتين (تترنيكس يورتيكا) فى مصر

محمد سلمى[١] ، أكرم أبوزيد[٢] ، أيات الجمال[١] ، أحمد أبوغالية[٢]

[١]محطة البحوث الزراعية بالإسماعيلية، معهد بحوث وقاية النباتات، الإسماعيلية، مصر

[٢] قسم علم الحيوان، كلية العلوم، جامعة قناة السويس، الإسماعيلية ٤١٥٢٢، مصر

الملخص العربي

يعتبر اكاروس العنكبوت ذو البقعتين من اهم الافات التي تؤثر على المحاصيل في مصر وعلى الرغم من أن المكافحة الحيوية لم تأخذ دورها في تصدرعمليات المكافحة في حين ان استخدام المبيدات الاكاروسية يتم بكميات كبيرة وقد هدفت هذه الدراسة إلى قياس كفاءة اثنين من المبيدات الاكاروسية وهما الابامكتين والكلورفينبير وهما من اكثر المبيدات استخداما فى مكافحة الاكاروس فى مصر مما تسبب عنه العديد من المشاكل عند التطبيق الحقلي. كما هدفت إلى قياس ميكانيكيات المقاومة التي يتسبب عنها مقاومة الاكاروس لفعل هذه المبيدات على الاكاروس في مصر. في هذه الدرسة تم اختبار حساسية اثنين من المبيدات الاكاروسية وهما الابامكتين والكلورفينبير علي سلالات اكاروسية حقلية تم جمعها من المراكز المختلفة بمحافظة الاسماعيلية ومقارنتها بالسلاله الحساسة المعملية. تم عمل الاختبارات الجينية على السلالات المختلفة من الاكاروس باستخدام تفاعل البلمرة المتسلسل الكيفى. اظهرت النتائج وجود طفرات لجينات المقاومة عند دراسة الاختلافات في الصفات الوراثية للمادة الوراثية بسلالات الاكاروس المختلفة وبمقارنتها بالسلالة المعملية الحساسة التى لم تتعرض للمبيدات مطلقا. واظهرت الدراسة ان استخدام مبيدي الابامكتين والكلورفينبير ادي الى ظهور طفرات في الاكاروس ادت الى زيادة ظاهرة المقاومة لهذين المبيدين في السلالات الحقلية التى تم معاملتها مقارنة بالسلالة الحساسة المعملية. وتعتبر هذه احدى الطرق الهامة التى يمكن الاستعانة بها في اكتشاف ظاهرة المقاومة لفعل هذة المبيدات. وخلاصه القول أن تلك الدراسة يمكن أن تكون قاعدة لدراسات أخرى تلقى الضوء على مشكله المقاومة لمبيدات سلالات الآفات مثل العنكبوت الأحمر ذى البقعتين. لذا ينصح بأجراء أبحاث أخرى جديدة لمحاوله فهم أكثر للأثار الجانبية للمقاومة الناتجة عن استخدام الأباميكتن والكلوروفينبير وغيرها من مبيدات الاكاروسات.