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Investigation of a PCR-based Method for Insecticide Resistance Monitoring

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Abstract: The use of RAPD-PCR was investigated for its suitability in pesticide resistance monitoring. A high salt and Chelex 100 (BioRad) resin methods were used to extract DNA from susceptible and propargite-resistant strains of *Tetranychus urticae* Koch. The high salt method of DNA extraction was found suitable for extracting DNA from individual adult females of *T. urticae*. The DNA products obtained with the primer B8 were reproducible but inconsistant causing heterogeneity of PCR banding patterns. The inconsistant banding patterns could be due to the unknown genotypes of the females used in the analyses as it could not be confirmed that the susceptible and propargite-resistant strains of *T. urticae* were of *SS* (homozygous susceptible) and *RR* (homozygous resistant) genotypes. A second reason for the inconsistency of RAPD bands in this study could be the presence of non-mite DNA in the samples. These problems, when resolved, could make RAPD-PCR a potential monitoring tool for pesticide resistance detection and subsequent management.

Key words: DNA extraction, RAPD-PCR, propargite, pesticide resistance, *Tetranychus urticae*

Introduction

Pesticide resistance is an increasingly urgent worldwide problem and more than 500 species of arthropods, 100 pathogens, 60 weed biotypes and one nematode are resistant to at least one pesticide (Georghiou and Legunes-Tejeda, 1991). These numbers are increasing day by day and because of widespread resistance of crop and livestock pests to pesticides, agricultural productivity can be jeopardized. Insecticide resistance is one of the greatest challenges facing applied entomologists today because of the ever widening circle of cross and multiple resistance among arthropod pests, the diminishing number of effective chemical insecticides and acaricides, and the exponentially increasing cost of developing new pesticides (Metcalf, 1980).

Bioassays have been the basic method used for documenting levels of insecticide or acaricide resistance in field populations on which management decisions are based. Conventional detection of resistance is based on insecticide susceptibility tests that consist of dose mortality experiments usually performed in the laboratory. Resistance management requires more effective techniques for detecting resistance in its early stages of development. It is in the early stages of resistance development that heterozygotes are abundant. The more or less intermediate response of those heterozygotes to the dose-mortality tests make them difficult to detect. Molecular diagnostics have been postulated to increase accuracy and to reduce the variability associated with insecticide bioassays that result from both intrinsic (genetic structure) and extrinsic (bioassay conditions, sample size, etc.) factors (Brown and Brogdon, 1987; Ffrench-Constant and Roush, 1990).

In recent years the polymerase chain reaction (PCR) has enabled molecular analysis to become an integral part of biosystematics, population biology and ecology (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Saiki, 1989). Large amounts of specific DNA fragments of defined length can be synthesized from very small amounts of starting material (genomic DNA). The sequence of the isolated DNA can be subsequently analyzed for variability at a range of evolutionary scales (Wong *et al.*, 1978; Gyllensten and Erlich, 1988). For an individual organism as small as a mite the amount of extracted DNA is too small to be used in conventional molecular methods. PCR can be performed on mite DNA to obtain larger amounts for further analyses.

PCR is based on the *in vitro* enzymatic amplification of a DNA fragment flanked by two oligonucleotide primers (synthetic DNA usually 20-30 nucleotides long) that bind to opposite strands of the target sequence. The DNA to be used as template in PCR could be extracted by several methods [e.g. high salt method; Chelex 100 resin (Edward and Hoy, 1993; Katakura *et al.*, 1997); phenol extraction (Kaliszewski *et al.*, 1992)]. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers by *Thermus aquaticus* (Taq) DNA Polymerase results in the exponential accumulation of the specific target fragments of DNA in a few hours.

Williams *et al.* (1990) reported the development of a PCR technique that uses a single primer, 10 bases long of arbitrary sequence, that hybridizes randomly to unknown regions of genomic DNA. A major advantage of this technique is that it requires no prior knowledge of an organism DNA sequence. This technique is called RAPD for ` randomly amplified polymorphic DNA'. RAPD-PCR provides a very versatile and widely applied biotechnology technique in entomology. RAPD-PCR has been used for the identification of individuals from the propargite-resistant strain of *T. urticae* in the present study.

Roehrdanz *et al.* (1993) utilized RAPD-PCR to obtain DNA banding patterns that can be used as genetic markers suitable for species and strain identification in *Diaeretiella rapae* (McIntosh) and *Aphidius matricariae* (Hal.), both are parasitic Hymenoptera. RAPD-PCR has also been used for detecting cyclodiene resistance in the mosquito, *Aedes aegypti* Linnaeus (Ffrench-Constant *et al.*, 1994) and *Tribolium castaneum* Herbst (Andreev *et al.*, 1994). RAPD markers have also been found for aphids (Black *et al.*, 1992), grasshoppers (Chapco *et al.*, 1992), mosquitoes (Ballinger-Crabtree, 1992), whiteflies (Perring *et al.*, 1993) and lady beetles (Roehrdanz, 1992). Field *et al.* (1996) also used PCR-REN technique for insecticide resistance detection in *Myzus persicae* (Sulzer).

The reported research work has been conducted during 1996-97 at Lincoln University, New Zealand, with the objectives to 1) develop a method of DNA extraction from an individual adult *T. urticae* female, and 2) determine the suitability of RAPD-PCR to differentiate between the susceptible and propargite-resistant strains of *T. urticae*.

Materials and Methods

Sources of the susceptible and propargite-resistant strains of *T. urticae*: A susceptible strain of twospotted spider mite (*T. urticae*) was collected from wild hosts from the Lincoln University organic production area. No pesticide of any type had been applied in this area for approximately 20 years. A resistant strain of *T. urticae* was air-freighted from an Auckland (New Zealand) glasshouse where there had been intensive use of miticides. Both strains were reared on French dwarf bean (*Phaseolus vulgaris*, cv. 'Tendergreen') in separate controlled temperature (CT) rooms at 21± 3 °C, 60± 15% RH and a 16L:8D photoperiod.

Extraction of mite DNA: Two methods of DNA extraction were tried.

High salt method: Individual adult female mites from a fresh culture were placed singly in Eppendorf tubes. Thirty microliter of

extraction buffer (24.3 µl dl water, 3.0 µl 10x SET, 1.2 µl 25% SDS and 1.5 µl Proteinase K) was added to each tube and the mites were squashed with the blunt end of a sterile glass needle. The tubes were placed in a 50° C water bath for 3 hours; mixed gently by inversion every 15-20 minutes. The digest was removed and 12.9 µl of 5 M NaCl was added to each tube. Tubes were rotated gently to mix and precipitate the protein.
Tubes were centrifuged at 4000 g for 15 minutes at 20° C and the supernatant was removed without disturbing the pellet. Two volumes of 96% ethanol were slowly added to each tube. The tubes were left standing for 2-3 minutes to precipitate the DNA. Tubes were centrifuged at 6500 g for 30 seconds at 20° C and the ethanol was discarded. Each DNA pellet was washed with 50 µl of 70 % ethanol. The tubes were turned so that the pellet was on the inner side of the rotor and spun at 6500 g rpm for another 30 seconds. Ethanol was discarded and the tubes were air dried. The DNA was dissolved in 30 µl TE and left in the refridgerator overnight.

Chelex 100 (BioRad) resin extraction method: An individual adult female mite was homogenized in 40 µl of 5% chelex (50 mg of Chelex 100 resin added to 1000 µl dl water) in PCR tubes using the blunt end of a sterile glass rod. The tubes were vortexed at high speed for 15 seconds and then left standing for 30 minutes. The samples were heated at 56 °C for 30 minutes and then vortexed for 15 seconds. The samples were incubated at 95° C for 15 minutes then vortexed for another 15 seconds. The tubes were centrifuged at 6500 g for 5 minutes to pellet the resin. The supernatant was removed into 0.5 ml tubes and stored in the freezer at -20 ° C overnight.

Amplification of target DNA: The following protocol was used to make 20 µl of PCR reaction.
The volumes of chemicals [dNTPs (Boehringer), buffer (Boehringer) and primers (Operon)] required were worked out on a PCR sheet. A reagent master solution equivalent to 11.775 µl dl water, 2.5 µl 10x *Taq* Buffer, 2.5 µl dNTP (1mM), 0.625 µl $MgCl_2$ (20 mM) was made. The required volume of reagent master was taken out for later diluting the *Taq* polymerase to 1 µl per reaction.
Primer (0.4 µl per reaction) was added to the reagent master. When using different primers, the primer was added directly to the PCR tubes. A total of 42 10-mer primers (A1-A12, A14-A20, K01-K05, B1, B3-B18 and B20) were used. The required volumes of reagent master were aliquoted into labelled PCR tubes. DNA (1.0 µl) was added into the PCR tubes; TE was added into the negative controls. The tubes were vortexed and spun down briefly.
A hot start protocol (D'Aquila *et al.*, 1991) was used to ensure sufficient denaturation of the DNA template. The PCR tubes were placed into a thermal cycler (Perkin Elmer, PE 2400), preheated to 95 ° C for four minutes. *Taq* (2.5 units) (0.2 µl) was added to the remaining reagent master solution, vortexed and spun down briefly. Diluted *Taq* (2 µl) was added to each PCR tube at 71 ° C. DNA amplification was achieved using the following thermal protocol.

Steps	Temperature (° C)	Duration	
Additional denaturation	95	4 minutes	
Denaturation	93	15 seconds	x 40
Annealing	37	15 seconds	x 40
Extension	71	15 seconds	x 40
Additional extension	72	5 minutes	

Separating the PCR products on an agarose gel: Agarose (Boehringer, LE) (0.45 g) was added to 30 ml of 0.5X TBE to make 1.5% solution. The solution was boiled until transparent, poured into a gel tray with a well comb and allowed to solidify (about 30 minutes). The gel was transferred to a submerged gel electrophoresis tank containing 0.5X TBE and the well comb was gently removed. Five microliter of each PCR product, mixed with 1 µl of loading dye (5:1), was loaded in the wells and electrophoresed alongside a molecular weight marker (100 bp ladder, Gibco-BRL) at a rate of 5 v/cm for 90 minutes. The separated PCR products were visualized on a UV trans illuminator and a polaroid photograph was taken as a permanent record.

Results

DNA extraction: Both the high salt and chelex 100 chelating resin technique were found successful in extracting DNA from a single adult *T. urticae* female and gave high molecular weight DNA. The concentration of DNA extracted using the high salt method was sufficient for PCR amplification and was used in subsequent studies.

RAPD-PCR: Using the PCR conditions described above, all of the primers from kit A (except A6 and A17) and kit K tested were found successful in amplifying *T. urticae* DNA. Six (B1, 3, 6, 9, 14 and 16) out of the 18 primers used from the kit B were unable to produce any band. Only primer B8 (Fig. 1A) gave banding patterns that could be used to distinguish between the susceptible and propargite-resistant strains of *T. urticae.* The primer B8 was, therefore, selected for further screening of the susceptible and propargite-resistant individuals.

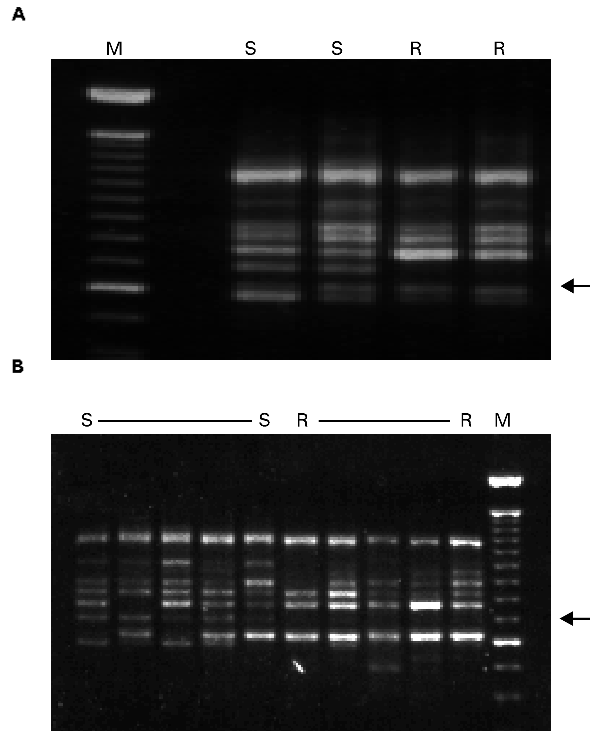

Fig. 1: The band patterns produced by primer B8
A: Initial PCR products indicating different patters for susceptible and propargite-resistant strains fo *Tetranychus urticae.*
B: Subsequent samples that to amplify discriminatory bands (S-susceptible strain, R-resitant strain, M-molecular weight.

Differentiation of susceptible and propargite-resistant strains of *T. urticae*: The PCR products obtained with primer B8 were reproducible but inconsistent (Fig. 1B) within the susceptible and propargite-resistant strains of *T. urticae.* No bands appeared to be unique to either strain. As a consequence of the inconsistency in banding pattern further experiments were abandoned until the suggested improvements could be made.

Discussion

Although, DNA extraction from individual mites is the first step towards applying RAPD-PCR for monitoring of acaricide resistance, it has not been reported in the literature. Kaliszewski *et al.* (1992), however, has described DNA extraction, using a phenol extraction method, from a bulk sample of 5-10 *T. urticae* females. Edward and Hoy (1993) were successful in extracting DNA from two parasitoids *Trioxys pallidus* and *Diglyphus begini* using the Chelex 100 chelating resin method which produced consistent yields. This method has also recently been extensively used for DNA extraction from pathogens: *Plasmodium falciparum* (Zhang *et al.*, 1995); powdery mildew (Hirata and Takamatsu, 1996); *Trypanosoma* spp. (Katakura *et al.*, 1997). Although both the methods were found successful in extracting DNA from individual *T. urticae* adult females, the high salt method (using NaCl) gave the most consistent yields. This method is appropriate for DNA extraction from other species of mites (*Panonychus ulmi*) and other small insects.

While performing RAPD-PCR, an additional denaturation step at the start of reaction (to ensure complete denaturation of the template DNA) and an additional extension step after completion of all the cycles (to ensure complete extension of any partial fragments that may be generated by premature termination) were added to the normal PCR protocol. Kaliszewski *et al.* (1992) emphasized the need for additional denaturation and extension steps.

There could be several reasons for the heterogeneity of the PCR banding patterns. Within each strain, the genotypes of the females used in the analyses were unknown. In other words it was not confirmed that the susceptible and propargite-resistant strains of *T. urticae* were of *SS* and *RR* genotypes. The importance of correct genotyping was addressed by Ffrench-Constant *et al.* (1994) when they developed PASA for cyclodiene resistance in *Drosophila melanogester* and *D. simulans* where the genotypes of the individuals were known. Prior to their examination of endosulfan resistance in *H. hampei* using PASA, the resistant strains used were selected to homozygosity by 13 generations. However, they could not be sure if the susceptible PASA primer successfully amplified all the susceptible alleles present due to the difficulty in ensuring that *H. hampei* strains were homozygous for the same susceptible allele. Steichen and Ffrench-Constant (1994) suggested the use of both the PCR-REN and PASA in combination for resistance monitoring. PCR-REN can be used to determine resistance frequency and PASA to identify the particular alleles involved.

A second reason for the inconsistency of RAPD bands in this study could be the presence of non-mite DNA in the samples. For example, the gut of the mite may contain DNA from the host, prey or gut micro-organisms (Persing *et al.*, 1990; Houck *et al.*, 1991). Kaliszewski *et al.* (1992) suggested the need to starve mites before DNA extraction or designing primers which will specifically amplify mite sequences. Johanowicz and Hoy (1996) reported the amplification of *Wolbachia* DNA with *T. urticae* DNA using RAPD-PCR and mentioned the need to develop a method to eliminate amplification of transient *Wolbachia* from gut contents. Similarly, Black *et al.* (1992) detected the presence of two endoparasitic wasps, *Lysiphlebus testaceipes* (Cresson) and *Diaeretiella rapae* (McIntosh), in the bodies of aphids using RAPD-PCR.

Ffrench-Constant *et al.* (1995) discussed the potential applications of the PCR-based techniques and compared them with the use of insecticide bioassays. They concluded that, although such techniques will probably never replace bioassays for routine monitoring, they can more readily address several fundamental questions related to the evolution and spread of specific resistance alleles in insect populations. Difficulties in developing molecular markers for resistance monitoring may arise through the inability to correctly genotype individual organisms and the potential inconsistency and reproducibility of results in case of RAPDs.

Genetic information concerning propargite-resistance loci and subsequent development of molecular tools is, however, possible through sequence analysis, including the development of RAPD markers such as those developed for *Pseudomonas* resistant gene in tomato (Martin *et al.*, 1991); gall midge, *Orseolia oryzae*, resistant gene (gm2) (Nair *et al.*, 1995) and (Gm4t) (Nair *et al.*, 1996) in rice; cyclodiene resistance in *A. aegypti* (Ffrench-Constant *et al.*, 1994) and *T. castaneum* (Andreev *et al.*, 1994). This approach of sequence analysis and development of RAPD markers, whilst currently expensive to undertake, may in future provide information and forecasting of propargite-resistance at population levels and of life stages that is not possible using conventional bioassay techniques. Clear differentiation between the *RR, SS* and hybrid genotypes and the ability to detect resistance in DNA extracted from eggs and larvae could facilitate the early detection of resistance in field infestations. Therefore, the use of PCR-based techniques, e.g., RAPD, REN and PASA could, in future, be used as very powerful monitoring tools in resistance detection and subsequent management.

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