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Role of Cytochrome P450 gene in insecticide susceptibility of the whitefly, *Bemisia tabaci* (Homoptera, Aleyrodidae) in Egyptian governorates

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Abstract
The sweet potato whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) is one of the most damaging pests world-wide. The neonicotinoid insecticides, imidacloprid, acetamiprid, thiamethoxam, dinutefuram and the insect growth-regulating insecticides (IGRs), buprofezin and pyriproxyfen were tested against whitefly adults collected from seven Egyptian governorates. Buprofezin is recommended over pyriproxyfen due to its low LC_{50} values in all the tested strains. Acetamiprid showed the higher potency to whiteflies in Behira, Gharbeia, Dakahleia, and Menia governorates due to its low LC_{50} values in comparison with the other neonicotinoids. To detect insect response to insecticide treatment, the mixed function oxidase activity was measured in laboratory and field collected samples. CYP6 gene was detected in all of the collected samples. A fragment of (852 bp) was obtained using CYP6 degenerate primer. PCR product yielded partial P450 sequence with 99% identity to *B. tabaci* CYP6CX1v1. Nucleotide variations were detected among the collected samples which may be one of the reasons for the insensitivity of Cytochrome P450 to neonicotinoids application in some governorates.

1. Introduction

The sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) is considered as one of the most damaging pests resulting in huge losses in numerous crops world-wide. It is considered to be a highly cryptic species complex, with 24 described biotypes (Perring, 2001). The biotypes differ in host range, host plant adaptability, phytotoxicity, insecticide resistance and virus-transmission capabilities (Jones, 2003). It has been shown to transmit 111 virus species, some of which are of high economic importance (Roditakis *et al.* 2005).

In Egypt, various classes of insecticides are extensively used by growers to control *B. tabaci*. In field crops, residual insecticide concentrations usually occur after the initial insecticide application. These concentrations depend on methods of insecticide applications, degradation by environmental factors, such as rainfall, temperature and sunlight (Desneux *et al*. 2005, Biondi *et al*. 2012, Vanaclocha *et al*. 2013) and, in the

case of systemic products, how fast is the plant growth (Zhang *et al*. 2011, He *et al*. 2013).

The neonicotinoid insecticides, imidacloprid, acetamiprid, thiamethoxam, dinutefuram and the insect growth-regulating insecticides (IGRs), buprofezin and pyriproxyfen, play critical roles in controlling whiteflies in variable regions of the world (Denholm *et al.* 1998). Buprofezin and pyriproxyfen have provided the foundation for successful resistance management, they were recommended to be used once per season against whiteflies in cotton for those strains that found to be resistant to them under both laboratory and field exposure conditions. Resistance to imidacloprid and cross-resistance to thiamethoxam and acetamiprid was first reported in the Almeria region of southern Spain (Cahill *et al.* 1996, Rauch and Nauen 2003). Whiteflies with reduced susceptibility to imidacloprid have subsequently been reported from Australia, Brazil, Crete, Germany, Israel, Italy, Mexico and Morocco, (Nauen and Denholm 2005). An up-to 82-fold resistance to imidacloprid was selected by Prabhaker *et al.* (1997) in the laboratory. Whitefly resistance to buprofezin and pyriproxyfen has been extensively characterized in Israel (Horowitz *et al*. 2005) and has resulted in decrease in use of these insecticides in some areas.

Neonicotinoids are nicotinic acetylcholine receptor (nAChR) agonists that are characterized by having high potency with low mammalian toxicity. It is used extensively for both crop protection and animal health applications. It is a nicotinic acetylcholine receptor (nAChR) agonist that combines high potency with low mammalian toxicity (Tomizawa and Casida, 2005). Resistance has been attributed to oxidative detoxification by cytochrome P450 monooxygenases (P450s) (Rauch and Nauen, 2003). P450s (encoded by CYP genes) constitute a multigenic superfamily of enzymes (Nelson *et al.,* 1996) In insects, CYP genes mostly belonging to microsomal CYP4, CYP6, CYP9 and mitochondria CYP12 families, have frequently been associated with detoxification processes giving tolerance to insecticides (Scott, 1999; Feyereisen, 1999) or plant toxins (Berenbaum, 2002) There are three independent evidences for linking enhanced P450 activity with resistance to neonicotinoids in B. tabaci. Firstly, pre-exposure to P450 inhibitors such as piperonyl butoxide increased the toxicity of imidacloprid against a highly resistant strain (Nauen *et al.,* 2002). Secondly, close correlation was found between degree of resistance in several strains and elevated monooxygenase activity. Thirdly, in vivo metabolism studies of [^{14}C] imidacloprid showed the main metabolite in imidacloprid-resistant strains to be 5-hydroxyimidacloprid, a product of oxidative degradation that was below the detection limit in a reference susceptible strain (Rauch and Nauen, 2003).

Monooxygenase activity increased 5–6-fold in highly resistant strains (RF~1,000). Only monooxygenase activity correlated with imidacloprid, thiamethoxam and acetamiprid resistance and, therefore, monooxygenases seem to be the only enzyme system responsible for neonicotinoid resistance in B. tabaci Q- and B-types.

Pyriproxyfen is a juvenile hormone analog with relatively low mammalian toxicity. The utility of pyriproxyfen in whitefly management was based on suppression of embryogenesis and adult formation in B. tabaci (Ishaaya and Horowitz, 1992) and Trialeurodes vaporariorum (Westwood) (Ishaaya *et al*., 1994). Buprofezin is a chitin synthesis inhibiting growth regulator that has been effectively used to control B. tabaci on cotton and other crops (Ishaaya *et al.,* 1988; Horowitz and Ishaaya, 1994).

2. Materials and Methods

2.1. Whiteflies

Seven insecticide-resistant field strains of *B. tabaci* (Genn.) were collected from different localities at 7 governorates in Egypt (table 1) covering the whole country in their distribution (Fig 1). Once a collection site had been located, samples were collected for species identification using the key by Martin *et al*. (2000). An insecticide-susceptible strain (SUD-S) ,used as control, was collected from Behira governorate in the year 2000 and was long-reared in laboratory of the Central Agricultural Pesticides Laboratory, Agricultural Research Centre. It provided the base-line data for establishing the resistance status of Egyptian samples. This strain was maintained on plant only without insecticide exposure under16 hr light photoperiod at 26°C as described by Coudriet *et al*. (1985). For the toxicological tests, adult whiteflies were collected in the early morning hours using a mouth aspirator into large pots with ventilated lids modified to fit the aspirator. Insects were collected from at least ten different sampling spots at each site. Whiteflies were transported to the laboratory in a cool box and used within a few hours after collection for the toxicological tests. Samples comprised at least 20 individuals and were stored at -20°C for molecular analysis.

Table 1. *Origin of the collected B. tabaci strains from Egyptian Governorates.*

Strain Symbol	Governorate	Latitude	Longitude
B	Behira	30.514	30.343
G	Gharbia	30.875	31.033
K	Qalyubia	30.329	31.216
D	Dakahlia	31.083	31.491
M	Menoufia	30.597	30.987
F	Fayoum	29.417	30.712
Mn	Menia	30.712	29.974

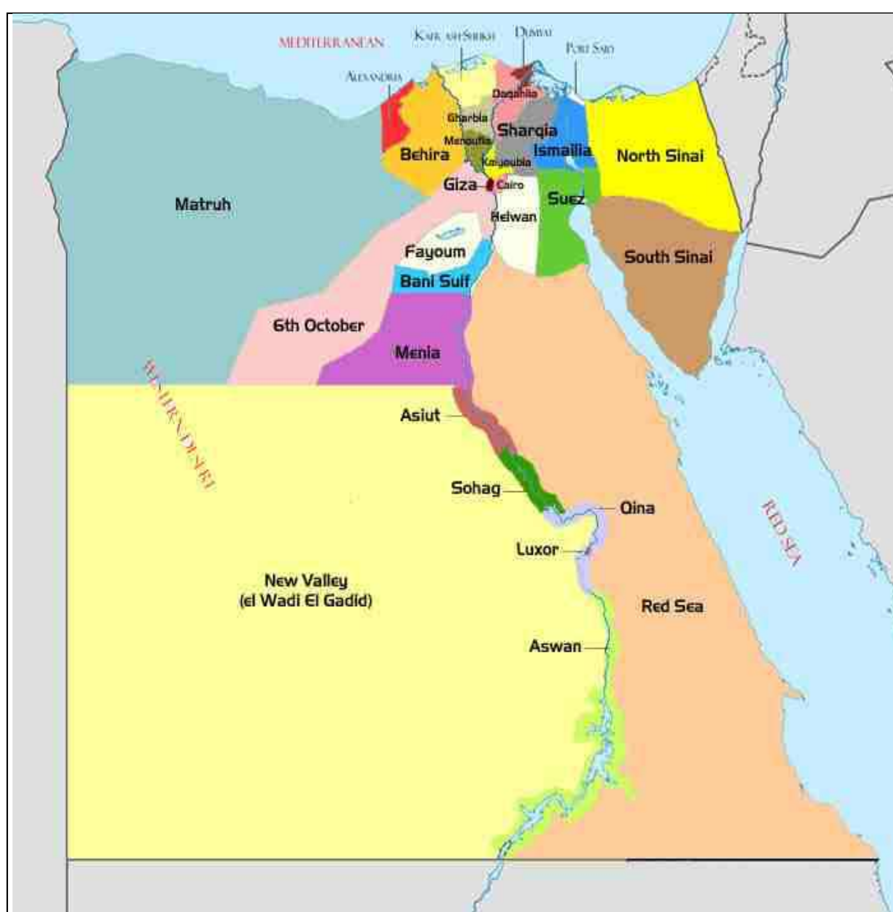

Figure 1. *Egyptian map showing the distribution of the governorates; the whitefly collection sites are Behira, Gharbia, Qalyubia, Dakahlia, Menoufia, Fayoum and Menia governorates.*

2.2. Insecticides

Six commercial insecticide formulations were used, namely: Neonicotinoids: Dinotefuran (MTI, 446, 4%SG), Imidacloprid (Confidor, 20%SL), Acetamiprid (Mospilan, 20% SP), Thiamethoxam (Actara, 25% WP).Insect growth regulators (IGR): Buprofezin (Applaud, 25% SC), Pyriproxyfen (Admiral, 100g/L).

2.3. Bioassays

Bioassay methods for obtaining concentration-mortality response lines were followed using a leaf-dip method according to Prabhaker *et al.* (1985) with some modifications. Cotton leave discs were dipped for 5 sec. in 100 ml of the desired concentration of each insecticide used and were air dried. Treated leaves were placed with their abaxial surface downwards onto a bed of thin layer of 2 % agar in small Petri dish placed in a small cage. Control groups were performed using diluents of insecticides only. Twenty adults were placed onto each leaf disc. Six concentrations were tested for each insecticide and there were five replicates per treatment. The dishes were inverted for the insects to orientate normally and placed in a large controlled environment room at 25±1°C, 50–60%RH and 16:8 hr light: dark, photoperiod.. Handling mortality was estimated within 1 hr. Mortality was recorded after 48 hr and was corrected according to Abbott's formula (1925). Data were analyzed for LC_{50} determination using Probit analysis according to Finney (1971). Resistance ratios (RR) for the collected strains were obtained by dividing their calculated LC_{50} values by that of the susceptible laboratory reference population. Resistance factors calculated in this way can represent both natural variations in tolerance as well as genetically determined resistance mechanisms; this is because of the spatial distribution of the tested governorate populations.

2.4. Enzymatic Assay

2.4.1. Preparation of Tissue Homogenates

Control and treated insects from all the governorates were subjected to measure the mixed function oxidase (MFO) activity. The insects were homogenized in distilled water (50mg/ml) using chilled glass Teflon homogenizer (ST-2 Mechanic- Preczyina, Poland). Homogenates were centrifuged at 8000rpm for 15 min. at 5°C in a cooling centrifuge. The supernatant was kept at -20° C till use.

2.4.2. Oxidase Activity

P-nitroanisole oxidative demethylation was assayed to determine the mixed function oxidase activity according to

the method of Hansen and Hodgson (1971) with slight modification. The standard incubation mixture contained 1 ml sodium phosphate buffer (0.1 M, pH 7.6), 1.5 ml enzyme solution. 0.2 ml NADPH (1 mM final concentration), 0.2 ml glucose-6-phosphate (G.6.P, final concentration, 1 mM) and 50 ug glucose-6-phosphate dehydrogenase (G-6PD). Reaction was initiated by the addition of p-nitroanisole in 10 ul aceton to give a final concentration of 0.8 mM and was incubated for 30 min. at 37° C. Incubation period was determined by addition of 1 ml HCl (1N). P-nitrophenol was extracted with CHCl3 and 0.5 N NaOH and absorbance of NaOH solution was measured at 405 nm using Double beam UV/ visible spectrophotometer (spectronic1201, Milton Roy Co., USA). An extinction coefficient of 14.28 mM-1cm-1 was used to calculate 4-nitrophenol concentration. The activity was expressed as nanomoles substrate oxidized/min./mg protein. There were three replicates per treatment. Means were analyzed by student's t-test (α=0.05).

2.5. RT-PCR and Direct Sequencing of Partial P450 Gene Transcript

2.5.1. RNA Extraction and cDNA Synthesis

Total RNA of the control, laboratory susceptible and treated insects from field collected resistant strains of *B. tabaci* was extracted using Wizard® Plus SV Total RNA isolation System. Fresh or frozen tissue (−80°C) was ground into a fine powder in liquid N_2 before the extraction buffer was added (1 mL of buffer to 1 g tissue). This method was rapid and produced a large amount of high quality and intact RNA. To judge the integrity and overall quality of a total RNA preparation, the total RNA extracted was examined on native agarose gel electrophoresis (1% agarose gel) and on denaturing formaldehyde/agarose gel (1.2% agarose in MOPS buffer) (Lehrach *et al.*, 1977). First strand cDNA was synthesized using RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas, cat # K1632). First strand cDNA was synthesized using random primer and 1 µg of total RNA.

2.5.2. Isolation of P450 Gene from the CYP6 Family using Degenerate Primers PCR-Strategy

cDNA region corresponding to P450 gene was amplified by PCR using two degenerate primers based on the conserved amino acid sequences of CYP4 and CYP6 families of P450 genes. (CYP4: Forward, 5'-TTY ATG TTY GAR GGI YAY GAY-3'; reverse 5'-AA YTT YTG ICC DAT RCA RTT-3', CYP6: forward 5'-TTY RCE YTI TAY GAR YTI GC-3'; reverse, 5'-RCA RTT YCK BGG ICC ITC ICC RAA-3', Bioneer). One step PCR kit (Biobasic, cat # 705, Canada) was used as the PCR reaction mixture. The amplification reaction was carried out using the following PCR reaction conditions: (1) an initial denaturation step at 95°C (3min); (2) 35 cycles of denaturation step at 95°C (30 sec); (3) primer-specific annealing temperature at 46°C (1 min); (4) primer extension at 72°C (1 min) and (5) final extension at 72°C (10min). PCR products were analyzed on 1% agarose gel in 1× Tris-borate EDTA (TBE) buffer (178 mM Tris base, 178 mM boric acid and 1 mM EDTA). PCR results were analyzed by Alphainnotech gel documentation system.

2.5. 3. Purification and Cloning of PCR Product

The PCR product was extracted and purified from the agarose gel with Wizard® Plus SV DNA purification and clean up system (Promega, U.S.A., cat#, A9281). The purity and integrity of the PCR product was verified after extraction and purified by agarose gel electrophoresis. The purified DNA fragments were subcloned into pGEM-T easy vector system, according to the manufacturer's instructions, then the(P450/pGEM-T) construct was introduced into the Mach1™- T1® *E. coli* chemically competent cells (Invitrogen, cat. No. ATCC#9637). Screening and selection of white/blue colonies was performed. The plasmids containing (P450/pGEM-T) construct were mini prepared using Wizard® Plus SV Mini Preps DNA Purification System (Promega, cat #A1340), according to the manufacturer's instructions.

2.6. DNA Sequencing and Phylogenetic Analyses

Sequencing was done using ready reaction Kit (Applied Bio-systems, USA) in conjunction with ABI-PRISM and ABI-PRISM big dye terminator cycler model 310 DNA automated sequencer. The sequences were provided by MACROGEN Company. The obtained sequences were analyzed for similarities to other known sequences found in the GenBank database using the BLAST program of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.,* 1997). Phylogenetic analysis was conducted using Mega 5 software in order to investigate evolutionary relationships among the putative P450 proteins identified in *B. tabaci* and other selected sequences (table 2) Multiple alignment of sequences was performed using the multiple alignment program CLUSTAL X (1.81) (Thompson *et al.*, 1997).Tree construction was performed by the neighbor-joining method, using PAUP* 4.0b10 (Swofford, 2002). The reliability of the trees was tested by the bootstrap procedure with 1000 replications.

3. Results

3.1. Bioassay

The seven *B. tabaci* strains from Behira, Gharbia, Dakahlia, Menofia, Kalyobia, Fayoum, and Menia governorates were tested for insecticide susceptibility against a laboratory susceptible strain using neonicotinoids: imidacloprid, dinotefuran, acetamiprid, thiamethoxam, insect growth regulators (IGR): buprofezin and pyriproxyfen through calculating their respective LC_{50} values (table 2).

Table 2. *Susceptibility of Laboratory and Field strains of adult Whitefly, B. tabaci to tested insecticides.*

Insecticide	Toxicity	Governorates							
		Lab	B	G	D	M	K	F	Mn
Imidacloprid	LC_{50}	0.8	2.1	1.1	1.4	0.9	1.3	1.8	0.7
	Slope	0.5	0.2	0.1	0.1	0.3	0.1	0.1	0.2
	(S.E.)	(0.1)	(1.3)	(1.6)	(1.4)	(1.6)	(1.5)	(1.6)	(1.9)
	R.R.	-	2.65	1.38	1.75	1.1	1.59	2.24	0.83
Dinotefuran	LC_{50}	1.0	2.4	1.1	0.9	1.0	2.0	0.8	2.1
	Slope	0.7	0.4	0.2	0.2	0.2	0.1	0.2	0.2
	(S.E.)	(0.3)	(1.5)	(1.6)	(1.4)	(1.6)	(1.7)	(1.4)	(1.3)
	R.R.	-	2.36	1.1	0.9	1.0	2.0	0.8	2.1
Acetamiprid	LC_{50}	0.7	1.3	0.7	0.8	1.0	1.5	1.1	0.5
	Slope	0.5	0.3	0.2	0.2	0.1	0.1	0.2	0.3
	(S.E.)	(0.9)	(1.4)	(1.3)	(1.5)	(1.5)	(1.3)	(1.3)	(1.3)
	R.R.	-	1.9	1.0	1.1	1.4	2.1	1.6	0.7
Thiamethoxam	LC_{50}	1.4	4.0	2.3	2.0	1.9	4.1	3.8	1.1
	Slope	1.4	0.2	0.3	0.1	0.3	0.2	0.3	0.2
	(S.E.)	(0.5)	(1.5)	(1.4)	(1.4)	(1.3)	(1.3)	(1.2)	(1.4)
	R.R.	-	2.9	1.6	1.4	1.4	2.9	2.7	0.8
Buprofezin	LC_{50}	3.5	10.0	8.5	12	6.7	16	15	12
	Slope	1.1	0.3	0.2	0.2	0.1	0.2	0.3	0.3
	(S.E.)	(0.3)	(1.3)	(1.3)	(1.3)	(1.2)	(1.1)	(1.4)	(1.3)
	R.R.	-	2.9	2.4	3.4	1.9	4.6	4.3	3.4
Pyreproxyfen	LC_{50}	4.0	122	102	66	87	77.5	56.1	42
	Slope	0.5	0.1	0.3	0.2	0.2	0.1	0.4	0.2
	(S.E.)	(0.1)	(1.4)	(1.2)	(1.4)	(1.2)	(1.2)	(1.3)	(1.3)
	R.R.	-	30.5	25.5	16.5	21.8	19.4	14.0	10.5

R.R. (Resistance Ratio) =LC_{50} of field strain/LC_{50} of lab strain.

Concerning susceptibility to the tested neonicotinoid insecticides and pyriproxyfen (IGR), the strain from Behira showed the highest resistance ratio in comparison to susceptible laboratory strain. The strain from Kalyobia showed the highest resistance ratio using buprofezin (IGR).

The strain from Menia was more susceptible to imidacloprid, acetamiprid, and thiamethoxam than laboratory strain. It was also more susceptible than the other strains but still higher than the control laboratory group when using pyriproxyfen insecticides. The strains from Fayoum and Dakahlia were also more susceptible to dinotefuran than laboratory strain.

Generally, when using IGRs in controlling the whitefly, B. tabaci in the previously mentioned governorates, buprofezin is recommended over pyriproxyfen due to its low LC_{50} values in all the tested strains. On the other hand, when using neonicotinoids, acetamiprid showed the higher potency to whiteflies in Behira, Gharbia, Dakahlia, and Menia governorates due to its low LC_{50} values in comparison with the other neonicotinoids.

3.2. Oxidase Assay

The activity of mixed function oxidase enzyme was assayed in laboratory and field strains collected from different localities at 7 governorates as an indication of resistance towards the insecticides applied at these areas that were falling into the category of neonicotinoids. The insects collected from Menofia, Gharbia, and Fayoum showed enzyme activity lower than that of susceptible lab strain (Fig. 2). On the other hand, the enzyme activity showed significantly higher values in insects collected from Behira, Menia, and Dakahlia (P≤0.05).

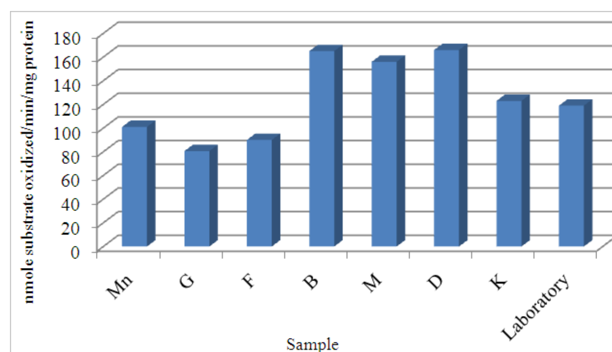

Figure 2. *Mixed function oxidase activity in Laboratory and Field strains of B. tabaci.*

3.3. RT-PCR and Sequencing of Partial P450 Gene Transcript

A fragment of (852 bp) was obtained using CYP4 and CYP6 degenerate primer. Sequencing and blasting of the cloned fragment was inserted against non-redundant nucleotide database of NCBI (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), which showed that the amplified sequence is corresponding to CYP6 family. PCR product yielded partial P450 sequence with 99% identity to B. tabaci CYP6CX1v1 cytochrome P450 sequence on the Genbank (accession number, D8V034).The product was obtained from both laboratory susceptible and field strains

collected from the previously mentioned seven governorates in Egypt.

3.4. Characterization of *B. Tabaci* CYP450 Partial cDNA and its Position in a Molecular Phylogeny

A PCR amplification approach using degenerate primers derived from conserved domains of insect P450 genes permitted cloning of the isolated 852 bp partial cDNA sequence for laboratory susceptible B. tabaci CYP6 that encodes a deduced 281-aa peptide with a molecular mass of approximately 31.817KDa and an estimated pI value of 9.75. The complete nucleotide sequence of *B. tabaci* P450 messenger RNA which is 2187 bp nucleotide long are deposited in GenBank, EMBL, DDBJ, PDB sequences database under accession number (EF675190.1).

Blastx comparisons and ClustalW alignment with other insect P450 further confirmed its identity which is also supported by the protein molecular mass and isoelectric point calculations.

The whole collected strains from the seven governorates were divided into two groups. First, the strains collected from Menofeia, Gharbeia and Menia shared the same sequence with laboratory susceptible strains that was used as control group (Fig. 3). All of these samples shared the same identity and sequence with *B. tabaci* CYP6CX1V1 on the Genbank. On the other hand, the second group represents samples collected from Dakahleia, Fayoum, Behira and Kalyobeia that showed differences in sequence from that of the control group. There were 25 negatively charged proteins (Asp, Gly) and 43 positively charged proteins in amino acid sequence composition of P450 from the second control group in comparison with 26 and 39, respectively for the first group.

Concerning those strains that showed differences in sequence from that of control, based on the high nucleotide similarity between the sequences from field resistant strains and that of control, it is likely that these four sequence variants may represent allele of the same gene. Nonetheless, the fragments clearly represent portions of CYP6 genes because of the amino acid sequence identity to members of the same gene family in other insects (Table 3), and their placement in the phylogenetic analysis within the CYP6 family (Fig. 4).

The phylogenetic analysis placed four of the fragments in one identical monophyletic group (Fayoum, Qalyubia, Dakahlia, Behira) and the other three fragments (Gharbia, Menoufia, Menia) together with control laboratory strain with CYP6CX1V1. Hence, the eight genes may be members of two different CYP6 subfamilies (Fig.3).

Homology analysis of amino acid sequence revealed that the protein encoded by B. tabaci CYP6 has the highest amino acid identity with CYP6 from the Formosan subterranean termite (36%), table (3).

Table 3. *Result of homology search of insect Cytochrome P450 molecule revealed by Genbank database. Sequences were chosen for multiple alignments with B. tabaci translated PCR amplified fragment.*

Entry	organism	Length	Id %	Score	E-value
R4UNB5_COPFO	Coptotermes formosanus (Formosan subterranean termite)	234	36	399	3.00E-43
A9QUS5_LIPBO	Liposcelis bostrychophila (Booklouse)	527	30	373	2.00E-37
N6SZ03_9CUCU	Dendroctonus ponderosae (mountain pine beetle)	530	29	371	5.00E-37
B0WVF0_CULQU	Culex quinquefasciatus (Southern house mosquito) (Culex pungens)	495	33	369	7.00E-37
Q16LY9_AEDAE	Aedes aegypti (Yellowfever mosquito) (Culex aegypti)	504	31	363	5.00E-36
F5CAR2_FRAOC	Frankliniella occidentalis (Western flower thrips)	509	32	359	2.00E-35
C6H0J3_NILLU	Nilaparvata lugens (Brown planthopper)	560	29	351	2.00E-34
E7CGB1_MYZPE	Myzus persicae (Green peach aphid) (Aphis persicae)	511	34	394	3.00E-40
D8V034_BEMTA	Bemisia tabaci Cytochrome P450 CYP6CX1v1	518	99	1436	0

The amino acid substitutions found between laboratory susceptible strain, used as control, and that of the field collected insecticide resistant strain (Behira) were presented in table (4). These substitutions may be the reason for the insensitivity of Cytochrome P450 to neonicotinoids application which is revealed from the high resistance ratios (Table 1). The same situation is found with similar sequence from strains of Dakahlia, Fayoum, Qalyubia.

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                 40         50         60         70         80         90        100
F          TGCAACCCCA ACTGAGCTAC TGGCGCCCGC GCGGTGTGCC CTACATCTCG CCGGTCAAGT GTTTTTCCTT
B          TGCAACCCCA ACTGAGCTAC TGGCGCCCGC GCGGTGTGCC CTACATCTCG CCGGTCAAGT GTTTTTCCTT
Q          TGCAACCCCA ACTGAGCTAC TGGCGCCCGC GCGGTGTGCC CTACATCTCG CCGGTCAAGT GTTTTTCCTT
D          TGCAACCCCA ACTGAGCTAC TGGCGCCCGC GCGGTGTGCC CTACATCTCG CCGGTCAAGT GTTTTTCCTT
CYP6CX1V1  TGCAAGACCA ACTGAGCTAC TGGCGCAAGC GCGGTGTGCC CTACATCTCG CCTATCAAGT GTTTTTCCTT
CONTROL    TGCAAGACCA ACTGAGCTAC TGGCGCAAGC GCGGTGTGCC CTACATCTCG CCTATCAAGT GTTTTTCCTT
M          TGCAAGACCA ACTGAGCTAC TGGCGCAAGC GCGGTGTGCC CTACATCTCG CCTATCAAGT GTTTTTCCTT
G          TGCAAGACCA ACTGAGCTAC TGGCGCAAGC GCGGTGTGCC CTACATCTCG CCTATCAAGT GTTTTTCCTT
Mn         TGCAAGACCA ACTGAGCTAC TGGCGCAAGC GCGGTGTGCC CTACATCTCG CCTATCAAGT GTTTTTCCTT
                110        120        130        140        150        160        170
F          CACGAGGGCC CAAACACTCC GGACGAAACC AGTGTTGGAG CGACGATAGA AATGTACGAG CTCCTGGAG
B          CACGAGGGCC CAAACACTCC GGACGAAACC AGTGTTGGAG CGACGATAGA AATGTACGAG CTCCTGGAG
Q          CACGAGGGCC CAAACACTCC GGACGAAACC AGTGTTGGAG CGACGATAGA AATGTACGAG CTCCTGGAG
D          CACGAGGGCC CAAACACTCC GGACGAAACC AGTGTTGGAG CGACGATAGA AATGTACGAG CTCCTGGAG
CYP6CX1V1  CACGATGGCC CAAACACTCC GGACGAAATC AGTGTTGGAG CGACGAAAGA AATGTACGAG CTCCTGGAG
CONTROL    CACGATGGCC CAAACACTCC GGACGAAATC AGTGTTGGAG CGACGAAAGA AATGTACGAG CTCCTGGAG
M          CACGATGGCC CAAACACTCC GGACGAAATC AGTGTTGGAG CGACGAAAGA AATGTACGAG CTCCTGGAG
G          CACGATGGCC CAAACACTCC GGACGAAATC AGTGTTGGAG CGACGAAAGA AATGTACGAG CTCCTGGAG
Mn         CACGATGGCC CAAACACTCC GGACGAAATC AGTGTTGGAG CGACGAAAGA AATGTACGAG CTCCTGGAG
                 180
F          CGACGATAGA
B          CGACGATAGA
Q          CGACGATAGA
D          CGACGATAGA
CYP6CX1V1  CGACGAAAGA
CONTROL    CGACGAAAGA
M          CGACGAAAGA
G          CGACGAAAGA
Mn         CGACGAAAGA
                 540        550        560        570        580        590        610
F          GGAGGGGGTG CCTTGGGTAT CCAGTGCTTC GCCATCAAAG ATCCGGATAA CTGCCAGATG TGCAGCAGAT
B          GGAGGGGGTG CCTTGGGTAT CCAGTGCTTC GCCATCAAAG ATCCGGATAA CTGCCAGATG TGCAGCAGAT
Q          GGAGGGGGTG CCTTGGGTAT CCAGTGCTTC GCCATCAAAG ATCCGGATAA CTGCCAGATG TGCAGCAGAT
D          GGAGGGGGTG CCTTGGGTAT CCAGTGCTTC GCCATCAAAG ATCCGGATAA CTGCCAGATG TGCAGCAGAT
CYP6CX1V1  GGAGCTTGTG CCTTGGGTAT CCAGTGCAAC GCCATCAAAG ATCCGGAAAA CTGTCAGATG TGCAGCAGAT
CONTROL    GGAGCTTGTG CCTTGGGTAT CCAGTGCAAC GCCATCAAAG ATCCGGAAAA CTGTCAGATG TGCAGCAGAT
M          GGAGCTTGTG CCTTGGGTAT CCAGTGCAAC GCCATCAAAG ATCCGGAAAA CTGTCAGATG TGCAGCAGAT
G          GGAGCTTGTG CCTTGGGTAT CCAGTGCAAC GCCATCAAAG ATCCGGAAAA CTGTCAGATG TGCAGCAGAT
Mn         GGAGCTTGTG CCTTGGGTAT CCAGTGCAAC GCCATCAAAG ATCCGGAAAA CTGTCAGATG TGCAGCAGAT
                  620        630        640        650        660        670        680
F          TTTTAGGCCC AATTGGAGGT TCTCCCTGCA GATCCTACTG GCAGCGATTC ATCCGAAGCT GGCTTTGCTC
B          TTTTAGGCCC AATTGGAGGT TCTCCCTGCA GATCCTACTG GCAGCGATTC ATCCGAAGCT GGCTTTGCTC
Q          TTTTAGGCCC AATTGGAGGT TCTCCCTGCA GATCCTACTG GCAGCGATTC ATCCGAAGCT GGCTTTGCTC
D          TTTTAGGCCC AATTGGAGGT TCTCCCTGCA GATCCTACTG GCAGCGATTC ATCCGAAGCT GGCTTTGCTC
CYP6CX1V1  TTTTAGGATC AATTGGAGGT TCTCCCTGCA GATCCTACTG TCAGCGATTC ATCCGAAGCT GGCTTTGCTC
CONTROL    TTTTAGGATC AATTGGAGGT TCTCCCTGCA GATCCTACTG TCAGCGATTC ATCCGAAGCT GGCTTTGCTC
M          TTTTAGGATC AATTGGAGGT TCTCCCTGCA GATCCTACTG TCAGCGATTC ATCCGAAGCT GGCTTTGCTC
G          TTTTAGGATC AATTGGAGGT TCTCCCTGCA GATCCTACTG TCAGCGATTC ATCCGAAGCT GGCTTTGCTC
Mn         TTTTAGGATC AATTGGAGGT TCTCCCTGCA GATCCTACTG TCAGCGATTC ATCCGAAGCT GGCTTTGCTC
                 690        700        710        720        730        740        750
F          CCCAGCTTGA GCAGAGGGAC TAGTGAAGTC ACAAAATTCC TGACTTCAGT AACCAAAGAA GCAATGGATG
B          CCCAGCTTGA GCAGAGGGAC TAGTGAAGTC ACAAAATTCC TGACTTCAGT AACCAAAGAA GCAATGGATG
Q          CCCAGCTTGA GCAGAGGGAC TAGTGAAGTC ACAAAATTCC TGACTTCAGT AACCAAAGAA GCAATGGATG
D          CCCAGCTTGA GCAGAGGGAC TAGTGAAGTC ACAAAATTCC TGACTTCAGT AACCAAAGAA GCAATGGATG
CYP6CX1V1  TTCAGCTTGA GCAGAGGGAC TAGTGAAGTG ACAAAATTCC TGACTTCAGT AACTAAAGAA GCAATGGATG
CONTROL    TTCAGCTTGA GCAGAGGGAC TAGTGAAGTG ACAAAATTCC TGACTTCAGT AACTAAAGAA GCAATGGATG
M          TTCAGCTTGA GCAGAGGGAC TAGTGAAGTG ACAAAATTCC TGACTTCAGT AACTAAAGAA GCAATGGATG
G          TTCAGCTTGA GCAGAGGGAC TAGTGAAGTG ACAAAATTCC TGACTTCAGT AACTAAAGAA GCAATGGATG
Mn         TTCAGCTTGA GCAGAGGGAC TAGTGAAGTG ACAAAATTCC TGACTTCAGT AACTAAAGAA GCAATGGATG

              760        770          780      790          800        810        820
F          TTAAGAAGAA AGCTGGATCGT GCCAGGAAA GATTTCCTGC AAACTATGAT GAGTGCTGGT GATACCGAGA
B          TTAAGAAGAA AGCTGGATCGT GCCAGGAAA GATTTCCTGC AAACTATGAT GAGTGCTGGT GATACCGAGA
Q          TTAAGAAGAA AGCTGGATCGT GCCAGGAAA GATTTCCTGC AAACTATGAT GAGTGCTGGT GATACCGAGA
D          TTAAGAAGAA AGCTGGATCGT GCCAGGAAA GATTTCCTGC AAACTATGAT GAGTGCTGGT GATACCGAGA
CYP6CX1V1  TTAAGAAGAA AGCTGGATCGT -CCAGGAAA GATTTCCTTC AAATTATGAT GAGTGCTGGT GATACCGAGA
CONTROL    TTAAGAAGAA AGCTGGATCGT -CCAGGAAA GATTTCCTTC AAATTATGAT GAGTGCTGGT GATACCGAGA
M          TTAAGAAGAA AGCTGGATCGT -CCAGGAAA GATTTCCTTC AAATTATGAT GAGTGCTGGT GATACCGAGA
G          TTAAGAAGAA AGCTGGATCGT -CCAGGAAA GATTTCCTTC AAATTATGAT GAGTGCTGGT GATACCGAGA
Mn         TTAAGAAGAA AGCTGGATCGT -CCAGGAAA GATTTCCTTC AAATTATGAT GAGTGCTGGT GATACCGAGA
                830
F          TTGAGAAGAG C
B          TTGAGAAGAG C
Q          TTGAGAAGAG C
D          TTGAGAAGAG C
CYP6CX1V1  TTGAGAAGAG G
CONTROL    TTGAGAAGAG G
M          TTGAGAAGAG G
G          TTGAGAAGAG G
Mn         TTGAGAAGAG G
```

Figure 3. *Multiple sequence alignment to neucleotide sequence of laboratory and field strains of B. tabaci Cytochrome P450 isolated fragment with sequence from genbank. Nucleotide variation sites only were shown and highlighted.*

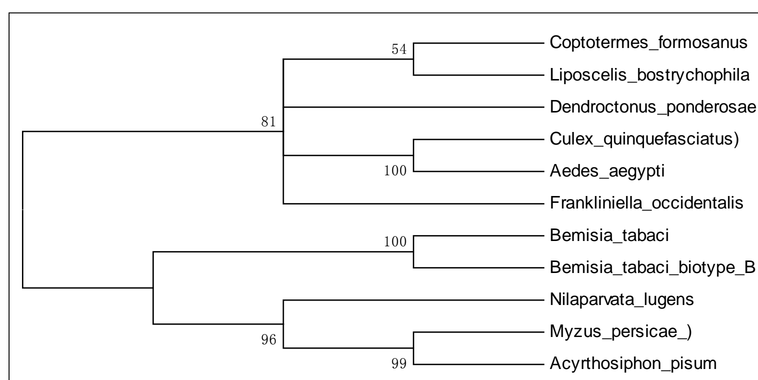

Figure 4. *Neighbour-joining phylogeny of B. tabaci (Bt) P450s deduced amino acid sequences and selected P450s. Bootstrap values next to nodes represent the percentage of 1000 replicate trees that preserved the corresponding clade. GenBank accession numbers of CYP450s are listed in Table 3.*

Table 4. *Amino acid substitution in resistant and susceptible B. tabaci strains.*

Amino acid position	Control(susceptible)	Collected(resistant)
12	K	N
13	T	P
20	S	R
28	L	R
36	W	G
179	L	G
187	T	S
193	K	I
195	V	A
207,228	S	P
217	C	W
245	L	P
258	P	A
259	G	R
261,271	I	D
262	S	F
263	F	L
264	K	Q
265	L	T
268	V	S
269	L	A
270	V	G
271	I	D
272	P	T
273	R	E
274	L	I
275	R	E
276	R	K
277	G	S
280	E	V

4. Discussion

This study showed that *B. tabaci* biotype B is prevalent in all of the surveyed sites and there was variation in insecticide resistance in *B. tabaci* collected in Egypt. The population collected from Menia governorate was the most susceptible against almost all insecticides among the Egyptian populations. The ranking of the populations according to the exposure to insecticides was the same as that observed in the level of resistance with the insecticides.

In Beheira populations, a significant correlation of the resistance toward the tested neonicotinoids and pyreproxyfen, but not buprofezin was found.

In Menia and Beheira, the largest area in the selected governorates, the lowest and highest resistance was exhibited against neonicotinoids by the population (RR = 0.7, 2.9, respectively). The latter resistance level was approximately 4 times higher than that of the other population, indicating a strong selection against neonicotinoids when moving towards Upper Egypt.

No major problems with efficacy of neonicotinoids against *B. tabaci* since most of the samples were susceptible to slightly resistant (RR≤2.5) which doesn't impair field performance against whiteflies. Susceptibility of field collected whiteflies to the used neonicotinoids remained high and unchanged along the whole tested Egyptian governorates.

Concerning use of insect growth regulators, buprofezin is recommended over pyreproxyfen in controlling whiteflies. This is attributed to the high resistance ratio of the whole collected populations towards the latter.

B. tabaci colonizes a wide range of wild host plant species (Calvitti and Remotti, 1998, Attique *et al.* 2003) and gene flow between treated and untreated populations may be the origin of discrepancies between insecticide use and level of resistance. Insecticide resistance is mainly based on mutations of the target gene decreasing the affinity to the respective insecticide and on increased detoxification by acetylcholine esterases, cytochrome P450-dependant monooxygenases or glutathione *S*-transferases .

In *B. tabaci*, resistance to neonicotinoids involves, in addition to the insensitivity of the synapse acetylcholinesterase (Byrne and Devonshire, 1997, Anthony *et al.* 1998) the enhanced detoxification of the insecticides by non-specific esterases and cytochrome P450-dependant monooxygenases (Dittrich et al. 1990, Bloch and Wool, 1994).

Overexpression of cytochrome P450-dependant monooxygenases was also found to be responsible for the resistance of *B. tabaci* to neonicotinoids (Rauch and Nauen, 2003). Detoxification by esterases and cytochrome P450-dependant monooxygenases has also been implicated in pyrethroid resistance in *B tabaci*. (Byrne *et al*. 2000, Shchukin and Wool, 1994).

Resistance against the insecticide, imidacloprid, has been reported by Rauch and Nauen, 2003 for populations collected from Spain, Germany and Israel.

The current status of *B. tabaci* resistance to neonicotinoid insecticides requires urgent attention. Pest management in the greenhouse crops heavily relies on chemical control, and particularly on neonicotinoids. An average of 30 insecticide applications per crop season does not allow the design of a rational insecticide rotation scheme. The presence of highly susceptible *B. tabaci* pool in wild host plants could possibly be a major advantage regarding the implementation of insecticide resistance management in the area.

Development of resistance depends in part on the occurrence of 'refugia' for susceptible populations (Georghiou, 1994) and on gene flow among populations colonizing different habitats in a given area. The biotype status as well as genetic exchanges between *B. tabaci* populations collected on different plant species and on greenhouse or outdoor crops are currently been examined using microsatellite markers. 40 Studies on the genetic structure of the *B. tabaci* population together with the identification of resistance genes will allow a better understanding of the evolution of resistance. The development of a resistance management scheme is being considerate through the area-wide implementation of integrated pest management.

The impact of metabolizing enzymes such as esterases, glutathione S-transferases, and cytochrome P450-dependent monooxygenases in neonicotinoid resistance was studied biochemically with artificial substrates. Monooxygenase activity was increased 2–3-fold in moderately resistant strains (RF ~30) and even 5–6-fold in highly resistant strains (RF ~1,000). Only monooxygenase activity correlated with imidacloprid, thiamethoxam and acetamiprid resistance and, therefore, monooxygenases seem to be the only enzyme system responsible for neonicotinoid resistance in B. tabaci Q- and B-types (Rauch and Nein, 2003).

These results are encouraging for the farmers' point of view in the sense that they can use all these insecticides to control the pest. But despite of these, the sole dependency on neonicotinoids should be strictly avoided because both imidacloprid and acetamiprid have similar mode of action and possess strong cross resistance. Diafenthiuron is a relatively newer entry in the market and present studies indicate absence of its cross resistance with neonicotinoids and endosulfan. Therefore it may successfully be used in the whitefly spray program in order to reduce the frequency of neonicotinoids application.

Overall, our results show a direct causal relationship between the nucleotide variations detected in CYP450 gene of this species and qualitative alterations of the activity of MFO enzymes as the basis for variation in resistance between the collected samples. Our results also provide a basis for further comparisons of insecticide resistance phenomena seen in other species and different biotypes.

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