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Association of cytochrome P450 enzyme with reduced susceptibility to neonicotinoids in *Bemisia tabaci* (Gennadius) populations from India

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Abstract
Neonicotinoid is the major class of insecticides used for management of sucking pests in cotton. The present investigation was done to know the susceptibility level of *Bemisia tabaci* to neonicotinoids and its association with detoxifying enzyme, cytochrome P450. In the present study five populations of *B. tabaci* were collected representing two regions/zones of country *viz.,* North (Delhi, Punjab and Rajasthan) and Central (Madhya Pradesh and Maharashtra) with one location from each state. The locations were New Delhi, Sriganganagar (RJ), Ludhiana (PJ), Khandwa (MP), and Amravati (MH). The populations were collected and bioassayed with neonicotinoids two consecutive years (2013-14 and 2014-15) and their cytochrome P450 levels were quantified. The populations from Sriganganagar, Ludhiana and Amravati showed highly reduced susceptibility with LC_{50} values ranging from 1199 to 1874 mg/lit during 2013-14 and 1315 to 1717 mg/lit during 2014-15 for all three neonicotinoids tested whereas, Khandwa showed moderate level of tolerance with LC_{50} values 717 to 1012 mg/lit and 854 to 973 mg/lit during first and second year of evaluation respectively. New Delhi populations showed low level of tolerance with LC_{50} values below 400 mg/lit and 500 mg/lit during first and second year of evaluation respectively. A strong correlation between bioassay results and cytochrome P450 level was observed. The populations were compared with laboratory susceptible strain collected from wild host, *Leucaena leucocephala*. All the five populations belong to Asia II 1 genetic group whereas lab susceptible strain belongs to Asia II 7 genetic group. Cytochrome P450 is the key enzyme involved in neonicotinoid detoxification and using synergists that inhibit the P450 activity and field efficacy of this group may be enhanced.

Keywords: *Bemisia tabaci*, cytochrome P450, enzymatic detoxification, insecticide, neonicotinoids, susceptibility

1. Introduction

Cotton is a major commercial crop of global significance being grown in more than 70 countries of the world. India is the largest cotton growing country with an area of 11.20 m ha with a production of 35.00 million bales of seed cotton during 2016 [1]. But the average productivity of the country is only 540 kg lint/ha which is far below the world average of 766 kg lint/ha [2]. Pest scenario in cotton has been changing over the past few decades, which is being the major constraint in cotton production system. After introduction of Bt cotton in India during 2002, cotton crop has witnessed significant changes in its pest scenario. Among the sucking pests whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is considered to be most devastating pest. *B. tabaci* is a phloem-feeding insect causing severe crop losses both directly by feeding on plant sap and indirectly by excretion of honeydew that favours sooty mould development and transmission of plant viruses [3]. Due to severity of damage caused by *B. tabaci*, it has been controlled predominantly with chemical insecticides. However, as a result of extensive application of synthetic insecticides, *B. tabaci* has developed a high degree of resistance to a wide range of insecticides, including carbamates, organophosphates, pyrethroids, insect growth regulators (IGRs), and neonicotinoids [4-13]. Whiteflies are assuming serious proportions causing widespread damage in Northern cotton growing zone of the country [14]. As a result the crop has been subjected to maximum pesticide exposure resulting in insect resistance to insecticides, pesticide residues, and resurgence of minor pests and caused immense problems to cultivators [7-14].

Evolution of resistance to insecticides by insect pests is a problem of global concern [15]. Insecticide resistance is mainly due to three basic mechanisms *viz*., decreased penetration of insecticides into insect system, enhanced detoxification of insecticides, and target site insensitivity. Among these, detoxifying enzymes play an important role in the development of resistance. Cytochrome P450 monooxygenases play a key role in conferring neonicotinoid resistance [16]. Currently neonicotinoids are being widely used for whitefly management in cotton ecosystem. Hence the present investigation was conducted to study the status and level susceptibility in various populations and role of monooxygenases in the development of neonicotinoid tolerance in *Bemisia tabaci* species complex on cotton.

2. Materials and Methods

2.1 Origin and maintenance of *Bemisia tabaci* population

The populations of *B. tabaci* were collected from cotton crop of five different locations falling under two major cotton growing regions of India [North region: Sriganganagar (Rajasthan), Ludhiana (Punjab) and Delhi; Central region; Khandwa (Madhya Pradesh) and Amravati (Maharashtra)]. The collections were made in august-september months in 2013 and 2014. While collecting, Zee walk mode was followed. Adult whiteflies were collected early in the morning using an aspirator; green leaves were plucked to collect immature stages. The insects were transferred in ventilated cages containing leaflets inserted into wet sponges. Collected populations were kept in rearing cages for the emergence of fresh adults and the 'puparia' were sampled for valid species authentication [17]. Laboratory susceptible population of *B. tabaci* was collected from, *Leucaena leucocephala*, a wild host of *B. tabaci*. As this population was collected from the insecticide free environment in the field so it is reared in laboratory to designate as susceptible population. The respective populations were raised in insecticide free exposure conditions on cotton (*Gossypium hirsutum* L) seedlings, at 27±2ºC temperature, 14:10 h (L:D) photoperiod and 60-70% relative humidity in insect rearing chamber at Division of Entomology, ICAR-IARI, New Delhi.

2.2 Insecticide bioassay

The required amount of technical grade insecticides [imidacloprid (98%), thiamethoxam (98%) and acetamiprid (96.5%)] were weighed (w/w), diluted in acetone and subsequently, different concentrations of insecticides were prepared by serial dilution in deionized water containing 0.1% of non-ionic wetting agent, triton x-100. The experiments were repeated two years consecutively to know the status of neonicotinoid susceptibility in *B. tabaci*. Bioassays were done through the leaf dip method with slight modification [18]. Cotton leaves with petiole from seven to fifteen days old seedlings were immersed in serially diluted solutions for 20 sec, and allowed to air dry on paper towel, and these were placed after inserting the petiole in agar slant (2%) in petri plate (90mm x 15mm), leaves dipped in emulsifier water were used as control. The adults were momentarily anaesthetized using CO_2 and were transferred in batches of 20-30 on to the treated leaves, the plates were sealed with ventilated lids. All such assays were replicated three times for each concentration, a minimum of five concentrations for each insecticide were used for bioassay. Mortality observations were recorded at 24 hours after treatment and adults showing no sign of movement were scored as dead. Mortality data for all five populations and laboratory susceptible strain against all three neonicotinoids were recorded for further calculation of LC_{50} value. Resistance ratio (RR) was calculated as given below [9].

$$\text{Resistance ratio} = \frac{LC_{50} \text{ value of particular location population}}{LC_{50} \text{ value of lab susceptible population}}$$

2.3 Cytochrome P450 (general oxidase) assay

Cytochrome P450 activity was quantified and expressed in terms of general oxidase level, which is an indirect measure of cytochrome P450 by using heme peroxidation method which is considered as a reliable tool for comparing differences in general oxidase levels based on hemeprotein levels. In this heme peroxidation TMBZ was used as a substrate and H_2O_2 as co-substrate. With the presence of H_2O_2 the microsomal oxidases use the TMBZ and develops two oxidized TMBZ molecules [19-20].

For general oxidase assays, five flies were separated in three replications and homogenates were prepared using a hand held homogenizer with a plastic pestle in 50 µl ice-cold potassium phosphate buffer (0.625 M, pH 7.2), containing 0.1% (w/v) Triton X-100. The reaction mixture consisted of 80 µl of 0.625 M potassium phosphate buffer (pH 7.2), 20 µl of enzyme source (homogenate), 200 µl TMBZ solution, 25 µl of H_2O_2 (3.0%) and giving a final volume of 325 µl. The substrate solution was prepared by dissolving 2 mg of TMBZ in 2.5 ml of methanol and 7.5 ml of 0.25 M sodium acetate buffer (pH 5.0). Absorbance was read at 620 nm against blanks (wells containing all reaction components, except the enzyme source) in a GEN5 absorbance microplate reader (Molecular Devices) after 5 min. of incubation. A standard curve for heme peroxidase activity was prepared using different concentrations of cytochrome C. The activity of Cytochrome P450 (general oxidase) obtained from plate reading was expressed as nano moles of cytochrome P450 per milligram of protein by using the standard curve of cytochrome C.

2.4 Protein estimation

The total Protein content of homogenate was determined using the Coomassie brilliant blue G-250 dye (CBBG) method [21] with bovine serum albumin (BSA) as the standard. Absorbance was recorded at 595 nm.

2.5 Genomic DNA extraction and mtCOI gene amplification

Genomic DNA was extracted from single *B. tabaci* adult using DNeasy blood and tissue kit (*Qiagen GmbH, Germany*) according to manufacturer's protocol. The mtCOI region was amplified using forward primer CI–J–2195 (3'–TTGATTTTTTGGTCATCCAGAAGT–5') in combination with a reverse primer TL2–N–3014 (3'–TCCAATGCACTAATCTGCCATATTA–5') [22-23]. The PCR mixtures consisted of 10 pmol of primer with 1 µl each, 12.5 µl of mastermix, 8.5 µl of nuclease free water and 2 µl of template DNA from individual insect. The PCR cycling conditions were initial denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing for 40 sec at 54ºC and extension of 40 sec at 72ºC and followed by final extension at 72ºC for 5 min in a thermal cycler (Biorad, Germany). The amplified products were resolved by electrophoresis using 1% agarose gel with a 1X TAE buffer in a submerged electrophoresis system (Biorad, USA) at 70v; stained with ethidium bromide (1ug/ml) and

visualized in a gel documentation system (UVP, UK). The PCR products were purified and sequenced [24].

2.6 Data analysis
Bioassay data were analysed to calculate the LC_{50} values using log–dose probit analysis [25] using PoloPlus 2.0 (LeOra Software, Petaluma, CA). Resistance ratios (RR) were computed at a given response level (LC_{50}) to test the folds of resistance to the insecticides in the evaluated populations compared with laboratory susceptible strain. Activities of general oxidase among the different populations were analyzed using analysis of variance (ANOVA) and differences between treatments were tested using Tukey's test ($P<0.05$).For identification of genetic group, mtCOI sequence obtained from each population was subjected to homology search using *Basic Local Alignment Search Tool* (nBLAST) algorithm at NCBI (http://www.ncbi.nlm.nih.gov).

3. Results
3.1 Insecticide bioassay
The results of bioassays revealed that all populations collected from five different locations showed considerably lower susceptibility to neonicotinoids when compared with laboratory susceptible strain. Based on LC_{50} values a high degree of tolerance was observed in populations collected from Sriganganagar (RJ), Ludhiana (PJ) and Amravati (MH) for both the consecutive years. The populations collected from Khandwa (MP) region showed moderate level of tolerance, whereas populations from New Delhi showed moderate to low level of tolerance when compared with other four populations. Based on the available results, the populations were categorized as highly tolerant (>1000 folds), moderately tolerant (500-1000 folds) and less tolerant (<500 folds).

Results of dose-response relationship of *B. tabaci* populations collected from various locations for the first year (2013-14) had been presented in Table 1. During the first year of study imidacloprid showed highest value of LC_{50} (1462 mg/l) for Amravati population followed by populations from Sriganganagar (1404 mg/l) and Ludhiana (1197 mg/l). Laboratory susceptible population of *B. tabaci* showed 3 mg/lit median lethal value for imidacloprid whereas New Delhi and Khandwa population were having 279 and 783 mg/lit LC_{50} values respectively. For acetamiprid, populations from Ludhiana and Sriganganagar gave higher LC_{50} values i.e., 1502 and 1540 mg/l respectively, whereas Amravati population showed value of 1272 mg/l. Again New Delhi and Khandwa population showed lower LC_{50} value for acetamiprid. For thiamethoxam, Ludhiana population registered lowest susceptibility with highest LC_{50} value (1874 mg/l). Sriganganagar and Amravati populations recorded LC_{50} values of 1233 and 1199 mg/l. Whereas Khandwa population showed lethal value of 1012 mg/lit.

Similar trend was observed during the second year of study (2014-15) for which the probit estimates are presented in Table 2. For the second year Ludhiana population recorded highest LC_{50} value of 1717 mg/l for imidacloprid followed by Sriganganagar (1610 mg/l) and Amravati (1315 mg/l). For acetamiprid Sriganganagar and Amravati populations recorded high LC_{50} value of 1702 mg/l and 1597 mg/l, respectively. Whereas, LC_{50} value for Ludhiana population was recorded to be 1430 mg/l. In case of thiamethoxam Ludhiana population recorded highest LC_{50} value of 1589 mg/l followed by Amravati and Sriganganagar with the LC_{50} values of 1497 mg/l and 1438 mg/l, respectively.

Populations from Khandwa showed moderate level of tolerance with the LC_{50} values of 783 mg/l, 717 mg/l, 1012 mg/l for first year 2013-14 and 973 mg/l 854 mg/l 925 mg/l for second year 2014-15 for imidacloprid, acetamiprid and thiamethoxam respectively. Whereas, New Delhi population showed moderate to low level of tolerance to all the three neonicotinoids, tested probit estimates are presented in Tables 1 and 2.

Table 1: Toxicity of neonicotinoids against field populations of *Bemisia tabaci* species complex during 2013-14.

Population	DF	Slope ±SE	χ^2	LC_{50} value mg/l (CI 95%)	Fiducial limit (mg/l)	RT
Imidacloprid						
New Delhi	3	1.969±0.179	0.276	279	231 to 333	93
Ludhiana	3	1.243±0.174	1.885	1197	858 to 1922	397.66
Sriganganagar	3	1.150±0.167	5.380	1404	704 to 9350	468
Amravati	3	1.074±0.159	1.828	1462	1068 to 2271	487.33
Khandwa	3	1.427±0.231	3.490	783	432 to 1932	261
Lab susceptible	3	1.604±0.239	2.236	3	2.3-3.8	1
Acetamiprid						
New Delhi	3	1.235±0.143	3.246	342	185 to 594	85.5
Ludhiana	3	1.307±0.140	1.841	1502	1178 to 2080	375.5
Sriganganagar	3	1.477±0.163	3.163	1540	1025 to 2948	385
Amravati	3	1.374±0.204	3.760	1272	741 to 3708	318
Khandwa	4	1.012±0.183	0.867	717	440 to 1412	179.25
Lab susceptible	3	1.573±0.228	2.544	4	3.1-5.1	1
Thiamethoxam						
New Delhi	3	1.054±0.189	1.013	382	255 to 586	127.33
Ludhiana	3	1.331±0.176	5.172	1874	1021 to 8969	624.66
Sriganganagar	3	1.247±0.142	4.953	1233	727 to 3185	411
Amravati	3	1.565±0.162	2.192	1199	952 to 1607	399.66
Khandwa	3	1.088±0.226	1.826	1012	638 to 2085	337.33
Lab susceptible	3	1.77±0.245	1.577	3	2.4-3.8	1

RT- Relative tolerance

Table 2: Toxicity of neonicotinoids against field populations of *Bemisia tabaci* species complex during 2014-15.

Population	DF	Slope ±SE	χ^2	LC_{50} value mg/l (CI 95%)	Fiducial limit (mg/l)	RT
Imidacloprid						
New Delhi	3	2.39±0.435	0.522	488	394-580	162.66
Ludhiana	4	2.039±0.297	1.286	1717	1414-1811	572.33
Sriganganagar	4	2.079±0.315	2.93	1610	1304-2189	536.67
Amravati	4	1.97±0.276	2.817	1315	1081-1717	438.33
Khandwa	4	1.847±0.288	1.356	973	801-1207	324.33
Lab susceptible	3	1.604±0.239	2.236	3	2.3-3.8	1
Acetamiprid						
New Delhi	3	2.29±0.399	0.651	466	377-553	116.5
Ludhiana	4	2.101±0.301	1.849	1430	1183-1862	357.5
Sriganganagar	4	2.021±0.280	1.675	1702	1402-2240	425.5
Amravati	4	2.051±0.332	1.336	1597	1300-2188	399.25
Khandwa	4	1.937±0.284	1.98	854	707-1037	213.5
Lab susceptible	3	1.573±0.228	2.544	4	3.1-5.1	1
Thiamethoxam						
New Delhi	3	2.384±0.38	0.457	486	401-571	162
Ludhiana	4	2.047±0.335	2.867	1589	1291-2192	529.67
Sriganganagar	4	2.137±0.316	1.75	1438	1206-1840	479.33
Amravati	4	1.934±0.297	1.94	1497	1228-1985	499
Khandwa	4	1.968±0.293	1.177	925	762-1126	308.33
Lab susceptible	3	1.771±0.245	1.577	3	2.4-3.8	1

RT- Relative tolerance

3.2 Variations in cytochrome P450 monooxygenases level in populations of *Bemisia tabaci*

Results of cytochrome levels in collected populations of *B. tabaci* showed (Table 3) that Amravati population had maximum enzyme activity with cytochrome value of 595.19 nmol/mg of protein for 2013-14 followed by populations from Sriganganagar (494.20 nmol/mg of protein) and Ludhiana (482.02 nmol/mg of protein). Comparatively lower activity was recorded in New Delhi (289.26 nmol/mg of protein) and Khandwa population (404.16 nmol/mg of protein). Relative activity was 5-7 times in Ludhiana, Sriganganagar and Amravati populations as compared to New Delhi population in both the consecutive years. Similar trend of cytochrome P450 activity was observed in the second year (2014-15) as well for the populations from the same locations (Table 3).

3.3 Identification of genetic groups of *B. tabaci* populations

All the populations *viz*., Sriganganagar (RJ), Ludhiana (PJ), Khandwa (MP), Amravati (MH) and Delhi found to cluster with Asia II 1 genetic group, whereas laboratory susceptible population collected from wild host, *Leucaena leucocephala* was identified to be Asia II 7 genetic group.

Table 3: Cytochrome P450 values in of different field collected populations of *Bemisia tabaci* species complex.

Population	2013-14		2014-15	
	Cytochrome P450 Monooxygenase (nmol/mg of protein ±SE)	Relative activity	Cytochrome P450 Monooxygenase (nmol/mg of protein ±SE)	Relative activity
New Delhi	289.26±33.93^{ab}	3.42	360.50±83.27^{abc}	4.26
Ludhiana	482.02±12.84^{bc}	5.70	465.48±68.93^{bc}	5.51
Sriganganagar	494.20±98.03^{bc}	5.85	503.21±82.87^{bc}	5.95
Amravati	595.19±95.65^{bc}	7.04	669.27±71.27^{c}	7.92
Khandwa	404.16±68.83^{abc}	4.78	393.70±35.28^{abc}	4.66
Lab susceptible	84.54±21.57^{a}	1.00	84.54±21.57^{a}	1.00

Values in the table followed by the same letters are not significantly different at P= 0.05 after Tukey's HSD (honest significant difference) test.

4. Discussion

The present study gives the susceptibility variations in *B. tabaci* populations to neonicotinoids from India and its correlation with metabolic enzyme like cytochrome P450. In Indian context neonicotinoids as a major group used for management of *B. tabaci*, high level of tolerance to neonicotinoids has been found in the present study. Similar studies on *B. tabaci* in southern Spain have shown that extensive use of neonicotinoids has lead to selection of resistant phenotypes. Extensive use of these novel products resulted in excessive pressure on the product in the region [7, 16, 26-27]. The cases of resistance have been documented from field populations of *B. tabaci* from China [28]. Similarly, reports are available for exhibiting more than 100 fold resistance to imidacloprid and comparable resistance to acetamiprid and thiamethoxam [16, 29]. High level of cross resistance to imidacloprid, acetamiprid and thiamethoxam has also been detected from Italy and Germany [16, 27, 30]. Reduced susceptibility of whiteflies to imidacloprid and other neonicotinoids have consequently been reported from other countries of world viz., Australia, Brazil, Crete, Germany, Israel, Italy, Mexico, Guatemala, Arizona and Morocco [31-34]. High levels of resistance were observed in Q biotype of *B. tabaci* to neonicotinoids around the world [34-37]. It has been observed that variations in the susceptibility to insecticide may be due to the type and frequency of insecticide usage on a particular location, cropping pattern and other management practices adapted against insect control. Similar study was conducted by Prabhakar [38] on imidacloprid resistance in *B. tabaci* collected from melon field under continuous selection pressure shows that variation in susceptibility was due to geographical differences as well as field level selection pressure existing among the populations.

It is evident from the above results that the populations having

relatively higher tolerance to neonicotinoids also show enhanced levels of cytochrome. This may be due to extensive use of neonicotinoid group of insecticides under field conditions for whitefly management in cotton. In India *Bt* cotton seeds are normally prior treated with imidacloprid which acts as impetus for development of tolerance to imidacloprid, this coupled with foliar sprays of imidacloprid and related neonicotinoids for management of *B. tabaci* under field conditions, resulting in selection of resistant individuals. Studies on neonicotinoid resistance in *B. tabaci* have shown that overexpression of monooxygenases was the key in detoxification and no evidence for target-site resistance has been found in whiteflies [7, 16]. Pre-exposure of whiteflies to synergists like piperonyl butoxide that inhibit monooxygenases intern increased the toxicity of imidacloprid against a resistant strain, suggesting an involvement of cytochrome P-450 dependent monooxygenases in conferring resistance [7, 16]. Present findings are supported by the work of Prabhakar [39] that suggests selection pressure by the systemic insecticides towards resistance build up is much higher when used as foliar spray due to longer residual activity, exposure of all stages of a pest as well as survival at sublethal dosages leading to selection for resistance over time. Differences in ecological factors and management practices among the various regions from which whiteflies originated may explain some of the observed variations in pattern of dynamics and development of relative tolerance to neonicotinoids. In addition commonality of chemical structure-relationships or mode of action within a class of insecticide chemistry could influence the resistance development in the pest [40, 41]. Imidacloprid resistance in *B. tabaci* populations collected from glasshouse conferred by enhanced oxidative detoxification by over expression of CYP6CM1 gene for the monooxygenase [42]. Similar study on field population also confirmed that resistance to imidacloprid in a *B. tabaci* conferred by CYP6CX1 gene responsible for overexpression of cytochrome P450 monooxygenase [43]. Neonicotinoids resistance in *B. tabaci* linked with an enhanced detoxification by monooxygenases is also documented by many researchers [44-46]. *B. tabaci* populations collected from Egypt were evaluated for their tolerance to neonicotinoids and also compared with laboratory strain. A close correlation between neonicotinoid susceptibility and cytochrome activity was found in the study [47]. Among the various factors contributing to insecticide susceptibility variations, the detoxifying enzymes play a major role. Over production of detoxifying enzymes coupled with prevalence of target site insensitivity in field populations could be responsible for tolerance to insecticides. Cytochrome P450 monooxygenases are the major enzymes responsible for neonicotinoid detoxification providing resistance to this group of insecticides.

5. Conclusion

The Present investigation gives an insight into the status of neonicotinoid susceptibility in Indian whitefly populations. High degree of tolerance to neonicotinoid insecticides in populations from Amaravati, Sriganganagar and Ludhiana can be attributed to the increased levels of monooxygenase, suggesting the involvement of cytochrome P450 in imparting neonicotinoid tolerance. As neonicotinoids represent quarter share in world insecticide usage representing the quantum of selection pressure operating under field conditions. Proper understanding of this mechanism will set novel targets for pest management which will be helpful in overcoming insecticide resistance. 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.

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