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RELATIVE TOXICITYOF FIPRONIL AND FLONICAMID AGAINST *BEMISIA TABACI* (GENNADIUS) ON COTTON

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ABSTRACT

The cotton whitefly *Bemisia tabaci* (Gennadius) is the most destructive sucking pest of many field and protected crops. It has developed resistance against almost all insecticides including neonicotinoids. This study evaluates fipronil and flonicamid against *B. tabaci* on cotton and relative detoxification enzyme system is quantified. The populations maintained in laboratory conditions on cotton collected from Sriganganagar (Rajasthan), Ludhiana (Punjab), Khandwa (Madhya Pradesh), Amravati (Maharashtra) and Delhi are included. Of these, the New Delhi showed a high degree of toxicity followed by the Khandwa, Ludhiana, Sriganganagar and Amravati ones. Fipronil was more effective than flonicamid. The CYP450 monooxygenases levels were found to be higher in Amravati population (793.70 nmol/mg of protein) followed by Sriganganagar (460.39 nmol/mg of protein), Ludhiana (456.69 nmol/mg of protein), Khandwa (380.12 nmol/mg of protein) and New Delhi ones (279.32 nmol/mg of protein). With increase in the CYP450 monooxygenases level, the degree of resistance also increased. However, enzymes like esterase and GST did not show significant changes.

Key words: *Bemisia tabaci*, cotton, fipronil, Flonicamid, New Delhi, Sriganganagar, Ludhiana, Khandwa, Amravati, esterase, GST, cytochrome P450

The cotton whitefly *Bemisia tabaci* (Gennadius) is considered as the most destructive sucking pest (Xie et al., 2014; Brown et al., 1994, 1995). It is regarded as a complex species with at least 34 morphologically identical species (Liu et al., 2012; Pan et al. 2012). In India, as early as 1920s, *B. tabaci* had become one of the serious pests of cotton (Prabhaker et al., 1989), and it is being controlled by conventional insecticides, and this has resulted in resurgence of sucking pests such as aphids, leafhoppers, thrips and whitefly (Patil et al., 1986). Among these, whitefly (*B. tabaci* Genn.) has become the most notorious. Continuous and indiscriminate use of insecticides has resulted in resistance development, affecting their field efficacy (Ahmad et al., 1999, 2000, 2002). Whiteflies have also been shown to develop resistance against neonicotinoids, pyriproxyfen, buprofezin, etc. (Prabhaker et al., 1997; Denholm et al., 1998; Horowitz and Ishaaya, 1994; Horowitz, 1994; Horowitz et al., 1999; Horowitz et al., 2002).

To overcome the problems of resistance, exploration of novel molecules is essential, and fipronil and flonicamid have been found to be effective (Chaton et al., 2001; Tomlin 2006). It acts on γ-aminobutyric acid (GABA), thereby blocking chloride channels (Grant et al., 1990; Scharf and Siegfrie, 1999; Bloomquist 2001). It has greater affinity to the target site, resulting in a high selectivity (Grant et al., 1990; Scharf and Siegfrie, 1999; Bloomquist 2001; Hainzal and Casida, 1996). Fipronil is observed effective against diamondback moth (*Plutella xylostella*), *Spodoptera* and *Heliothis* species, Colorado potato beetle, whiteflies, and against household pests, such as termites and cockroaches (Grant et al., 1990; Hamon et al., 1996). Flonicamid is another new molecule taking more time to reach maximum levels of mortality (Morita et al., 2007). It inhibits feeding of homopteran insects and death occurs from starvation (http://www.iraconline.org/eClassification/). It gave also good control of cotton aphids in field condition (Hancock, 2003).

Development of resistance is a serious problem (Rough and Tabashnik, 1990). It is essential to study the status of the detoxifying enzymes and biochemical factors in the development of resistance. Insecticides such as pyrethroids, carbamates and organophosphates are generally detoxified by two major enzymes, esterases and glutathione s-transferase whereas CYP450 monooxygenases is responsible for detoxification of

converted into nmol CDNB conjugated/min/fly using the molar extinction coefficient (εmolar) of the resulting 2, 4−dinitrophenyl−glutatione: $\varepsilon_{340nm} = 9.6\ mM^{-1}\ cm^{-1}$ (Habig et al. 1997).

$$\text{CDNB-GS in nmol/min/fly} = \frac{\text{ABS (increase in absorbance after time period) x Volume of total solution used} \times \text{Conversion factor (From mM to nmol- } 10^6)}{\text{Extinction coefficient of 2, 4−dinitrophenyl−glutatione x Time of run x Number of flies taken} \times \text{Path length correction}}$$

$$\text{CDNB-GS in nmol/min/fly} = \frac{\text{Increase in absorbance after 10 min x } 0.2 \times 10^6}{9.6 \times 10 \times 10 \times 0.524}$$

The activity of Cytochrome P450 was expressed in terms of general oxidase level, which is an indirect measure of cytochrome P450 by using heme peroxidation (Brogdon et al*.,* 1997). The heme peroxidation method has been considered as a reliable tool for comparing differences in general oxidase levels based on hemeprotein levels (Casimiro et al*.,* 2006; Penilla et al*.,* 2007). The heme constitutes the majority of cytochrome P450 in nonblood fed insects, quantification of heme activity has been used to compare the levels of cytochrome P450 on the basis of general oxidase levels (Brogdon et al., 1997). In heme peroxidation, TMBZ was used as a substrate and H_2O_2 as co-substrate (Brogdon et al., 1997; Penilla et al*.,* 2007). With the presence of H_2O_2, the microsomal oxidases use the TMBZ and developed two oxidized TMBZ molecules.

Five adult females were homogenized with 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 comprised of 80 µl of 0.625 M potassium phosphate buffer (pH 7.2), 20 µl of enzyme source, 200 µl TMBZ solution, 25 µl of H_2O_2 (3.0%) and giving a final volume of 325 µl. The substrate solution was made 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). Wells containing all reaction components, except the enzyme source was used as blank and absorbance was read at 620 nm in GEN5 absorbance microplate reader after 5 min of incubation. The measurements were replicated thrice. A standard curve for heme peroxidase activity was prepared using different concentrations of cytochrome C. Cytochrome P450 (general oxidase) activity obtained from plate reading was expressed as nanomoles of cytochrome P450 per milligram of protein by using the standard curve of cytochrome C.

The total protein content of homogenate was determined using the Coomassie brilliant blue G-250 dye (CBBG) method as described by Bradford (1976) with bovine serum albumin (BSA) as the standard. Absorbance was recorded at 595 nm. Bioassay data were computed by using PoloPlus 2.0 (LeOra Software, Petaluma, CA) for estimation of LC50 value using log–dose probit analysis (Finney, 1971). Resistance ratios (RR) were analysed at a given response level (LC_{50}) to test the folds of resistance to the insecticides in the evaluated populations compared with the most susceptible population.

RESULTS AND DISCUSSION

Bioassays and resistance ratio

The populations of *B. tabaci* collected from the five locations were bioassayed with fipronil and flonicamid for a period of 24 hr. Based on LC_{50} values, the insecticides showed better efficacy against *B. tabaci* of all the populations. When the population of New Delhi treated with fipronil, the LC_{50} value was found to be 6.56 mg/l as compared to 23.35 mg/l of LC_{50} value when the same treatment was given with flonicamid. A high degree of tolerance was observed in Amravati population and its LC_{50} value was found as 20.80 mg/l when treated with fipronil whereas in case of flonicamid treatment with the same population, 749.915 mg/l (LC_{50} value) was obtained. The population of New Delhi showed highest toxicity followed by Khandwa, Ludhiana, Sriganganagar and Amravati in both the insecticides. Among the two insecticides, fipronil gave better efficacy as compared to flonicamid.

The resistance ratio (RR) has also been computed at the LD_{50} level as RR equals LD_{50} of particular location divided by LD_{50} of New Delhi population in a view of keeping New Delhi population as susceptible strain. The resistance was found to be highest for Amravati population (3-fold) followed by Sriganganagar (2.6-fold), Ludhiana (2-fold) and Khandwa (1-fold) against fipronil bioassayed. The Amravati strain which showed the highest resistance to fipronil was also highly resistant to flonicamid (750-fold). The least resistance was observed in the population of Khandwa (2-fold) followed by Ludhiana (8-fold) and Sriganganagar (12-fold) as compared to New Delhi population (Table 1).

A level of resistance to fipronil was observed in *B. tabaci* with LC_{50} values of 21.808-43.768 mg/l

Table 1. Relative toxicity of fipronil and Flonicamid to populations of *B. tabaci*- 24 hr period

Population	n	df	Slope ±SE	χ^2	LC_{50} value mg/l (CI 95%)	Fiducial limit	RR
Fipronil Ludhiana	354	3	1.907±0.364	3.301	11.953	3.100 to 17.953	1.82
Sriganganagar	423	3	1.966±0.322	3.489	17.323	8.705 to 23.932	2.63
Khandwa	382	3	0.925±0.321	0.185	9.047	1.970 to 15.124	1.37
Amravati	348	3	1.683±0.452	0.181	20.806	11.663 to 30.004	3.17
New Delhi	305	3	1.386±0.346	0.727	6.566	2.712 to 9.927	1
Flonicamid							
Ludhiana	360	4	1.382±0.256	1.289	188.458	121.646 to 244.759	8.06
Sriganganagar	410	4	1.029±0.210	1.779	279.461	203.197 to 434.833	11.96
Khandwa	313	3	1.093±0.316	1.018	41.882	28.407 to 63.634	1.79
Amravati	320	3	1.155±0.403	0.669	749.915	493.138 to 2059.542	32.10
New Delhi	309	3	1.351±0.293	2.830	23.355	15.340 to 30.742	1

n-Number of insects taken; df= Degrees of Freedom; χ^2 = Goodness of fit; CI= Confidence Interval; RR= Resistance Ratio

Table 2. Variations in detoxifying enzymes in *B. tabaci* on cotton

Population	Esterase activity in mOD/min	RA	GST activity in nmol/min/fly	RA	Cytochrome P450 monooxygenase activity in nmol/mg of protein	RA
Ludhiana	*3.54±0.29	1.16	*225.58±15.40	1.45	*456.69±35.97	1.64
Sriganganagar	3.34±0.29	1.09	257.44±20.67	1.66	460.39±35.63	1.65
Khandwa	5.09±0.18	1.66	273.24±23.26	1.76	380.12±48.11	1.36
Amravati	5.49±0.78	1.79	175.73±7.87	1.13	793.70±22.23	2.84
New Delhi	3.06±0.27	1	155.25±11.01	1	279.32±30.01	1

*Mean ± Standard Deviation, RA= Relative Activity

(DuanXiaoDong, 2010). However, the LC_{50} value for cotton whitefly against fipronil was 3.51 mg/l and the fiducial limit of 2.55–4.83 mg/l (Xie et al., 2010). Flonicamid exhibited low to no insecticidal activity on eggs, emerging crawlers and second-instar nymphs of *B. tabaci* at the maximum registered label rate (RLR_{max} = 125 mg/l). In long-term cage experiments, flonicamid at the RLR_{max} caused 95% mortality to *B. tabaci* 10 days after treatment and delayed population growth by one generation (32 days). The insecticide significantly delayed nymphal development by increasing the development time (DT_{50}) of treated insects by 7.2 days (Roditakis et al. 2013). In case of *Aphis punicae*, the LC_{50} value for flonicamid after 24 h was found to be 0.05 mg/ml (Rouhani et al. 2013). Different crops like Japanese radish, eggplant, wheat, Chinese cabbage seedlings were treated with flonicamid against different aphid species like *M. persicae, Aphis gossypii, Rhopalosiphum erysimi* and *Schizaphis graminum*, the LC_{50} values ranged between 0.64 and 2.01 mg/l (Morita et al., 2007). Sadeghi et al. (2009) reported that flonicamid showed high toxicity against first-instar *A. pisum* nymphs with an LC_{50} of 20.4 g/ml after 24 hours, and of 0.24 g/ml after 72 hours of treatment.

Variability in detoxifying enzymes

The various levels of detoxifying enzymes of *B. tabaci* populations were depicted in Table 2. In the present work, enzyme systems such as esterase, glutathione S-transferases (GST) and cytochrome P450 monooxygenase (CYP450) were estimated. The CYP450 levels were found to be higher in Amravati (793.70 nmol/mg of protein) followed by Sriganganagar (460.39 nmol/mg of protein), Ludhiana (456.69 nmol/mg of protein) and Khandwa (380.12 nmol/mg of protein) populations. The New Delhi population was observed to be the least CYP450 level and was recorded as 279.32 nmol/mg of protein. With the increase in CYP450 level, the degree of resistance was also increased. The data is in connection with the insecticide bioassayed in which Amravati population was having the highest degree of resistance against fipronil and flonicamid followed by Sriganganagar, Ludhiana, Khandwa and New Delhi (Fig. 1). In case of esterase

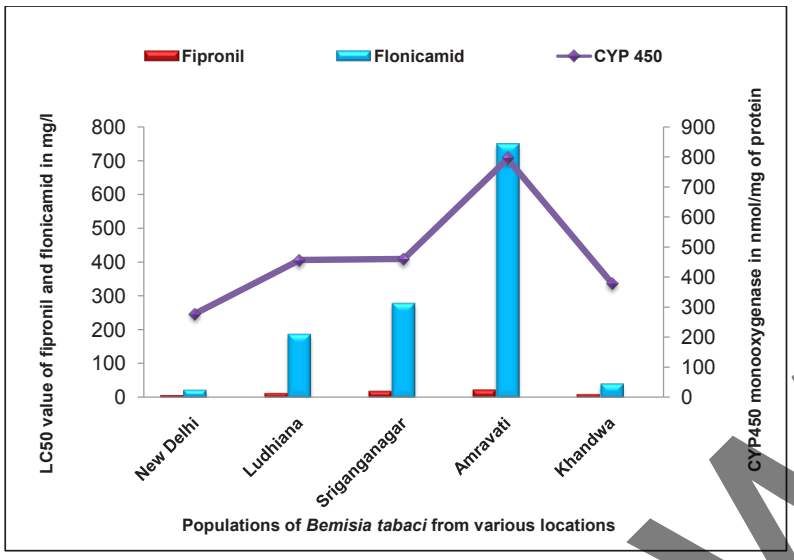

Fig. 1. Cytochrome P450 enzyme vs. resistance to fipronil and flonicamid in *B.tabaci* on cotton

activity, the Amravati population showed the highest level (5.49 mOD/min) and New Delhi population (3.06 mOD/min) showed the least level but the same trend was not followed for the rest of the populations. However, in GST activity, the highest activity was found in Khandwa population (273.24 nmol/min/fly) and the least activity recorded in the population of 175.73 nmol/min/fly.

The physiological adaptations of western corn rootworm (*Diabrotica virgifera virgifera*) populations responsible for resistance to a number of insecticides from different neurotoxic classes include elevated esterase-based hydrolysis and cytochrome P450-based oxidation (Miota et al., 1998; Scharf et al., 1999). In addition, insecticide resistance and related mechanisms have been identifed in both adults and larvae of rootworm (Wright et al., 2000). The microsomal monooxygenase detoxification system appears to be intricately involved in the metabolism of fipronil, as formation of the sulfone metabolite is dependent on this enzyme system. Brookhart and Bushey (1994) reported fipronil-sulfone was the predominant metabolite formed from [^{14}C] fipronil when orally administered to the southern armyworm, *Spodoptera eridania* (Cram).

In vitro metabolism results indicated cytochrome P450 monooxygenases were responsible for the conversion of fipronil to its sulfone form in European corn borer (Durham et al., 2002). The metabolic pathway generated increased toxicity of the parent compound. Similar observation was observed in larvae of both *Chilo suppressalis* and *Sesamia inferens* that toxicity levels of fipronil metabolites were greater than parent compound, fipronil due to monooxygenases (Fang et al., 2008). The high level of toxic effects observed in *C. suppressalis* was believed to be the result of increased metabolic activity producing higher amounts of fipronil sulfone. The monooxygenase activity was responsible for neonicotinoid resistance in *B. tabaci* (Rauch and Nauen, 2003). Resistance in *B. tabaci* appears to be linked to enhanced oxidative detoxification of neonicotinoids due to overexpression of monooxygenases. No evidence for target-site resistance has been found in whiteflies (Nauen and Denholm, 2005).

GST was known to metabolize fipronil but there was little evidence that GST metabolism increases resistance to fipronil (Scharf et al., 2000). Kristensen et al. (2004) found increased GST levels in both fipronil-susceptible and tolerant strains of *Musca domestica* and Li et al. (2007) found no significant differences in GST levels between susceptible and resistant strains of *C. suppressalis*. There was also evidence that esterase activity might have played a role in fipronil resistance in *Sogatella fucifera and C. suppressalis* (Li et al., 2007; Tang et al., 2010). In *B. tabaci* species complex, the elevated esterase activity was correlated with OPs and pyrethroid resistance (Alon et al., 2008; Prabhaker et al., 1988; Dittrich et al., 1990; Byrne et al., 2000). However, the role of GST in *B. tabaci* species complex was less likely to be important in detoxification of OPs and pyrethroids (Zhang et al., 2011; Liang et al., 2014).

The present study reveals that fipronil and flonicamid showed better efficacy against *B. tabaci* on cotton. Of these, fipronil gave better efficacy. Populations from

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