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Different exposure methods to neonicotinoids influenced biochemical characteristics in cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae)

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We measured lipid, carbohydrate and protein content in three strains of cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae) from very resistant to neonicotinoids up to semi sensitive and sensitive strains in different exposure method of spraying to neonicotinoids. We observed changes in energy source rates at which each substrate was metabolised under starvation, selection and residue stress method spraying that assist in metabolisation of their biochemical parameters. These particular exposure methods influenced some of biochemical parameters in cotton aphid. Results indicated that Sugar, glycogen, total protein and lipid in aphids have significant changes. Studies showed that among three strains called Ag-R, Ag-M and Ag-D which have different susceptibilities to neonicotinoids, strain Ag-R was the most tolerated aphids in counter of imidacloprid and thiametoxam, strain Ag-D was somewhat more tolerated to these insecticides and strain Ag-M was the most sensitive strain which has the resistance factor of nearly 890 in starvation method and was different depending on the method of exposure. Among energy sources, the total lipid in susceptible strain were decreasing more than resistant strain, whereas total proteins were increasing in resistant strain compared with sensitive strain. Total Glycogen was affected significantly in the stress condition which caused an increase and was the most resistant strain.

Keywords: cotton aphid; adaptation; biochemical parameters

Introduction

The Cotton Aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) is one of the main pests of cotton and summer crops throughout the world causing significant problems due to sucking phloem sap and honeydew contamination directly and also virus transmission damage indirectly (Blackman and Eastop 1994). Among 4000 worldwide aphid species, very different feeding behaviours can be observed, from strictly monophagous to the species able to switch from one to plenty of host plants (Blackman and Eastop 1994). This capability to use a so large range of food sources has to be closely linked to high potential adaptation systems to cope with various defence mechanisms in host plants (Berenbaum and Zangerl 1999). The biochemical characteristic was involved as well as enzymatic activity in the metabolisation by several herbivores of a broad range of secondary metabolites (SM) from the host plant (Cohen et al. 1992). So, the kind of plants that change the aphid's biochemical parameters reveals that aphids imposed

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selection favouring increase or reduction on the energy sources concentration or density.

Imidacloprid and thiametoxam, like all of other neonicotinoids act, affect agonistically the insect nicotinic acetylcholine receptors. This was caused by blocking nerve transmission at cholinergic synapses, thus causing a reduced breakdown of imidacloprid and thiametoxam in nervous tissue and reduced ability to recover) Nauen et al. 1998).

Energy availability can limit the ability of organisms to survive under stressful conditions (Marron et al. 2003). In cotton aphid, laboratory experiments have revealed that energy storage patterns differ between populations separated with insecticide spraying with different method like selection, starvation and residue methods compared with population which is not exposed to insecticides, because aphids may use different sources of energy when exposed to insecticide stresses. Energy metabolism may also play a critical role in stress resistance. Reductions in metabolic rate will increase the amount of time aphids can survive and lose energy sources in exposure to neonicitinoids in stressful conditions. An important link from these studies is knowledge of which energetic substrates are actually consumed when aphids exposed to insecticide stresses. Measuring the rates of disappearance of energetic substrates (lipids, carbohydrates and proteins) to test the hypothesis that aphids with different neonicotinoid exposure method will contain greater levels of metabolic reserves, particularly carbohydrates, will help us to manage spraying method for controlling cotton aphids. The objective of the present work was to investigate the effect of bioassay method of spraying on tolerance of *A. gossypii* using a range of biochemical assays.

Materials and methods

Rearing conditions

Three cotton aphid colonies were obtained from a colony maintained on the Department of Plant Protection at the Faculty of Agriculture, University of Tehran; a greenhouse located in this faculty and from a greenhouse placed on the Mohammad-Shahr, Karaj, Iran, which were mostly sprayed by the insecticides of imidacloprid (35 SC Confidor®) and thiametoxame (50WG Actara®). We called after that the mentioned colonies as V, M and D, respectively. From these aphids, a single apterous partenogenically reproducing female was selected to establish a parent colony. These colonies were used as a source for all aphids used in our laboratory assays. Colonies were maintained with environmentally controlled conditions on three different free pesticide plants from Cucurbitacae, *Cucumis sativus* variety Sultan.

Bioassay methods

The bioassay procedures were used by three different bioassay approaches. In the first method (FAO Dip method), after dipping of apterous adults in chosen aqueous insecticidal for 5 s, aphids were transferred onto freshly excised mentioned plants leaves on 9 cm diameter Petri-dishes placed upside down on a layer of moist paper towel (direct method). In another method (Starvation method), dipped aphids in chosen insecticidal solutions were transferred on the Petri-dish without any water and food supplies (indirect method). In the third method, aphids were transferred onto the leaves that have been dipped in insecticide solutions and then one-day-old apterous aphids were transferred onto the toxic leaves on mentioned Petri-dishes placed upside down on a layer of moist paper towel (Residue method). All the bioassay described below were done in the

laboratory under controlled conditions, the temperature 23 ± 1 °C, under photoperiod 16:8 and 70% R.H. Three different bioassay experiments were designed for detecting the susceptibility of cotton aphid to imidacloprid and thiametoxam. Aphid survival was assessed after 24 h and probit analysis (LeOra Software 2007) was used to establish the lethal concentration required to kill 50% of the test population (LC_{50}).

Biochemical analysis

We used standard biochemical techniques to assay the amount of protein, lipid and carbohydrate present in aphids. Protein was assayed with the Bradford reagent, lipids with vanillin in phosphoric acid and carbohydrates with anthrone reagent, respectively, by Bradford (1976), Van Handel (1965) and Yuval et al. (1994) methods with some modifications. The analytical protocol was as follows: 20 aphids were homogenised individually in 200 µl of 2% Na_2SO_4. Lipids and sugars were extracted in 1300 µl of chloroform: methanol (1: 2). After that, they centrifuged for 10 min at 10,000 g. Samples were then dissolved in 500 µl of H_2SO_4 and incubated for 10 min at 90 °C. Samples of 30 µl were put into wells on ELIZA plates; together with 270 µl of vanillin reagent (600 mg vanillin dissolved in 100 ml distilled water and 400 ml 85% H_2SO_4). The plate was shaken for 30 min at room temperature, and then optical density was read at 530 nm .Total lipids in each aphid were calculated from standard curve.

To determine the amount of sugar in each aphid, 300 µl were taken from the chloroform: methanol extract. After adding 200 µl water, the sample was reacted for 10 min at 90 °C with 1 ml of anthrone reagent (500 mg anthrone dissolved in 500 ml concentrated H_2SO_4). Samples of 300 µl were then put into wells on ELISA plates and optical density was read at 630 nm. Total glycogen was calculated from standard curves.

To determine the qualities of total lipid contents, about 30 adult aphids were crushed in ethanol and centrifuged at 15,000 g for 10 min. Standard curve was used form cholesterol.

The glycogen content was determined from the pellet that resulted from the centrifugation of prepared sample in 10,000 g. After washing it with 400 µl of 80% methanol to remove any traces of sugar, 250 µl of water were added and heated for 5 min at 70 °C in order to extract the glycogen. From each tube, 200 µl were taken and incubated for 10 min at 0 °C with 1 ml anthrone reagent (this time 600 mg anthrone dissolved in 30 ml concentrated H_2SO_4). Samples of 300 µl were put into wells on ELIZA plates and optical density was read at 630 nm using a microplate reader (Beckman Coultter TM AD 340). Total glycogen was calculated from standard curves.

Haemolymph-dissolved protein was extracted in 1200 µl phosphate-buffered saline (PBS).Samples of 300 µl were taken and after adding 500 µl PBS, were reacted with 200 µl Bradford reagent. Samples of 300 µl were put into wells on ELIZA plates and optical density was read at 595 nm. Total dissolved protein in the haemolymph of each aphid was calculated from standard curve.

Statistical analysis

Data were analysed by means of analysis of variance (ANOVA) using the General Linear Model (GLM), model procedure with Minitab 18, and for bioassay experiments, data were analysed by probit analysis (Finney 1971) using the software package POLO-PC (LeOra Software 2007). Resistance Factor (RF) was calculated by dividing LC_{50} values computed for the resistant populations by the corresponding LC_{50}s for the

susceptible colony. $LC_{50}-$ values and their 95% confidential limits were calculated from probit regressions using the computer programme POLO-PC (LeOra Software 2007, Berkeley, CA).

Results

The RF (resistance factor means the proportion of LC_{50} of resistant strain per LC_{50} of sensitive strain) of strain Ag-R compared to Strain Ag-M was 890 in FAO Dip method and strain *R* was the most tolerate strain in counter of imidacloprid and thiametoxam, strain Ag-D was somewhat more tolerated to these insecticides and strain *M* was the most sensitive strain to these insecticides.

Activities of CaE (i.e. α-Naphthyl acetate), GSH (i.e. CDNB) and Cytochrome P_{450} (i.e. NADPH) have been used to determine insecticide resistance mechanisms and to monitor resistance of various aphid strains on different host plants (Gerami 2012) and the effects of activity of this detoxifying enzyme associated with the imidacloprid and thiametoxam on resistant strains and host plants were studied (Gerami 2012), and aphids resistant to insecticides were recognised by over-producing detoxifying esterases and increasing activities of CaE. The quantity and quality of this enzyme was associated with host plant upon which aphids feed (Gerami 2012). Also, qualities and quantitative changes of GST enzymes were related to the aphid feeding preference, depending on which the allelochemical presence in host plants were studied (Gerami 2012).

In our study, it is shown that different strains have different amount of biochemical parameters. It is also showed compared with no-enzymatic components that glucose content showed significant increase in resistant strain while in susceptible strain, the amount of sugar was reduced in counter of treatment stress. Total glycogen and protein levels were affected significantly in resistance strain. The sources of energy are decreased in starvation method more than other methods when aphids countered on spraying stress. LC_{50} in starvation method is more than other methods and in FAO Dip method is less than other methods for both of imidacloprid and thiamethoxam.

Discussion

Experiments with each of bioassay methods showed that the strain of Ag-R is the most resistant and strain of Ag-M is the most sensitive population. The RF (resistance factor means the proportion of LC_{50} of resistant strain per LC_{50} of sensitive strain) of strain Ag-R compared with Ag-M was different depending on the bioassay method related to imidacloprid and with thiametoxam (Tables 1 and 2). The slopes of dose–response curves were less steep for thiametoxam compared with imidacloprid. The slopes of dose–response curves in residue method was steeper compared with other bioassay methods especially in the case of Ag-R strain which suggests a considerable heterogeneity between the strains and different methods of exposure. Results in Tables 1 and 2 revealed that the results are strongly depending on the used exposure procedure. This finding is in accordance with Lowery and Smirle's (2003) studies. When LC_{50} values were compared, they were judged as a significant difference when the respective 95% CL did not overlap. In Nauen et al.'s studies (1998), the apparent tolerance of tobacco aphids to imidacloprid was attributed to a strong anti-feedant effect that resulted in reduced exposure to the toxicant during 1-d test period. When neonicotinoids were directly applied to aphids, mortality increased while in indirect exposure, the mortality lowered. Our findings suggested that the type of exposure method is very important

Table 1. LC_{50} of cotton aphid in different methods of exposure to imidacloprid.

	FAO Dip method (selection)				Starvation				Residue			
Population bioassay method*	LC_{50}	95% CL	Slope	RF	LC_{50}	95% CL	Slope	RF	LC_{50}	95% CL	Slope	RF
Ag-R	6080	5760-6321	1.33±0.2	894.1	8640	8129-9000	1.8±0.22	691.2	7600	7353-7860	1.64±0.07	690.9
Ag-M	6.8	4.2–9.1	1.01±0.22	–	12.5	9.2–14.3	1.13±0.24	–	11	8.7–13.6	1.08±0.2	–
Ag-D	231	201-276	1.22±0.25	26.3	385	330-411	1.38±0.03	22.44	370	331-402	1.165±0.18	20.54

Table 2. LC_{50} of cotton aphid in different methods of exposure to thiametoxam.

	FAO dip method (selection)				Starvation				Residue			
Population bioassay method*	LC_{50}	95%CL	Slope	RF	LC_{50}	95%CL	Slope	RF	LC_{50}	95%CL	Slope	RF
Ag-R	6900	6570–7230	1.4±0.01	492.85	9700	9409–9919	1.96±0.3	450.4	9100	8870–9430	1.9±0.1	801.6
Ag-M	14	11.2–17.1	1.25±0.01	–	20.2	18–23.2	1.5±0.01	–	12.1	8.9–14.1	1.11±0.03	–
Ag-D	225	211–243	1.1±0.02	30.66	183	175–196	1.4±0.03	32.97	276	256–298	0.89±0.006	53.005

*Ag- R: Resistant *Aphis gossypii*; *Ag- M: Sensitive *Aphis gossypii*; *Ag- D: Semi resistant *Aphis gossypii*; *95% CL: 95%Confidence Level; *RF: Resistance Factor= LC_{50} Resistant Strain/LC_{50} Sensitive Strain; *All of the application methods have the significantly differences ($p < 0.01$).

when aphids' populations assess for the quantity and quality of biochemical parameters against the neonicotinod insecticides. The findings outlined in this study should be considered when aphid populations are assessed for possible resistance against with imidacloprid and thiametoxam. It is clarified that imidacloprid and thiametoxam have a negative effect on the biochemical parameters on the starvation method. Negative effects can mostly attribute to the anti-feedant activity of these compounds and the protracted time to death. The results of this investigation contributed to a better understanding of the most suitable technique for assessing aphids' mortality after exposure to these insecticides and provide a baseline susceptibility to imidacloprid and thiametoxam in cotton aphid. Differences in the hardiness (tolerance) of different populations could strongly affect the result of resistance monitoring. The present investigation revealed that with short-term bioassay, the only reliable measure of imidacloprid and thiametoxam is that which is made using a FAO aphid-dip technique. In contrast to an FAO dip test, the leaf disc (starvation) bioassay mainly measures the reversible behavioural alternations in aphids which cause them to die due to starvation. If tolerance is measured in such kinds of bioassay, then it could be interpreted as behavioural tolerance, induced by the avoidance of imidacloprid and thiametoxam-treated plants or by starvation tolerance of the aphids as shown in our investigation. Thus, it is safer to choose the FAO dip technique for determining possible tolerance, because it focuses more on the fast-acting neourotoxicological mode of action which causes death with clearly visible symptoms resulting from interference with the nervous system. The FAO test protocol, which involves dipping adult aphids in solutions of insecticides, was developed to rapidly assess the toxicity of neurotoxins that easily penetrate the insect cuticle. This test might not provide an accurate measure of toxicity for compounds that act slowly or those that intoxicate mainly through oral ingestion. Toxicity of imidacloprid is mainly through oral ingestion (Ninsin and Tanaka 2005). Toxicity of imidacloprid to aphids' results primarily affects ingestion (Mullins 1993) and mortality is not fully expressed until several days after exposure. Findings outlined in the present study should be considered when aphid populations for possible resistance were affected by the neonicotinoids that have a more inconspicuous antifeeding response against sub-lethal concentrations, causing death by starvation over a longer period of time, because of their distinct mode of action.

As shown in Table 3, starvation and selection exposure are positively correlated with the Glycogen levels. Starvation-selected populations of cotton aphids accumulate high carbohydrate levels, as predicted from comparative studies. Selection-stressed populations store less lipid but much more glycogen than control populations as in Djawdan et al.'s (1998) studies. In the residue method, aphids use more lipids and less carbohydrate. So, it is of greater interest; in the present context, the relative contributions can be perturbed by insect biochemical parameters as a crucial agent such as elements using for providing energy that exert their activity by inhibition or induction of biochemical parameters. Choice tests (Leaf dip technique) showed that cotton aphids were also less susceptible to the anti-feedant potential of imidacloprid and thiametoxam in contact bioassays.

Survival insects under stress conditions can be maximised by two physiological mechanisms: increasing the storage or resources (energy or water) that are utilised during stress, or conserving resources by reducing the rates at which they are consumed (Telfer and Kunkel 1991). It is clear in our study that the mean content of biochemical characteristics of aphids with different susceptibility were surprisingly different ($df = 4$, $F = 30.323$, $p < 0.000$). In addition to rates of sources of energy consumption, both the

Table 3. Rates of energy sources use by *Aphis gossypii* in exposure with starvation, (FAO Dip method) selection and residue stress.

		Imidacloprid				Thiametoxam			
Population	Stress condition	FAO	Starvation	Residue	Control	FAO	Starvation	Residue	Control
Source of energy									
Ag-R	Lipid	16.02±0.4	11.6±0.4	23.6±0.4	33.4±0.4	15.3±0.4	11.6±0.4	23.6±0.4	33.4±0.4
	Protein	72.6±6.4	105.7±6.4	105.4±6.4	296.9±6.4	25.6±0.2	14.45±0.25	31.4±0.4	34.61±0.07
	Glycogen	90.03±0.94	7.4±0.94	17.08±0.9	11.5±0.9	157.7±0.9	11.5±0.9	10.07±0.9	7.25±0.03
	Sugar	309.4±7.2	172.7±7.2	358.5±7.2	742.6±7.2	225.7±1.7	140.5±2.4	247±8.5	912.85±11.3
Ag-M	Lipid	12.4±0.4	2.3±0.4	14.3±0.4	25.9±0.4	9.7±0.4	0.6±0.4	17.2±0.4	21.6±0.4
	Protein	35.85±6.4	21.3±6.4	119.03±6.4	132.4±6.4	10.5±0.4	0.23±0.03	22±1.3	25.5±0.19
	Glycogen	50.7±0.9	1.9±0.9	5.5±0.9	6.3±0.9	61.2±0.3	1.78±0.11	6.74±0.02	7.58±1.2
	Sugar	149.2±7.2	67.2±7.2	202.4±7.2	581.8±7.2	365.7±3.4	142.8±3.6	257.7±1.1	670.4±25.34
Ag-D	Lipid	18.6±0.4	7.7±0.4	20.5±0.4	29.3±0.4	15.1±0.4	9.4±0.4	20.3±0.4	29.3±0.4
	Protein	70.9±6.4	37.04±6.4	97.1±6.4	273.9±6.4	74.2±1.1	55.5±0.2	98.4±0.6	276.5±0.2
	Glycogen	67.4±0.9	7.5±0.9	12.7±0.9	7.2±0.9	154.4±1.1	6.75±0.04	11.24±0.01	6.8±0.2
	Sugar	255.1±7.2	155.1±7.2	256.1±7.2	555.1 ±7.2	260.2±14.5	180.7±2.2	97.2±0.3	665±15.3

amount and form of these sources of energy storage can affect aphid stress to neonicotinoids and resistance. In our study, among no-enzymatic components, interestingly these parameters are increased on resistance and semi-resistance strain more compared to sensitive strain. It may be mean; metabolism on resistance strain is more than the others. On the other hand, resistant strain needs more nutrition to overcome the insecticide treatments stress. As we have shown in our studies, total glycogen and protein content were affected in each strain. Glycogen was the most changeable parameter rather than other parameters. The amount of protein and glycogen in resistant strain are more than these parameters in sensitive strain. The total lipids were decreased whereas soluble protein and glycogen content showed a significant increase in resistant strain compared with other strains. Total lipids decrease probably because they are being routed to main metabolic pathway and utilised for energy production after imidacloprid and thiametoxam treatment. According to Downer and Matthews (1976), increased lipid storage will only help aphids to survive starvation if they actually metabolise lipids under these conditions. Because of its high-energy content, lipid is the primary stored nutrient in insects and most other animals (Downer and Matthews 1976). According to Mushtaq et al.'s (1986) studies, beetles utilised lipid and cholesterol in addition to glucose and glycogen under stress conditions. Our results also showed the relationship of resistance and energy using pattern. We observed that resistant strain use more sources of energy compared with sensitive strain. May be it depends on the fact that resistant strain need more energy to cope with chemical stress.

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