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Toxicity of neonicotinoids used in melon culture towards *Apis mellifera* L.

Whalamys Lourenço de Araújo[1]*, Maurício Sekiguchi de Godoy[2], Patrício Borges Maracajá[3], Wesley Adson Costa Coelho[4], Bárbara Karine de Albuquerque Silva[5], Adrian José Molina Rugama[2], Elton Lucio de Araújo[2] and Jacinto de Luna Batista[6]

[1]Agronomy Department, Federal University of Paraíba (UFPB), Paraíba, Brazil.
[2]Department of Plant Sciences, Federal University of Semiarid (UFERSA), Rio Grande do Norte, Brazil.
[3]Academic Unit of Agricultural Sciences (UAGRA), Federal University of Campina Grande, Campina Grande, Paraíba, Brazil.
[4]Veterinary Medicine Department, Federal University of Semiarid (UFERSA), Rio Grande do Norte, Brazil.
[5]Graduate Program in Plant Science, Federal University of Semiarid (UFERSA), Rio Grande do Norte, Brazil.
[6]Agricultural Science Center (CCA), Federal University of Paraíba (UFPB), Paraíba, Brazil.

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The cultivation of melon (*Cucumis melo* L.) is of great importance to the Brazilian economy, especially the semiarid regions of the Northeast region. Damage caused by pests have hindered the production, requiring that control measures be adopted, among them applications of chemical insecticides, including neonicotinoids. Studies have shown collateral damage to beneficial insects such as bees, important pollinators for 90% of angiosperms, especially melon. The objective of this study was to evaluate the toxicity on melon crops of neonicotinoids used to control pests related to honeybee, *Apis mellifera* L. Bioassays were performed in the laboratory. The mortality of specimens over time when contaminated with the products thiamethoxam, midaclopride and acetamiprid (two commercial products by different companies) was evaluated. The exposure of bees to the compounds was performed by food ingestion (sugar candy) contaminated with the lowest and highest doses recommended by the manufacturers. Regardless of the dose, all insecticides were toxic, decreasing up to 11 days the useful life of bees as compared to the control (water + sugar candy), which survived 18 days on average.

Key words: Contamination, pollinators, bees, *Cucumis melo* L.

INTRODUCTION

The cultivation of melon (*Cucumis melo* L.) is of great importance to the Brazilian economy. In recent years, it has the highest export volume to the international market. In 2014, 196,850 tonnes of the product were exported

*Correspondent author. E-mail: whalamys@hotmail.com

(Reetz et al., 2015). Northeastern semiarid regions are the main productive centers for its favorable climate and soil conditions (Lopes et al., 2012). However, such conditions have also favored pest insect attacks, hindering the production (Fernandes, 2016).

Control of melon pests is usually done with the use of chemicals, due to immediate positive results regarding the suppression of population of such organisms.

However, if used improperly, they cause major environmental problems such as food contamination, environmental pollution, poisoning and death of farmers, as well as extinction of animals (Ferrari, 1986). In many cases, they promote a selection of resistant population lineages, causing a greater crop dependence on pesticides, committing the fauna and in many cases decreasing the number of beneficial organisms (Chagas, 2016) such as pollinating insects, bees and wasps (Barbosa, 2016).

Several authors have investigated the deleterious effects of insecticides, mainly neonicotinoids, on pollinating insects, including bees (Bortolotti et al., 2003; Barnett et al., 2007; Thompson and MAUS, 2007; Schneider et al., 2012; Palmer et al., 2013; Sandrok et al., 2014; Barbosa, 2016; Pacífico da Silva et al., 2015). However, there are few studies focusing on the Brazilian Northeast region and more specifically on melon crops. The effects of insecticides on bees vary, including changes in the olfactory system, flight disorders, impaired immune response and reduced survival rate (Pacífico da Silva et al., 2016).

Considering the environmental impact the improper use of such insecticides may cause and considering bees as pollinators important to melon plants, although easily affected by pesticides in crops, the main objective of this study was to evaluate the toxic effects of neonicotinoids insecticides, used in a melon crop to control pests, on worker *Apis mellifera* L. via ingestion of food contaminated with the lowest and the highest doses of insecticides recommended by manufacturers.

MATERIALS AND METHODS

The bioassay was conducted at the Entomology Laboratory of the Center for Agricultural Sciences of the Federal University of Campina Grande (UFCG), Campus de Pombal, Paraíba State.

Bee specimens were previously collected in beehive frames from colonies of the Central Apiary of Entomology Laboratory (UFCG) located at the experimental farm of that institution in the city of São Domingos de Pombal.

In this apiary, hives are installed in rational, wooden Langstroth structures kept in the natural environment of the Caatinga semiarid, typical of the region. Before the collection of the specimens, the hives went through a "preparation" process: the collection and selection of insects for experimentation. This preparation consisted of managing the supply of energy of colonies using an artificial diet comprising water and sugar syrup in a 1:2 proportion enriched with 2.5% of Glicopan® (free amino acids) and 2.5% of Aminomix® (vitamins, minerals and amino acids). The supply was weekly provided in individual feeders with a capacity of 500 mL in order to stimulate the mass production of young bees in hives, reaching a population suitable for bioassays. The hives were analyzed to select bees for the experiment. The general appearance of the population was taken into account. Hives that had all nest spaces configured for the production of juveniles, number of adult bees able to cover 2/3 of the breeding area and a food collection activity that resulted in a return flow from the field above one hundred (100) bees per minute were considered suitable.

Among the frames containing bee nests from selected hives, the worker bees were removed during the pre-emergence to adulthood stage in order to obtain newly emerged adults. The frames were packed in a wooden box adapted to a motor vehicle to transport them to the laboratory. For toxicity testing, four neonicotinoids insecticides were selected. The products were applied at the lowest and highest concentrations recommended by the manufacturers for the control of insect-pests in melon crops. Insecticides and their respective trade names, active ingredients and concentrations used (doses in grams of the product commercial per liter – g.p.c./L) are described in Table 1. Each neonicotinoid dose represented one treatment. The control consisted of distilled water only.

For the contamination of food, a chemical solution of each product, with its respective doses, was incorporated into the honey using a dosage calculated based on weight/volume. Then, powdered sugar (100 g) was added to prepare the sugar candy, obtaining a homogeneous diet for each treatment: 30% of chemical solution in 100 mL of honey. The treatments were arranged in a completely randomized design with nine treatments and five replications. Each experimental unit consisted of 20 adult worker bees up to 48 h old. To constitute the plots, bees were transferred to wooden boxes (cages) (11.0 cm long x 11.0 cm wide x 7.0 cm high) containing holes on the sides and sealed with nylon screens for ventilation. The boxes were previously covered on its internal base with filter paper and had a transparent glass lid on the top.

For each cage, contaminated sugar candy (honey + sugar) (10 g) was offered, varying according to the treatment, but always in a makeshift feeder (sterilized pet bottle caps), covered with a steel mesh facilitating the access of bees and avoiding death by drowning. Beside the feeder, a cotton swab soaked in distilled water was added. It was also packed in sterilized pet bottle caps and used as a source of water. For the negative control, sugar candy without contamination by insecticide was used associated with the water source mentioned above. The bioassay was kept in a room at 25 ± 2°C, RH of 70 ± 10% and 12 h photoperiod. At 1, 2, 3, 6, 12, 24, 48, 72 and 96 h after the beginning of exposure of bees to the products, the toxicity of the insecticides was observed on the individuals until their death. For each treatment, the evaluated biological parameter was the mortality rate of the specimens by counting dead insects over time. Only bees that did not move, even when lightly touched with a slim-tip brush, were considered dead.

Statistical analysis was performed using the software GraphPadPrism (v.5 for Mac). The median of the survival time with a 95% confidence interval and the significant differences between groups were estimated using the Kaplan-Meier test followed by a log-rank test. A significance level of $p<0.05$ was adopted.

RESULTS AND DISCUSSION

After one hour with contaminated food supply, it has been found that highest dose (3.0 g.p.c./L of water) of the neonicotinoid insecticide thiamethoxam caused highly toxic effects to bees, presenting mortality of insects in all experimental plots (Figure 1A). The lowest dose of thiamethoxam (0.3 g.p.c./L of water), added to the sugar candy offered to bees, also showed a toxic effect, but only up to 24 h (Figure 1B).

Similar effects were found by Carvalho et al. (2009),

Table 1. Trade names, active ingredients and doses of insecticides from the neonicotinoidsgroup evaluated in toxicity tests using *A. mellifera* L. worker bees under laboratory conditions.

Trade name	Active ingredient	Dose (g.p.c./L of water)	
		Lowest	Highest
Actara 250 WG	Thiamethoxam	0.3	3.0
Evidence 700 WG	Imidacloprid	1.0	1.5
Mospilan	Acetamiprid	0.25	0.3
Orfeu	Acetamiprid	0.25	0.3

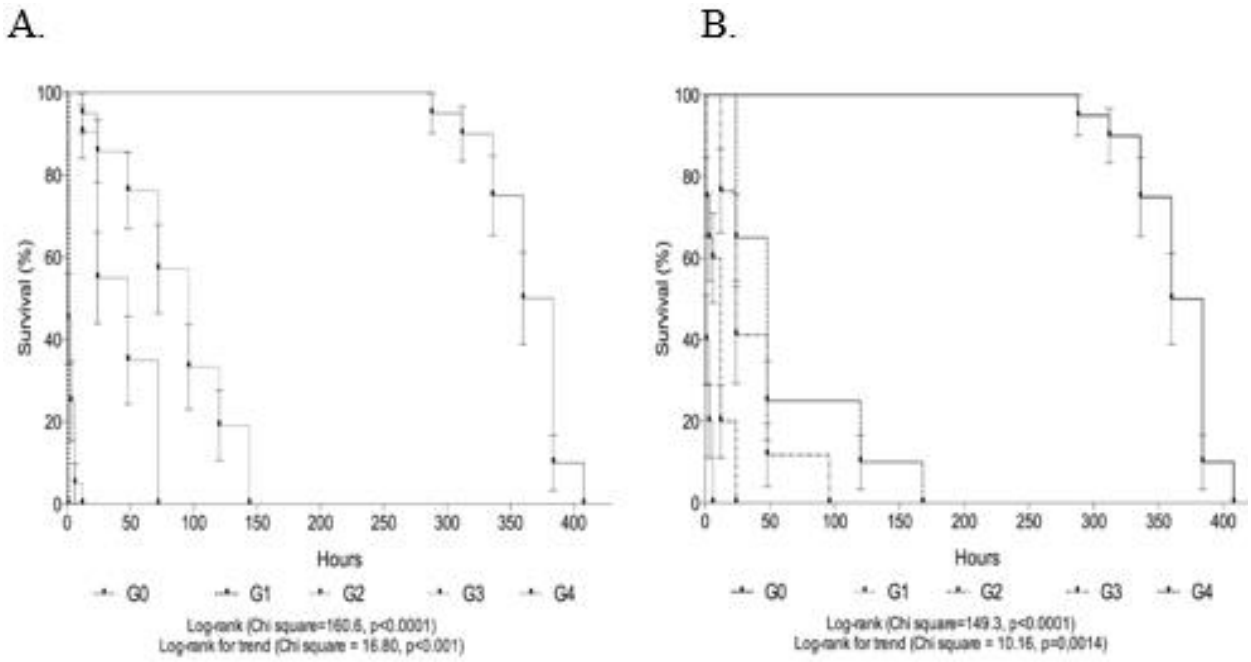

Figure 1. Survival rate (%) of *A. mellifera* L. worker bees fed with sugar candy contaminated with the highest or the lowest dose of thiamethoxam, imidacloprid and acetamiprid recommended by the manufacturers to melon crops. A) Highest doses. B) Lowest doses. Note: G0: control, sugar candy without insecticide + distilled water; G1: sugar candy + thiamethoxam; G2: sugar candy + imidacloprid; G3: sugar candy + Mospilan®/acetamiprid; G4: sugar candy +Orfeu®/acetamiprid

according to whom the movements of bees were disordered and shaking, and they died a few hours after being exposed to sub-lethal doses of thiamethoxam (150 g/L H_2O). Antunes-Kenyon and Kennedy (2001) observed mortalities in less than 3 h. Thiamethoxam caused an 89% mortality of individuals exposed to contaminated food and spraying of sugar candy containing the insecticide.

These results corroborate those found by Laurino et al. (2011), showing that, in addition to direct damage, the effects caused by thiamethoxam chemical molecules may have a cytotoxic action, causing vacuolation, that is, formation of clear vacuoles in the midgut of bees. This was also reported by Oliveira et al. (2013) on evaluating the deleterious effects of chemical molecules on the midgut of bees. Catae et al. (2014), after exposing *A. mellifera* L. to a diet containing a sub-lethal dose of thiamethoxam 1/10 of CL_{50} (0.0428 ngi.a./L of diet) for up to 8 days, reported that the continuous exposure to a sub-lethal dose of thiamethoxam may damage organs responsible for the metabolism of the insecticide molecule.

The neonicotinoid insecticide Evidence 700 WG®, which contains the active ingredient imidacloprid, at the highest and the lowest dose, 1.5 and 1.0 g.p.c./L, respectively, proved to be toxic to bees when applied together with food, similar to thiamethoxam. However, the mortality of bees occurred within 48 h (Figures 1A and B). Thirty minutes after an oral treatment with imidacloprid, Decourtye et al. (2004) found a deficiency in the olfactory

learning of bees contextualized by a proboscis extension response, that is, the chemical molecule affected the functioning of the insect's brain, causing mortality. Such toxicities significantly reflect on the life of these organisms, decreasing considerably as compared to their useful life, which may have an average of 960 h, equivalent to 40 days (Rocha, 2008). In this study, the control treatment had an average useful life of 18 days under laboratory conditions. The insecticides Orfeu® and Mospilan®, both with the same active ingredient (acetamiprid), at concentrations of 0.25 and 0.3 g.p.c./L , also proved toxic to bees. However, they were toxic after 78 h, reducing up to 11 days the useful life of bees as compared to the survival time of the control treatment.

Experimentally, chemical molecules caused early death of bees assuming that these organisms, if contaminated in field, would not return to their colony. It is assumed they would be vulnerable to attack from predators because of intoxication symptoms observed in this study.

The evaluation of toxicity by the tested insecticides to bees, with their respective doses, showed that if exposed to contaminated food, there is a fast death of some specimens. It would justify the non-return of bees to colonies because of a possible suppression of mass of these organisms in the area when such insecticides are used.

Pesticide poisoning may occur during the collection of nectar and contaminated pollen grains since neonicotinoids are chemicals with a contact and systemic action. They can be dispersed internally or deposited in different parts of the plant, consequently appearing in the floral components of melon when sprayed with such insecticides, since the systemic insecticide circulates with the sap (Mariconi, 1977).

Medrzycki et al. (2003), evaluating the effect of sub-lethal doses of imidacloprid on the behavior of bees, observed a decrease in mobility and communication among insects, which hindered their social behavior. Tome et al. (2012) also found changes in the behavior of stingless bees when fed with a diet contaminated with imidacloprid.

This is in line with reports by Freeman and Doherty (2006), who described the occurrence of toxicity of imidacloprid on adult bees, consequently decreasing activities such as pollen conduction frequency during foraging and formation of a number of operculated cells. Such events were probably caused by the effects (hyperarousal/tremors) of agonist acetylcholine molecules, active ingredients of neonicotinoids. Possibly, the side effects compromise the growth of colonies in the field by affecting the ability to search for food and exerting direct effects on the propagation of new individuals, as reported by Whitehorn et al. (2012).

Considering the above, the treatments with thiamethoxam and imidacloprid caused symptoms of poisoning shortly after the beginning of the intake of contaminated food, inducing a poor motor coordination (tremors) and prostration of intoxicated individuals at the bottom of the cages. The presence of regurgitations of food consumed was noted. It is evidenced by regurgitated remains in the glass plate of the boxes where the bees were accommodated. Such symptoms are supposedly a result of the action of the neurotoxic insecticides under evaluation. They act as acetylcholine agonists on the central nervous system of insects in nicotinic post-synaptic receptors (Nauen et al., 2001). However, they are not degraded by the enzyme acetylcholinesterase, which causes hyperarousal (tremors) and consequently insect death (Carvalho et al., 2009).

All treatments at their lowest doses showed symptoms of poisoning six h after exposure to contaminated food. However, they also decreased the survival time of bees, with average values of 6, 96 and 168 h (Figure 1B), related to intake of thiamethoxam, imidacloprid and acetamiprid, respectively. These were different times from those observed for the highest doses.

According to Iwasa et al. (2004), the neonicotinoid thiamethoxam has a nitro group (N-nitroguanidine), making this molecule on average 192 times more toxic to bees than molecules with a cyano group (N-cyano-amidine) such as acetamiprid. This probably justifies the difference in toxicity considering bee lethal times. For the authors, the low toxicity of acetamiprid in relation to other neonicotinoids studied in this work could be associated with the high detoxification capacity of such molecules mediated by enzymes dependent on the cytochrome P450 of nerve cells. The authors reported that the difference in toxicity, even between active ingredients of a same chemical group, such as imidacloprid, is approximately 400 times as compared to acetamiprid. The importance of toxicity studies using different plant protection products are thus evidenced even if they have the same mode of action on organisms in general.

Brunet et al. (2005) studied the metabolism of acetamiprid in *Apis mellifera* worker bees and reported that, when adult workers received acetamiprid containing radio isotopemarkers orally, more than 50% of the acetamiprid were metabolized in different parts of the insect's body (head, thorax, abdomen, hemolymph, midgut and rectum) in less than 30 min, indicating a very short half-life. During the first hours, the authors also found a higher frequency of acetamiprid on the nicotinic receptors of acetylcholine in the abdomen, thorax and head. This implies another hypothesis regarding the differences in toxicity responses of plant protection products of a same chemical group given to the bee population of this study.

For Iwasa et al. (2004), the products of the acetamiprid metabolism by *A. mellifera* do not have a high toxicity. This could explain the significant difference among insecticides of a same chemical group, a result of this study, and the toxic effects of acetamiprid, thiamethoxam and imidacloprid. This discrepancy may also be

elucidated by the lower affinity to the nicotinic acetylcholine receptors of acetamiprid as compared to imidacloprid (Tomizaw et al., 2000; Tomizawa and Casida, 2003).

Based on this information, it is assumed that differences in lethal times of the products evaluated in the present study indicate that lower concentrations may be less toxic to bees. However, there is a question regarding the toxic viability to insect-pests.

There are many challenges to be faced in this field in order to analyze the real situation of the insect and pesticides relationship. However, according to the results obtained and analyzed in this study, it is suggested that the application of neonicotinoid insecticides be performed not coinciding with the melon flowering period, so that there is no contamination of the plant reproductive parts and consequently pollinating bees, concomitantly present at this phenological stage of the crop.

Conclusions

Regardless of the dose of insecticide added to the diet (sugar candy), thiamethoxam, imidacloprid and acetamiprid are toxic to *A. mellifera* L. worker bees.

The insecticide, thiamethoxam is the most toxic to worker bees as compared to the other active ingredients evaluated, imidacloprid or acetamiprid, regardless of the dose added to the food (sugar candy).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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