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TOXIC EFFECTS OF NEONICOTINOIDS ON *APIS MELLIFERA* L. WORKERS (HYMENOPTARA: APIDAE)

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ABSTRACT

Neonicotinoid insecticides are applied in the cultures of melon (Cucumis melo L.) to control pests of insects. However, insecticides can have indirect effects on bees, which are important pollinators of melon crops. The objective of this work was to evaluate the toxicity of neonicotinoids to the bee Apis mellifera, Linnaeus. The bioassays were carried out at the Entomology Laboratory of the Center for Agro-Food Science and Technology (CCTA), Federal University of Campina Grande (UFCG), and repeated measures were taken in the mortality time for thiamethoxam, imidacloprid and acetamiprid products (two formulations: Orpheus and Mospilan). Sprays of chemical solutions containing the highest and lowest doses recommended by the manufacturers were sprayed manually (500mL capacity and 0.58mL flow rate and average application rate 1.5 ± 0.5mL solution/cm2). The work consisted of 9 treatments with five replicates. In each plot, we used ten adults of worker bees with 48 hours (h) of age. After spraying, the bees were transferred to a wooden cage (11.0 in length x 11.0 cm wide x 7.0 in height) with a glass cap and kept in an air-conditioned room at 25 ± 2°C, UR 70 ± 10% and 12h of photophase, disposed of in completely randomized design. Observations of the dead insects were made in the hours one, two, three, six, 12, 24, 48, 72 and 96 hours. Regardless of dosage, all insecticides evaluated were toxic to A. mellifera workers. Thiamethoxam showed higher toxicity among the evaluated active ingredients, killing all bees within 1h after spraying, regardless of the dosage.

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INTRODUCTION

Neonicotinoid are neurotoxic insecticides (Nauen *et al.,* 2001; Gallo *et al.,* 2002), applied to pest-control (LIMA *et al.,* 2012) of melon (*Cucumis melo* L.). Melon is an important crop in Brazil, leading the ranking of the exported volume of fresh fruit to the international market in recent years (Reetz *et al.,* 2015). Although, the use of neonicotinoid provid positive and immediate results in the population suppression of insect pests, there are evidence of collateral damage to populations of beneficial organisms (Peres *et al*. 2013; Chagas, 2017), as bees (Schneider *et al.,* 2012; Palmer *et al.,* 2013; Sandrok *et al.,* 2014; Catae *et al.,* 2014; Barbosa, 2017; Pacífico da Silva *et. al.*, 2015; Araújo *et al.,* 2017), which are pollinators of melon crops (Dantas *et al.,* 2013; Ribeiro, 2015). Neonicotinoids are applied via treatment of seeds, soil, water irrigation and leaf spraying (Thompsom, 2010; Blacquière *et al.,* 2012) in many crops used by bees (Whitehorn *et al.,* 2012; Godfray *et al.,* 2014). The imprecision in the application of insecticides and dose failures expose the bees to intoxication (PIMENTAL, 1995). The amount of applied insecticides sometimes does not reach the target, is lost by drift, leading to long-distance intoxication over non-target insects. Sublethal doses might influence bee learning, ability to orient, foraging capacity and

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fecundity (Thompson e Maus, 2007; Schneider *et al.,* 2012; Pacífico da Silva *et al.,* 2016), and the ingestion of contaminated forest resources (Fairbrother *et al.,* 2014). Given the possible damage that insecticides can cause to beneficial insects, especially bees, and the need to preserve these pollinating agents, the objective of this work was to evaluate the toxic effect of the neonicotinoid insecticides used in the melon for pest control on bees workers of *A. mellifera* L.

MATERIAL AND METHODS

The bioassays were realized at the Laboratory of Entomology, Center of Agricultural Sciences (CCA), Federal University of Campina Grande (UFCG), Campus of Pombal, Paraíba, Brazil. Bee species were taken from beehives at the Apiary of Laboratory of Entomology, at the city of São Domingos de Pombal, Paraíba, Brazil. At this Apiary, the beehives are installed in boxes composed by Langstroth type wooden structures, in the natural environment. Before sampling, beehives passed a process of "preparation" for obtaining and selecting insects for the experiment. The preparation consisted of feed the colonies with artificial diet, composed of water and sugar solution in a 1:2 ratio, enriched with 2.5% of Glicopan ® (product based on free amino acids) and 2.5% Aminomix ® (product composed of vitamins, minerals and, amino acids). They were delivered weekly in individual feeders with a capacity of 500 mL. The artificial feed was provided to stimulate bee reproduction, reaching the appropriate population state for bioassays. For the selection of bees, we analysed the general aspect of population at the beehives, regarding as appropriate those that presented all the spaces of the nest configured for production of young forms (brood), an amount of adult bees covering 2/3 of the breeding area and a food collection activity that resulted in a return flow above 100 (one hundred) bees per minute. From the selected beehives, we took the workers at pre-emergence to adulthood stage, to get newly emerged adults. The hives with bees were packed in a wooden box fitted in a motor vehicle to transport them to the Laboratory of Entomology. We used four neonicotinoid insecticides, applied in the lowest and highest recommended concentrations for control of insect pests in melon culture (Table 1). Each dose of the neonicotinoids represented a treatment; the control consisted only of distilled water. The bioassay was arranged in a completely randomized design with 09 treatments (Control, and the four neonicotinoid insecticides at low and high concentration). Fifty insects per treatment were used, distributed in five replicates with ten workers of newly emerged bees per plot. It was kept in an air-conditioned room at 25 ± 2°C, 70 ± 10% RH and 12h photophase.

The insecticides were applied via topical sprays directly on the bees, which were conditioned in Petri dishes of 10 cm in diameter. For the topical sprays, we useda manually pressurized spray, with a capacity of 500 mL, a flow rate of 0.58 mL/s, with an average application rate of 1.5 ± 0.5 mL of chemical solution/cm². After insecticide application, the bees were transferred to wooden boxes (11.0 cm long by 11.0 cm wide and 7.0 cm high). The boxes had holes in the sides sealed with nylon mesh for ventilation. Boxes were previously lined with filter paper at their inner base and contained a transparent glass lid at the top for observation, according to the methodology proposed by Maracajá *et al.* (2006) for bee toxicology bioassays. The bees were fed a food composed of 10 g of Cândi paste (WAHL, 1968) packed in a feeder, containing a piece of cotton soaked in distilled water. The action of insecticides on bees was observed in the range of one, two, three, six, 12, 24, 48, 72 and 96 hours after application. The biological parameter evaluated were the mortality rate over time, verifying the number of dead insects, and the behavior during the observed intervals. Statistical analysis was performed using the program Graph Pad Prism (v.5 for Mac). The Kaplan-Meier test followed by log-rank test were used to estimate the median survival time with 95% confidence interval, and the difference between groups. The level of significance was set at $p < 0.05$.

Table 1. Commercial names, active ingredients and doses of neonicotinoids insecticides applied in toxicity tests on workers of *Apis mellifera* L. under laboratory conditions

Commercial Name	Active Ingredients	Dose (g p.c./L of water)	
		Lowest	Highest
Actara 250 WG	Tiametoxam	0.3	3.0
Evidence 700 WG	Imidaclopride	1.0	1.5
Mospilan	Acetamipride	0.25	0.3
Orfeu	Acetamipride	0.25	0.3

RESULTS AND DISCUSSION

All insecticides were toxic to workers of *A. mellifera* L. Under the highest doses, the bees had a mortality of 100%, 24h after application (Figure 1). However, in the treatment of the active ingredient thiamethoxam, bees showed symptoms of intoxication up to 1 hour after direct contact with the chemical molecule, with 100% of mortality at the first hour, characterizing it as the most toxic to bees among the tested insecticides. The treatment with imidacloroprid was the second most deleterious for bees. Intoxication was observed in the early hours, showing hyperexcitation, paralysis, and disorientation, and all the bees died within six hours (Figure 1). The results from the two treatments with acetamiprid have a similar behavior, with intoxication at the first hours, but the bees resisted up to 24 hours after direct contact with the chemical molecule. The bees showed a mean longevity of 18 days, at the control treatment. Although the symptoms of intoxication were similarly observed in the first hours after their contact with the chemical molecules of the tested insecticides, the lifetime of the bees varied significantly between the control treatment and all chemicals, and these with each other.

These variations are probably related to the different forms of absorption of the chemical molecule by the cuticle of the insects, as the effective interaction of the chemical with the tegumentary layers, lipoproteins that are part of the exoskeleton, which can present a hydrophobic characteristic, delaying the absorption of insecticides with a polar chemical molecule. Also, tarsal contact contamination on contaminated surfaces or the respiratory tract (spiracles) may be different among tested insecticides. Our results show that these insecticides may have a strong effect on *A. mellifera*bees, which can potentially lead to serious ecological problems. The majority of neonicotinoids are applied via foliar spraying (Thopsom, 2010; Blacquière *et al.,* 2012), thus making bees that forage in the target crops susceptible to the application of the chemical solution, although they were initially manufactured and marketed for seed treatment and spraying on the seedlings. The foliar sprayingin the phenological phases of vegetative growth and reproduction leave residues in leaves and flowers, respectively, of the crop itselfor even of weeds found next to treated crops (Krupke *et al*., 2012).

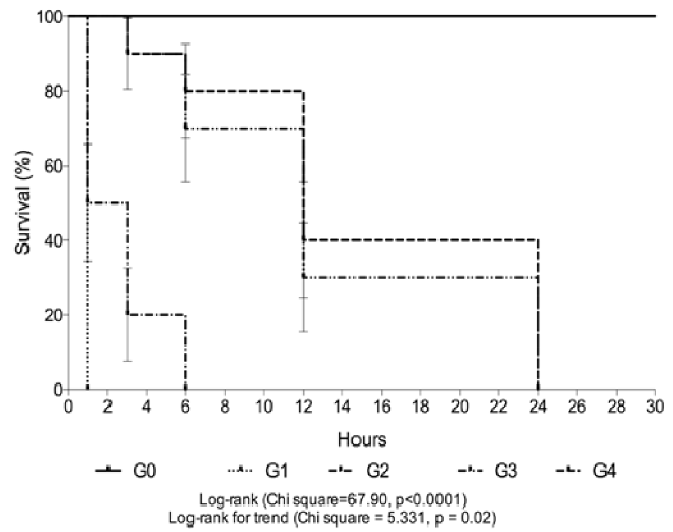

Figure 1. Survival (%) of *Apis mellifera* workers contaminated by direct spraying with the insecticides thiamethoxam, imidacloprid or acetamiprid, at the highest doses recommended by the manufacturers for the melon crop. Note: G0: control, solution consisting of distilled water; G1: thiamethoxam (3.0 g p.c./L H_2O); G2: imidacloprid (1.5 g p.c./L H_2O); G3: Mospilan® / acetamipride (0.3 g p.c./L H_2O); G4: Orfeu® / acetamipride (0.3 g p.c./L H_2O)

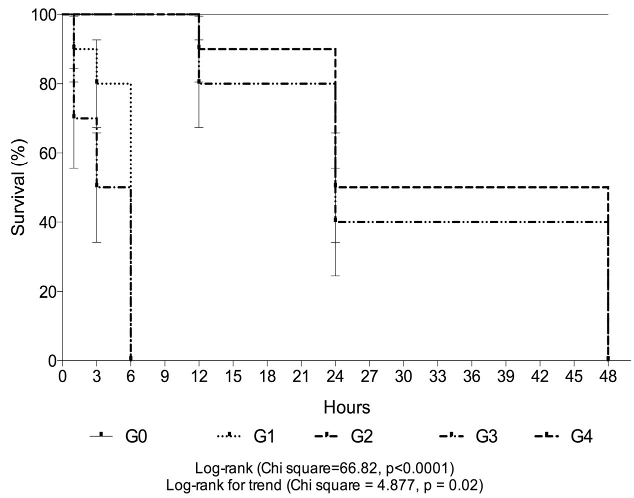

Figure 2. Survival (%) of *Apis mellifera* workers contaminated by direct spraying of solution with the insecticides thiamethoxam, imidacloprid or acetamiprid, at the lowest doses recommended by the manufacturers for the melon crop. Note: G0: control, solution consisting of distilled water; G1: thiamethoxam (0.3 g p.c./L H_2O); G2: imidacloprid (1.0 g p.c./L H_2O); G3: Mospilan® / acetamipride (0.25 g p.c./L H_2O); G4: Orfeu® / acetamipride (0.25 g p.c./L H_2O)

Residues of insecticides, such thiamethoxam, has been found in dead bees in apiaries (Krupke *et al*., 2012), which were probably contaminated by the application in crops for the control of insect pests. Our results corroborate with the idea that those kinds of insecticides have negative effects on non-target groups of species, like bees. Even at the lowest doses of the insecticides indicated by the manufacturers, the bees showed symptoms of intoxication within 1 hour after contamination, and 100% mortality is observed after 6 hours of exposure in the treatments containing thiamethoxam and imidacloprid, and after 48 hours in the treatments with acetamiprid. Thiamethoxam was the most toxic among the insecticides tested, followed by imidacloprid, even at the lowest doses. Such results corroborate with Noninato (2012), which found a 100% of bee mortality 6hours after exposure to thiamethoxam. Similar results were also observed by Carvalho *et al*. (2009) and Costa *et al*. (2014) for *A. mellifera* and others pecies of African bees.

Differently, Pereira (2010) applying a limit dose of 17 nd/bee (LD_{50}) reported the mortality of bees only during the first 8 hours, and after this, no bee was found dead, which suggests the complete metabolization of the toxic compound by the remaining bees. However, the dose used by Pereira (2010) is lower than that used in the presentstudy. At the present study, the concentrations are similar to those applied in the field, according to the manufacturer's recommendations, where the lowest doses are indicated to be applied when the insects are in the stage of establishment in the crop, and the higher doses when the population level reaches higher levels of pest control in the melon crop, mainly for the whitefly *Bemisia tabaci* (Gennadius) (Diptera: Aleyrodidae). The toxicity of imidacloprid was also found by Nauen *et al*. (2001b) and Schumuck *et al*. (2003), that indicated a LD_{50} of 49 to 102 ng/bees and 42 to 104 ng/bees, respectively.The high toxicity of imidacloprid is due tothe presence of a nitro functional group, which gives great affinity to the nicotinic acetylcholine receptor, causinga nervous collapse in the insect and, consequently, its death (Tomizawa and Casida 2003).

At the lowest doses, the insecticides containing acetamiprid (the products Mospilan® and Orfeu®) affected bees after the first hour. The first deaths were recorded only from the 12th hour after the contamination, reaching 100% mortality after 48 hours, unlike the other insecticides (thiamethoxam andimidacloprid), where the bees did not resist the exposure beyond 6h. Costa *et al*. (2014) also considered acetamiprid as toxic for *A. mellifera*, finding100% of mortality in up to 2 hours, differing only in lethal time. However, Iwasa *et al*. (2004) reported that when applied topically to bees, a dose of acetamipridem of 50 µg/bees was not toxic. For Iwasa *et al*. (2004), the toxicity of the neonicotinoids is altered as a function of the radical bound to the main nitrogen of the carbonic chain of the chemical molecule. Depending on the radical, its Lethal Dose (LD50) can vary in considerable terms, or until it forms another product. Thus, when with the main nitrogen of the carbonic chain exists a radical of a cyano chemical group (which occurs in the acetamiprid), it can replace the nitro group, in the constant disintegration reactions of the molecules, decreasing the toxicity of the molecule, as acetamiprid when compared to the other neonicotinoids. Regarding our results, we can conclude that even the lowest recommended dose may be detrimental to *Apis mellifera* survival, affecting its populations and its role as pollinators species. The care for a long-term sustainable management of crops requires the reduction of the use of insecticides of the neonicotinoid type to ensure the survival of pollinating species, or the use of insecticides with a narrower target, reducing the risk of contamination by non-target species.

Conclusion

Regardless of the exposure dose of the bees to the tested pesticides, thiamethoxam, imidacloprid, and acetamiprid are toxic to *Apis mellifera* workers. The insecticide thiamethoxam is the most toxic to the workers of Apis mellifera among the active ingredients tested.

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