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RESEARCH ARTICLE - BEES

Toxicity of Fenpyroximate, Difenoconazole and Mineral Oil on *Apis mellifera* L.

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Abstract

Bees of genus *Apis* are the main crop pollinators; however, the use of pesticides in agriculture may intoxicate them during foraging. In this study, we evaluated the toxic effects caused by difenoconazole (fungicide), fenpyroximate (acaricide) and mineral oil (adjuvant) used alone and associated (pesticide + adjuvant) on workers of *Apis mellifera* L. Bees were exposed to product doses recommended by manufacturers, orally and in contact on a contaminated surface in a controlled environment. All products presented low lethality, both in isolation and combination (except for difenoconazole via contact), however, they all showed toxic effects. The results showed that combination of pesticides with adjuvant augmented toxic effects.

Introduction

Insects are the main responsible for sexual reproduction of most species of cataloged angiosperms, increasing genetic variability of plants (Raven et al., 2001; Michener, 2007). In addition, insects are associated to better fruit and seed productivity and development (Kerr et al., 1996; Imperatriz-Fonseca & Nunes-Silva, 2010; Putra et al., 2014; Silva et al., 2015), play an essential role for maintenance and balance of ecosystems. In this context, studies seek to measure the actual value of services to the environment that pollinators have (Lonsdorf et al., 2009; Gallai et al., 2009; Giannini et al., 2015).

However, the growth of agriculture and deforestation, driven by the intensification of agricultural practices (Leblois et al., 2016), favors the emergence of pests and diseases, leading farmers to use pesticides to control insect pest populations (Ecobichon, 2001; Genersch, 2010; Coupe et al., 2012), which, in turn, affects both food production and the environment (Schreinemachers & Tipraqsa, 2012; Guedes et al., 2016).

Originally, the relationship between plant and pollinator was essentially positive; however, pollinators are suffering from the frequent use of pesticides in floral resources (Blacquière et al., 2012; Fürst et al., 2014; Morais et al., 2018). Bees are some of the main pollinating agents and have become the most affected individuals, due to the contact with contaminated sources that cause behavioral disorders and even death of individuals (Sandrock et al., 2014; Goulson, 2015).

Numerous factors can compromise colony development and perpetuate pests, parasites and pathogens (Genersch, 2010). Among them, the use of pesticides affects pests and bee species (Della Lucia et al., 2014; Rondeau et al., 2014).

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Studies on acaricide fenpyroximate (Dahlgren et al., 2012; Li-Byarlay et al., 2014) have reported its toxicity on *Apis mellifera* L. Syromyatnikov et al. (2017) and Kinasih et al. (2017) also described the effects of the fungicide difenoconazole on *Bombus terrestris* L. and *Trigona* (*Tetragonula*) *laeviceps* Smith.

Fenpyroximate is an acaricide belonging to the chemical group of pyrazoles, classified as highly toxic and very dangerous to the environment. Fungicide difenoconazole is a triazole, classified as extremely toxic and environmentally very dangerous. Mineral oil is an aliphatic hydrocarbon indicated to several crops, classified as low toxic and not dangerous to the environment and it can be applied as an adjuvant added to pesticide syrup (MAPA, 2018).

Studies have also proven that neurotoxic insecticides affect the immune system of the *A. mellifera*, favoring the onset of diseases (Brandt et al., 2016) and showing that secondary effects can be as damaging as the lethal ones. However, toxicity caused by secondary effects is more difficult to diagnose, as it does not present immediate lethality and may further promote the spread of the active principle within the colony. In this context, this work aimed to analyze toxicity (on survival and secondary effects) of pesticides (fenpyroximate, difenoconazole and mineral oil) on *A. mellifera* when exposed to the contaminated surface of *Citrus* leaves and the ingestion of candi paste, contaminated by these products.

Material and Methods

Products used and description of bioassays

Bioassays were carried out to test the effects of the maximum recommended doses (Table 1) of products for the phytosanitary control in *Citrus* crops on *A. mellifera*. For that, the formulated products used were fenpyroximate (50 g/L of the active ingredient in the formulated product), difenoconazole (250 g/L of the active ingredient in the formulated product) and mineral oil (756 g/L of the active ingredient in the formulated product). The products were purchased at a commercial store. Workers of *A. mellifera* were collected from three colonies kept in Langstroth boxes in an apiary at the Federal University of the Recôncavo da Bahia, Cruz das Almas, Brazil.

Plastic cages (30 cm in diameter and 4 cm high) were made for bioassays with holes in the closed caps with *voile* fabric for air circulation for comfort of the bees and two lateral holes to fit the feeders adapted from centrifuge microtubes (one with water and another with the candi paste).

Bees were exposed to the products as follows:

a) Exposure through ingestion - Before being exposed to contaminated food, the bees were kept for three hours without feed. For each treatment, the recommended dose was added to 100 mL of honey and confectionery sugar homogenized with a glass stick in a Becker to form the candi paste. Then, the paste was offered to the bees in microtubes inside the cages for 96 h.

b) Contact exposure to contaminated surfaces (by pesticides) - leaves of lemon *Citrus aurantifolia* var. thaiti, collected from a plant without phytosanitary treatment, were immersed for 5 min in the solution with each pesticide, which was diluted in water as described in Table 1 (adapted from Carvalho et al., 2009). Then, the leaves dried at room temperature for about two hours. As a control treatment, the leaves were immersed for 5 min in distilled water.

Assessment of toxicity (survival and secondary effects on bees)

All treatments with different types of exposure were installed in chamber type B.O.D. at temperature (30 ± 5 ºC) and relative humidity (70 ± 5%) controlled, with absence of light. We evaluated the mortality of each bee at intervals of one, two, three, four, five, six, nine, 12, 15, 18, 21, 24, 30, 36, 42, 48, 60, 72 and 96 h after the beginning of the treatments (Carvalho et al., 2009).The secondary effects were evaluated by means of bees observation: disorientation, paralysis, prostration, hyperexcitation, impaired motor coordination and agitation, according to Cox and Wilson (1984), and Carvalho et al. (2009).

Statistical analysis

A completely randomized design was used to measure the survival rate of the bees, with five treatments on different exposure methods (mineral oil, difenoconazole, fenpyroximate, difenoconazole + mineral oil, fenpyroximate

Table 1. Active principle, class, maximum dosages recommended by the manufacturer (DMR), target organism and chemical group of pesticides used in *citrus* orchards.

Active Principle	Class	DMR[1]	Target Organism	Chemical group
Difenoconazole	Fungicide	20 mL/100L	*Colletotrichum gloeosporioides*	Triazole
Fenpyroximate	Acaricide	100 mL/100L	*Brevipalpus phoenicis*	Pirazol
Mineral oil	Adjuvant	2 L/100L	*Orthezia praelonga*	Aliphatic hydrocarbons

[1]in accordance with Ministry of Agriculture, Livestock and Food Supply (MAPA), Brazil.

+ mineral oil) and with a control composed of distilled water with five repetitions. Each repetition was composed of 10 bees, totaling 50 bees per treatment and 300 bees per experiment, from colonies installed in a box of the Langstroth model.

The data were submitted to the survival analysis using the survival package and submitted to statistical analysis in R® software (R Development Core Team, 2016). Kaplan-Meier survival curves were generated to determine the proportion of surviving bees against times after application of pesticides by ingestion, contact with contaminated surface and topical contact. The Log Rank test was used to test the null hypothesis when the Kaplan-Meier curves were identical.

Results

Survival of bees

The survival of bees submitted to ingestion treatments presented significant differences according to the Log Rank test of Cox-Mantel ($\chi 2 = 8.8$, gl = 5, p <0.0001), with 80% of survival 96 hours after exposure to the active principle difenoconazole. Bees exposed only to fenpyroximate and difenoconazole + mineral oil had 94% and 92% survival rate, respectively, at 96 h of exposure. The survival of bees exposed to active principles mineral oil + fenpyroximate and only mineral oil were 92% and 84% at 72 and 60 h, respectively. Bees that were not submitted to the active products had a survival rate of 94% (Fig 1).

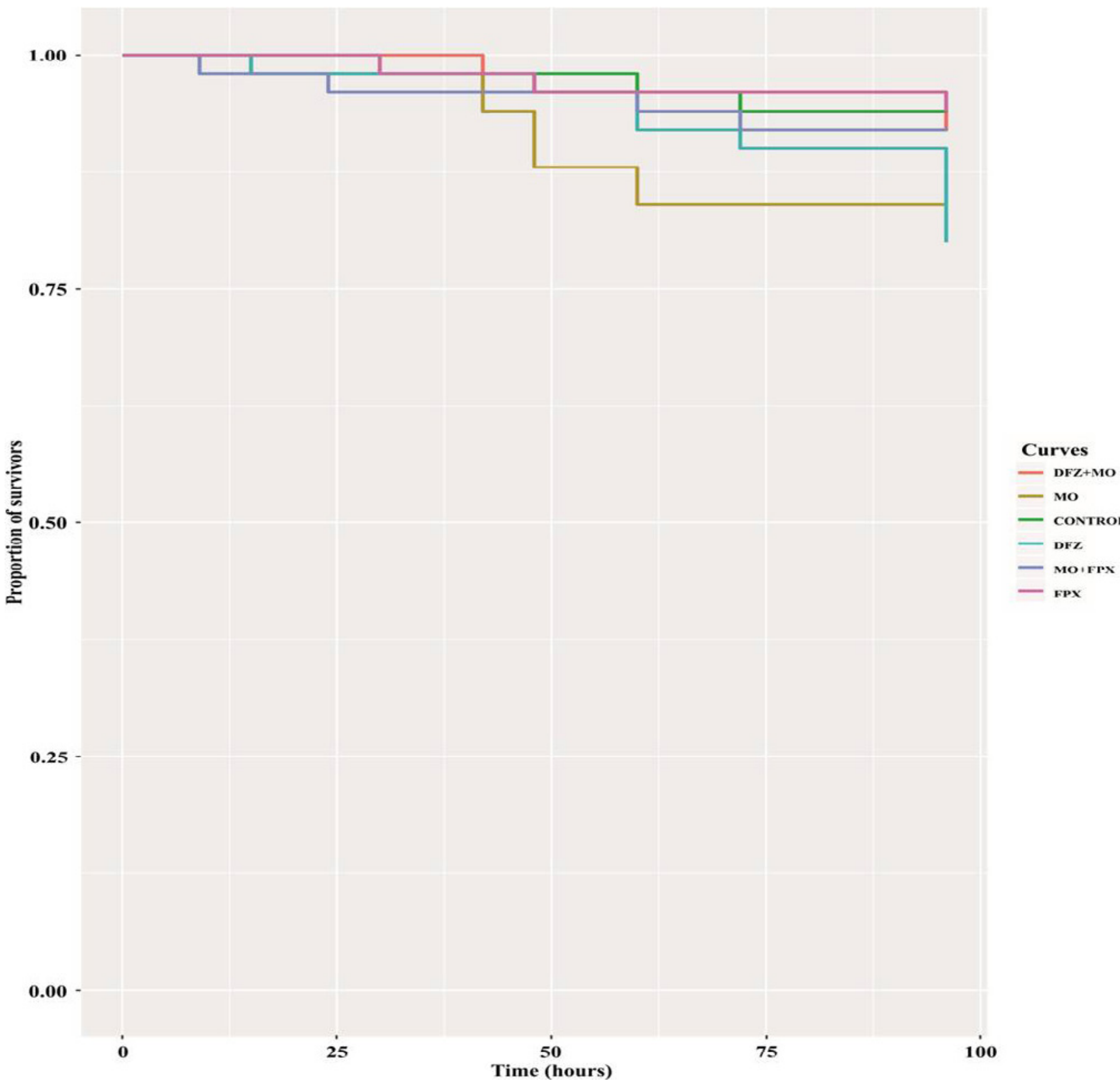

Fig 1. Survival curves plotted from exposure time (hours) via ingestion until death of each bee (*Apis mellifera*): Difenoconazole + Mineral oil (DFZ+MO); Mineral oil (MO); Control (CONTROL); Difenoconazole (DFZ); Mineral Oil + Fenpyroximate (MO+FPX); Fenpyroximate (FPX); . Curves indicate the median and 95%, respectively.

The survival curve of the Cox-Mantel Log Rank test showed significant differences in the survival rates between bees that were submitted to different pesticides applied by contact ($\chi 2 = 31.6$, gl = 5, $p < 0.0001$). There was mortality of 24% and 28% at 60 h after application of difenoconazole and mineral Oil + fenpyroximate, respectively. At 40 and 96 h, mortality of bees exposed only to fenpyroximate and to mineral oil was 22% and 24%, respectively. The survival rate was lower for bees exposed to difenoconazole + 50% mineral oil at 96 h. The survival rate of control bees was 96% (Fig 2).

Secondary effects on bees

During the study, behavioral changes of bees were observed in all treatments, except for the control, where bees had the same behavior throughout the evaluation period. Contact with mineral oil after 12 h of exposure to the product left the bees with impaired motor coordination, where the bees were unable to stay in the natural position and remained part of the time with the back to the ground.

Regardless of exposure type, difenoconazole showed effects after 15 h of evaluation, when workers of *A. mellifera* presented behaviors divergent to the control, such as agitation and changes in motor coordination. When difenoconazole was added to mineral oil, changes in motor coordination occurred after five hours of evaluation.

For fenpyroximate, bees submitted to the contact with this acaricide had alterations in the motor coordination after 12 h of exposure. Fenpyroximate added with mineral oil left

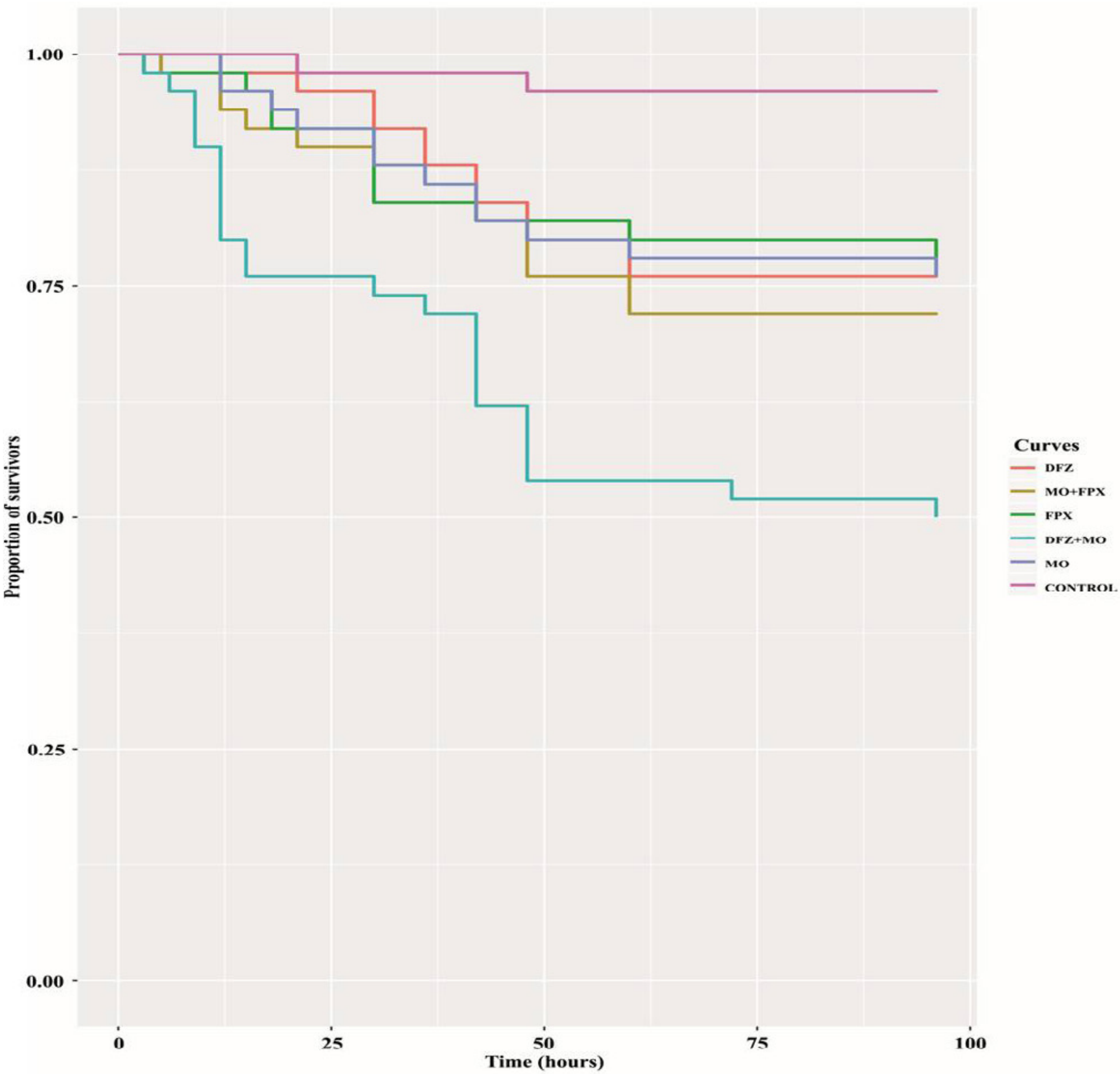

Fig 2. Survival curves plotted from exposure time (hours) via contact until death of each bee (*Apis mellifera*): Difenoconazole (DFZ); Mineral Oil + Fenpyroximate (MO+FPX); Fenpyroximate (FPX); Difenoconazole + Mineral Oil (DFZ+MO); Mineral Oil (MO); Control (CONTROL). Curves indicate the median and 95%, respectively.

the bees at first agitated and later inactive (they did not show any reactions and remained stopped all the time).

Pesticides appeared to have repellent action, since bees exposed to the contaminated food moved away from the food during a certain time. In contrast, bees consumed the food naturally in the control treatment during the evaluations.

Discussion

The application of difenoconazole, fenpyroximate and mineral oil in isolation had a little effect on bee survival or even in combination to the adjuvant (except difenoconazole via contact). However, secondary effects were evident. The products applied in combination with the adjuvant mineral oil had a faster and more noticeable action, causing agitation, changes in motor coordination, followed by prostration. Adjuvants enhance penetration and fixation to improve efficiency by reducing dispersion (Mullin et al., 2016). However, this combination has adverse physiological effects on non-target organisms, suggesting a negative point (Mesnage et al., 2014; Mullin, 2015). In this sense, Mesnage et al. (2013) reported that mixing pesticides with adjuvants may alter toxicity of pesticides.

Generally, adjuvants are considered biologically inert and are not evaluated as agents with toxicological potential for non-target organisms, especially when they are combined to pesticides (Ciarlo et al., 2012; Mesnage et al., 2013). However, Ciarlo et al. (2012) noted that adjuvants affect the olfactory ability of bees, which is essential for foraging. In our study, there was compromise of the search for food by the bees submitted to the treatments with pesticides. In addition, when pesticides were applied in combination, their effects were more pronounced.

Secondary effects can cause damage to adult bees, such as disorientation, which could compromise their return to the colony (Ingram et al., 2015; Silva et al., 2016). Furthermore, bees could transport the chemical products to colonies, accumulating pesticides in the food, which suggests poisoning the larvae when fed with these contaminated products (Johnson et al., 2010; Martinello et al., 2017).

Difenoconazole was more toxic to bees exposed to contact when associated with the adjuvant. Mineral oil may have augmented the effect of difenoconazole, since this adjuvant acts in the contact action mode (MAPA, 2018), while difenoconazole is a systemic action compound with high residual effect (Andrade Junior & Galbieri, 2014; Balardin, 2015). On the other hand, difenoconazole was classified as little toxic for *Trigona* (*Tetragonula*) *laeviceps* Smith by topical route (Kinasih et al., 2017), suggesting that this fungicide used without addition of adjuvants is considered nontoxic for bee survival. Nevertheless, the authors reported that this fungicide could cause secondary effects. Syromyatnikov et al. (2017) found that difenoconazole affected mitochondrial respiration and consequently reduced energy production in flight muscles of *Bombus terrestris* L.

Fenpyroximate applied in isolation or combination with the adjuvant did not significantly reduce bee survival in any of the contamination media evaluated. Fenpyroximate is an acaricide classified as highly dangerous to the environment and belonging to pyrazole, which is a chemical group of neurotoxic action. However, in our study, high toxicity regarding the survival of *A. mellifera* was not observed. On the other hand, Dahlgren et al. (2012) compared toxicity of fenpyroximate on workers and queens of *A. mellifera*, and reported that this acaricide was more toxic to bee workers than to queen. In our research, we also observed that bees exposed only to fenpyroximate showed changes in motor coordination; however, when exposed to fenpyroximate associated with mineral oil, the insects displayed agitation. Li-Byarlay et al. (2014) observed that the fenpyroximate could stimulate aggressiveness in bees of *A. mellifera*.

Bees could be, multiple times, exposed to the same active ingredient or more than one, which may affect the immune system by developing chronic problems, aggravating secondary effects (Whitehorn et al., 2012; Morais et al., 2018). Exposure of bees to pesticides could become even more harmful, since there is the possibility of repeated, simultaneous and synergistic exposure among and between different chemical groups (Luttik et al., 2012). In our study, despite low lethality of difenoconazole, fenpyroximate and mineral oil, except difenoconazole associated with mineral oil via contact, secondary effects were evident mainly when the products were associated with mineral oil.

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