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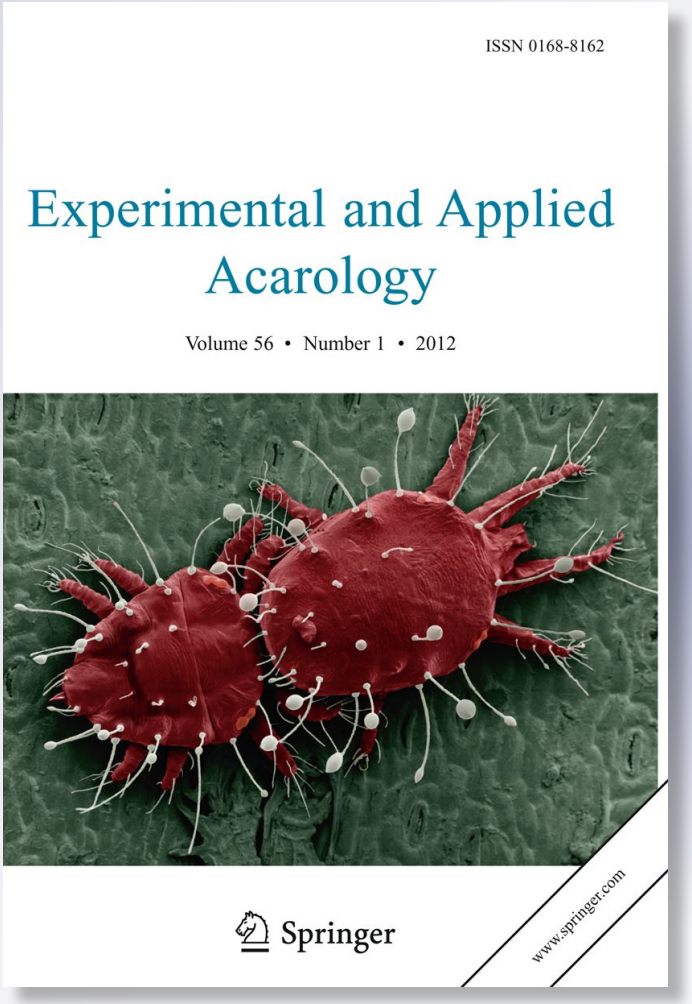

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Repellency of the oily extract of neem seeds (*Azadirachta indica*) against *Varroa destructor* (Acari: Varroidae)

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Abstract A crude oil extract of neem seed (*Azadirachta indica*, Sapindales: Meliaceae) was evaluated for repellency on *Varroa destructor* Anderson and Trueman. Burgerjon's tower was used to spray worker bee pupae with 0.0, 0.3, 0.7, 1.3, 2.6, 5.3, 10.6 and 21.1% neem extract concentrations. Sprayed pupae were attached to observation arenas and incubated at 32 ± 2°C and 70 ± 10% RH. The ability of *V. destructor* to locate and feed on treated and untreated pupae was monitored from 30 min to 72 h after spray. Higher and more stable repellency was achieved with 2.6, 5.3, 10.6 and 21.1% neem extract. At the highest concentration, 98% of *V. destructor* were prevented to settle on bee pupae, resulting in 100% *V. destructor* mortality at 72 h.

Keywords Honey bees · *Apis mellifera* · Neem · Sublethal effects

Introduction

Varroa destructor Anderson and Trueman (2000) is one of the most important mite pests of honey bees (De Jong 1990; Sammataro et al. 2000). Only a few products are available for its control, where the pyrethroids fluvalinate (Apistan® Basel, Switzerland) and flumehtrin (Bayvarol®, Leverkusen, Germany) are commonly used acaricides. The excessive use of these pesticides has resulted in the development of resistance in this mite (Elzen et al. 1999; Thompson et al. 2002; Rodríguez-Dehaibes et al. 2005). In addition, these pesticides

José Antonio Santizo-Rincón—deceased.

R. González-Gómez · G. Otero-Colina (✉) · C. B. Peña-Valdivia · J. A. Santizo-Rincón
Colegio de Postgraduados Campus Montecillo, Km. 36.5. Carretera México-Texcoco, 56230 Montecillo, Texcoco, Edo. de México, Mexico
e-mail: gotero@colpos.mx

J. A. Villanueva-Jiménez
Colegio de Postgraduados Campus Veracruz, Km. 88.5 Carretera Federal Xalapa-Veracruz, 91700 Manlio Fabio Altamirano, Veracruz, Mexico

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can be found contaminating wax and honey (Bogdanov et al. 1998; Wallner 1999; Bernardini and Gardi 2001).

Natural products such as essential oils and their components are known to exhibit pesticide activity; many of them have been evaluated through topical application, spraying and evaporation on *V. destructor* (Imdorf et al. 1999; Rosenkranz et al. 2010). Sublethal effects might be useful in the control of this mite, where the use of lower doses could interfere with mite ability to locate its host (Kraus et al. 1994). Colin et al. (1994) developed a protocol to identify sublethal effects on bee parasites with plant products.

Phytochemicals used for pest management are gaining importance. The neem tree *Azadirachta indica* A. Juss. (Meliaceae) is being used as source of several terpenoids. Azadirachtin (AZ) has been considered by many the most significant tetranortriterpenoid, acting as repellent, feeding inhibitor, and growth disruptor on arthropods (Schmutterer 1990; Mordue and Blackwell 1993). However, other triterpenoids like salannin, nimbin, deacetylnimbin, and nimbandiol, among others, show important sublethal effects on several insects, which at the end enhance the toxicity of neem oil (Stark and Walter 1995).

Melathopoulos et al. (2000a, b) observed a reduction in the infestations of *V. destructor* and the tracheal mite *Acarapis woodi* Rennie, after feeding bees with sugar syrup mixed with a neem extract. A neem-based pesticide topically applied or mixed with sugar syrup, and supplied as food, exhibited acute toxicity on *V. destructor* as well as reduction in the proportion of fecund females and viability of eggs (Peng et al. 2000). They also observed a reduction in *V. destructor* numbers when sprayed hives with neem oil; when neem oil with no AZ or other vegetable oils were used, *V. destructor* mortality in a lower proportion was achieved, meaning that mortality was due to the oil, and it also was caused by the AZ.

González-Gómez et al. (2006) did not find acute toxicity after spraying 0.3, 0.7 and 1.3% of neem oil extract on *V. destructor* females, worker bee pupae and adults; however, neem extracts exerted an important repellent effect on this mite, interfering with its ability to locate sprayed bee pupae and feed from them. As a follow up, this research proposed to explore the possibility of neem oil on having a long-lasting repellent effect at higher concentrations.

Materials and methods

Mites and insects

Varroa destructor females were extracted from drone cells using the trapping method developed by Maul (1983), and placed in plastic Petri dishes, where they received drone pupae as food. These mites were kept overnight in an incubator at 32 ± 2°C and 70 ± 10% RH, before moving them to the bioassay devices, a day later.

According to the classification of Jay (1963) and Rembold et al. (1980), worker bee pupae of the stages Pp (white body) and Pr (pink to purple eyes) were collected manually from capped cells, approximately 1 h before performing the bioassays.

Oily extract of neem

Neem seeds were collected in an orchard at Colegio de Postgraduados, Campus Veracruz, Municipality of Manlio F. Altamirano, Veracruz, Mexico. Neem oil was obtained by pressing seeds at a temperature lower than 35°C, using a stainless steel extractor, by applying a pressure of 1,406 kg cm^{-2}, and then stored at −3°C (González-Gómez et al. 2006).

Extract application

Mother solution used in bioassays comprised oil extract, distilled water and Tween 20 as emulsifier (1:1:1 w/w/w). Previous bioassays done by the authors (González-Gómez 2005; González-Gómez et al. 2006) found that the emulsifier Tween 20 did not exhibit repellent or toxic effect at concentrations similar to those used in this work. Treatments corresponded to 1, 2, 4, 8, 16, 32 and 64% of the mother solution, resulting in concentrations of 0.3, 0.7, 1.3, 2.6, 5.3, 10.6 and 21.1% of the crude original extract. As a control, distilled water was used. Neem extracts were sprayed on bee pupae using a Burgerjon's (1956) pulverization tower, calibrated to apply 1–2 mg cm^{-2} of each solution (Hassan 1985); this was achieved spraying 15 ml of the solution at a pressure of 0.7 kg cm^{-2}. To allow drop sedimentation, organisms were left inside the tower for 1 min after the spray.

Repellency choice test

As observation arenas, polyethylene Petri dishes were used following the design of Colin et al. (1994). Arenas had a zero (0) zone, corresponding to the centre of the dish, and two main zones (A and B) corresponding to both halves of the dish. Zones A and B had three subdivisions (a, b and c), delimiting two concentric areas on each extreme of the dish (Fig. 1). Ten *V. destructor* were placed in zone zero. On each extreme (zones Ac and Bc), three bee pupae were placed. Only pupae located in the zone Ac had been sprayed. Number of *V. destructor* on each zone and its subdivisions was registered 0.5, 1, 2, 4, 8, 24, 48 and 72 h after they were placed on zone 0. All treatments were replicated four times.

Repellency no-choice test

The same procedure for the repellency choice test was followed, except that all pupae in an observation arena were treated with the corresponding solutions. It accounted for the repellency effect of the treatments in absence of an alternative, untreated food source.

Azadirachtin content in the neem oil extract

AZ content was obtained as a relative indicator of the concentration of oil components; it was not intended to tag the repellent and toxic effect to this metabolite, but to the whole extract. The determination of AZ concentration was carried out by Laboratorio de Alta

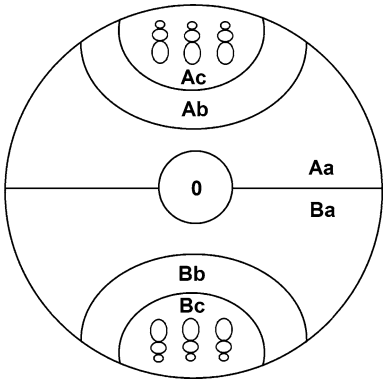

Fig. 1 Observation arena to test repellency of neem extracts. *A* and *B*, main divisions; *a*, *b* and *c*, concentric subdivisions around bee pupae; 0, central area where 10 *Varroa destructor* were placed

Tecnología de Orizaba, S. C., Mexico, following the method used by the Environment Protection Agency (Schneider and Ermel 1987), based on a HPLC modular system Perkin Elmer, ISO-9000 certified. Azadirachtin 95% (Sigma) was used as the standard.

Experimental design and statistical analysis

The first criterion used to evaluate treatment repellency was the allocation of mites on each bioassay zone. Allocation was transformed to position indexes (PI) using the formula given by Colin et al. (1994). Here, 140 is the maximum value and represents total repellency, whereas total attraction is a minimum value of 60; 100 is the intermediate value and is interpreted as the absence of any effect. As additional criteria, the number of mites found on bee pupae and the number of dead mites were recorded.

Data registered at each successive time were evaluated with one-way analysis of variance with Proc GLM (SAS 2000) on a completely randomized design; a Tukey test was used to separate means ($P \leq 0.05$). For the relation concentration-mortality correlation coefficients were estimated and Probit analysis was run at 24, 48 and 72 h, also using SAS (2000).

Results

Repellency choice test

Figure 2 shows PI′s on time for each neem oil treatment. The control exhibited PI values ranging from 97.5 to 103. Treatment 2% was never significantly different ($P \leq 0.05$) from the control while treatment 1% showed significant difference 4 h after *V. destructor* were exposed to bee pupae. The 4% neem oil extract presented clear repellency at 2, 4, and 24 h, with PI values around 120. In contrast, higher concentrations (8, 16, 32 and 64%) were significantly different from the control, as soon as 0.5 h after exposure to treated bee

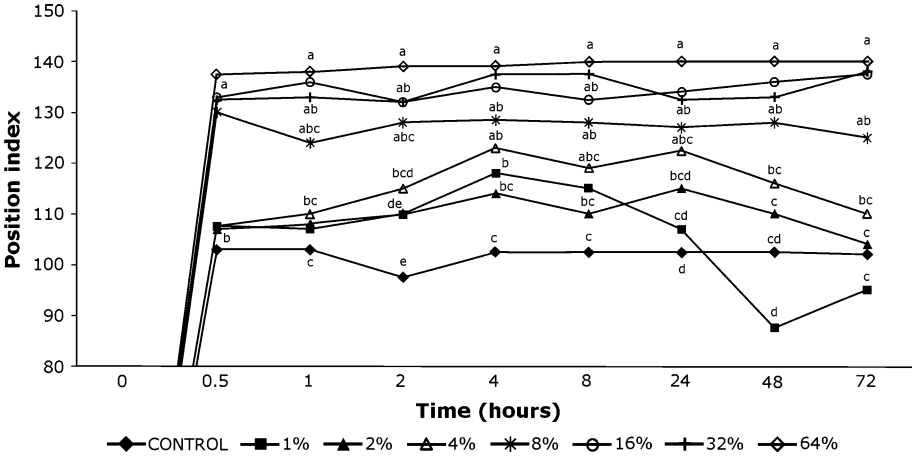

Fig. 2 Position index values after applications of several concentrations of oily extract of neem. The intersections marked with a *different letter* taken at the same time are significantly different (Tukey, ($P \leq 0.05$)

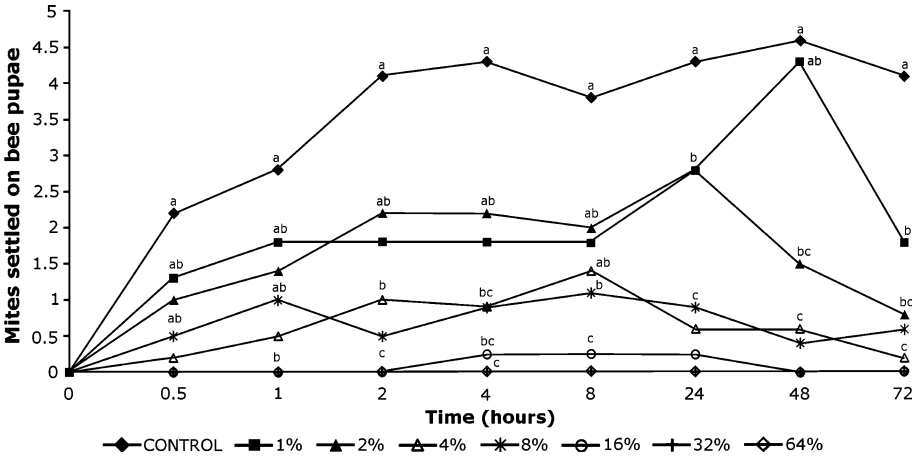

Fig. 3 Mean number of mites settled on bee pupae treated with neem extracts in the repellency test with choice. The intersections marked with a *different letter* taken at the same time are significantly different (Tukey, $P \leq 0.05$)

pupae, with PI ranging between 129 and 140. As concentration increased PI became higher and more durable. It was noteworthy that all *V. destructor* survived the entire observation time (72 h).

Figure 3 shows the mean number of *V. destructor* settled on bee pupae treated with different neem extract concentrations. After the first 2 h, four or more mites moved from zone zero towards the unsprayed pupae on both extremes of the Petri dish in the control. As neem concentrations increased, the number of mites settled on treated bee pupae was progressively lower; concentrations of 16, 32 and 64% had the most reliable values, significantly different from the control at all times, with the mean number of specimens settled on pupae fluctuating from 0 to 0.25.

Repellency no-choice test

Figure 4 shows the number of *V. destructor* that settled on bee pupae, during the observation time. The number of mites in the control was close to the total number of mites placed on zone 0, showing their ability to locate the pupae and feed from them. This value differed from the one observed in the control (approximate four mites) of the repellency choice test, because only specimens settled on the treated pupae were counted, ignoring the ones on the untreated pupae.

All neem concentrations resulted on a reduction in the number of *V. destructor* settled on pupae; however, at 4, 8 and 24 h, 1% neem extract was no significantly different ($P \leq 0.05$) from the control. The number of mites settled on pupae decreased with progressive concentrations. Two concentration groups could be characterized: in concentrations 1, 2, 4 and 8%, the numbers of mites on pupae per arena were from 3 to 6; on the other hand, concentrations 16, 32 and 64% had up to 2 mites settled on pupae. In all treatments the number of mites on treated bee pupae declined after 24 h of being exposed to neem residues. At 24 h mites also started to die.

The number of dead *V. destructor* in the no-choice test is shown in Fig. 5. Data start 24 h after treatments, before that time all mites were alive. All mites survived until the end

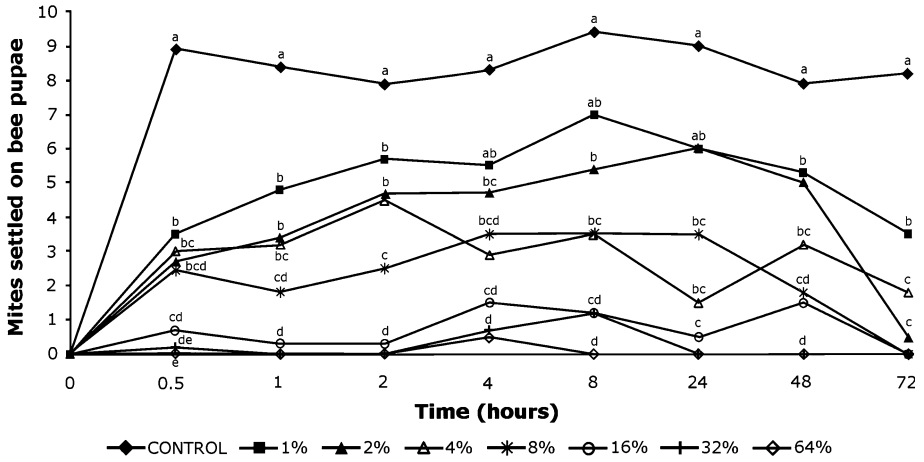

Fig. 4 Mean number of mites settled on bee pupae in a no-choice repellency test, after the application of different concentrations of oily extract of neem. The intersections marked with a *different letter* taken at the same time are significantly different (Tukey, $P \leq 0.05$)

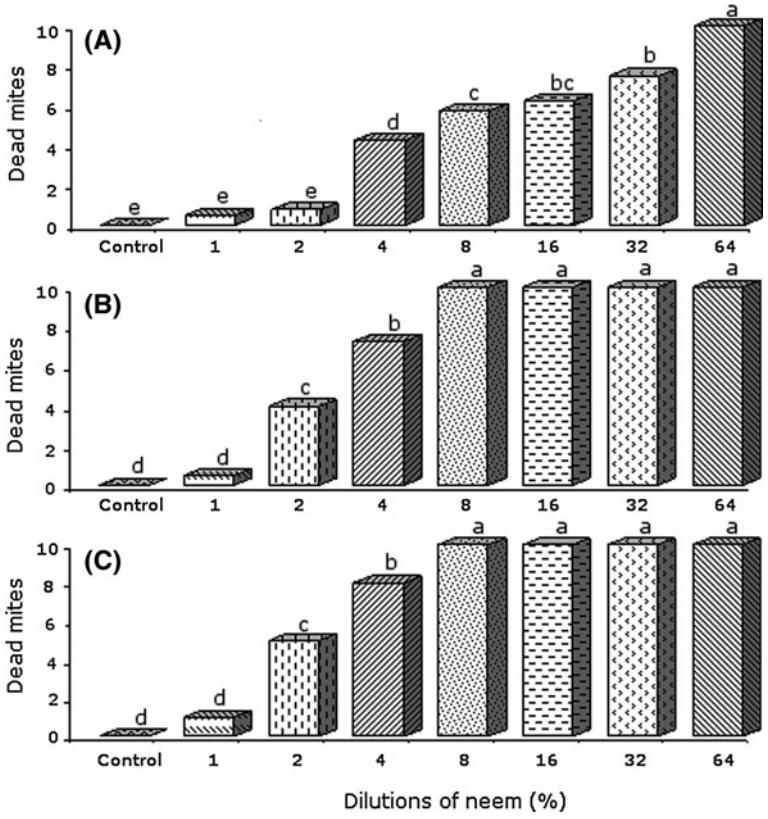

Fig. 5 Mean number of dead mites in a no-choice repellency test at **a** 24 h, **b** 48 h, and **c** 72 h. *Bars* marked with a *different letter* are significantly different (Tukey, $P \leq 0.05$)

of the study in the control. The correlation coefficient between concentration and mortality was positive and significant at all observation times ($r = 0.859$, 0.612, and 0.586 at 24, 48 and 72 h, respectively), indicating a strong association between variables. Mortality on 1% concentration at 72 h was not different from the control ($P \leq 0.05$); however at 48 h, 100% mortality resulted for concentrations 8, 16, 32 and 64%.

Lethal concentrations 70 (LC_{70}) and their fiducial limits 95% (in parenthesis) were estimated to be 13.3 (11.3–16), 3.6 (3.2–4.1) and 3% (2.6–3.4) of the mother solution, at 24, 48 and 72 h, respectively.

Azadirachtin content in the neem oil extract

The AZ concentration of the neem oil extract was 2,200 mg l^{-1}. The effects of repellency and mortality of mites were present at the neem concentrations of 8, 16, 32, and 64% (58.1, 116.2, 232.3, and 464.6 mg l^{-1} AZ, respectively). Concentration of other components of the neem extract was not determined, although they could also have a repellent or toxic effect (Gauvin et al. 2003).

Discussion

Previous research on the potential of neem extracts and AZ to control *V. destructor* provided discouraging results. Melathopoulos et al. (2000a) and Peng et al. (2000) directed their attention to determine acute toxicity of those products by a long term exposure (feeding, residual films or vapors) or topical application of high doses. More promising results were obtained by Whittington et al. (2000) and Melathopoulos et al. (2000b) by spray applications of neem extracts directly on bee colonies. In agreement with the proposal of Colin et al. (1994), the current work tried to explore the administration of lower quantities of a neem extract to determine sublethal effects, in this case interference with *V. destructor* behavior to locate larval bees during the short time period they are attractive to mite parasitization. Our results showed that neem has a higher potential to repel and/or control this mite.

Melathopoulos et al. (2000a) tested the toxicity of a residual film of neem on *V. destructor*; the estimated LC_{70} of different neem batches ranged from 1.1 to 7.9 mg/observation cage after 24 h exposure, and from 0.6 to 3.9 mg/observation cage, after 72 h exposure. Trying to compare our results with those of the above authors, LC_{70} were translated to weight of neem extract per area. Assuming that 1.5 mg cm^{-2} of neem dilution adhered to each bee pupae, and that a single pupa covers 1 cm^2, the LC_{70} estimated during the current work roughly corresponded to 0.2 and 0.04 mg neem, deposited on three pupae, with exposure times of 24 and 72 h, respectively. Although it is difficult to compare that LC_{70} because extraction and application methods are different, after our results it is suggested that less neem extract may be equally effective, provided it is in contact with bee pupae.

The interference of neem extracts on the ability of *V. destructor* to locate bee pupae and/or settle on them was shown in the repellency choice and no-choice tests (Figs. 3, 4). Even in the absence of another food source, a reduced number of mites could settle down on pupae and feed on them. This suggests that the elevated mortality of *V. destructor* observed in the higher concentrations (Fig. 5) was caused by their inability to find the food source; it is very likely that they died of starvation, as postulated in a previous study (González-

Gómez et al. 2006). Similarly, essential oils of *Salvia officinalis* and *Chenopodium* spp. evaluated by Colin et al. (1994) caused 90% of *V. destructor* mortality 16 h after exposure.

Repellency duration of neem extracts might be of main importance, since reproduction of *V. destructor* only occurs inside capped bee brood cells (Ifantidis 1983; Martin 1994, 1995). According to Ifantidis (1988) and Boot et al. (1992), brood cells become attractive to *V. destructor* females 24 h before being capped by worker bees, and a neem treatment during that period would protect them from being parasitized. However, bee colonies host brood of all ages, and mites surviving neem treatment would be able to locate and enter cells with brood of appropriate age to start a parasitic cycle. Thus, repeated applications of neem extracts using the longer lasting repellent concentrations (16, 32 and 64%) might be a winning strategy. However, concentrations so high might produce additional side effects on bees, not noted at the moment, besides the additional costs implied. Further research must be done to clarify this issue.

Neem oil extract used in this research contained AZ at concentrations that ranged from 58.1 to 464.6 mg l^{-1}, for dilutions of 8 and 64%, respectively. Similar concentrations did not cause negative effects to the bees; to this regard, Larson (1990) and Isman (1995) observed that direct contact of adult worker bees with 20 mg l^{-1} of AZ produced no negative effects. Naumann et al. (1994) reported that 150 mg l^{-1} AZ applied to control canola pests did not stop worker bees from foraging on this crop, and bees did not acquire detectable quantities of AZ under those conditions. Naumann and Isman (1996) also found that a neem-based product (Neem EC) applied at 100, 250 or 500 mg l^{-1} of AZ did not cause significant effects on longevity of surviving adults. AZ concentrations found here were similar to those used by the above authors (Schmutterer 1990), and these concentrations could be safe for honey bees. However, it is not possible to assume that repellency and toxicity caused by the neem oil extract is due to AZ itself, but to the complex of limonoids and oils present in the mixture, as suggested by Stark and Walter (1995), among others. Field data are required to confirm this hypothesis.

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