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CHARACTERISTICS OF IMIDACLOPRID TOXICITY IN TWO *APIS MELLIFERA* SUBSPECIES

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Abstract—Imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) belongs to a new chemical family of chloronicotinyl compounds whose mode of action on the insect nervous system differs from that of traditional neurotoxic products. Imidacloprid, a strong systemic compound, is effective against several sucking and mining pests. The acute toxicity of contact and oral applications on two *Apis mellifera* subspecies, *Apis mellifera mellifera* and *Apis mellifera caucasica,* was investigated. In all toxicological studies, each dose included three cages of 20 individuals and each study was replicated three times. The dose–mortality relation revealed some unusual characteristics. At low imidacloprid concentrations, a biphasic mortality appeared, particularly with the contact exposure route. At moderate doses, mortality profiles at 24 and 48 h were different only after oral application. Response kinetics showed that mortality was delayed at the higher doses of imidacloprid. After oral intoxication, the LD50 values of imidacloprid at 24 and 48 h were about 5 ng/bee for both *A. m. mellifera* and *A. m. caucasica.* After contact application, the LD50 values at 24 and 48 h were approximately 24 ng/bee for *A. m. mellifera* and 14 ng/bee for *A. m. caucasica.* Imidacloprid ranks among the more potent contact insecticides in this important pollinator species.

Keywords—Imidacloprid *Apis mellifera* Honeybees Lethal dose Insecticide

INTRODUCTION

The honeybee, *Apis mellifera,* is widely recognized as an insect of great agronomic, ecological, and scientific importance. It produces valuable products (honey, pollen, royal jelly, propolis, and wax) and plays a major role in crop pollination [1]. Because of agronomic and environmental problems, including loss of pollinators, efforts have been made by the European Plant Protection Organization and the European Community to improve the assessment of pesticide-related risks to bees [2,3].

Chloronicotinyl compounds are a recently discovered class of selective insecticides and include imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine), a nitroguanidine systemic molecule whose mode of action entails competing agonistically with the nicotinic acetylcholine receptor of insects [4–7]. Imidacloprid is extremely effective against sucking insects and some heteroptera, coleoptera, and lepidoptera species, whereas vertebrates appear to be relatively insensitive to it [8,9]. In contrast with nicotine, imidacloprid is a striking example of a product with high insecticide activity and low mammalian toxicity; the oral median lethal dose (LD50) in rat was about 450 mg/kg [10].

Major applications of imidacloprid include seed dressing, spraying, and the use of pills and granules [11]. With seed dressing, insects can be poisoned through the oral way by parent or metabolite compounds. With spraying, nectar can also be contaminated, and bees are poisoned either through direct contact with the product or through contact with its residues.

The purpose of this study was to examine the acute effects of imidacloprid to bee workers. Its intrinsic toxicity was studied by determining the laboratory-based median lethal dose (LD) after oral and contact applications in two *Apis mellifera* subspecies, *A. m. mellifera* and *A. m. caucasica.* Mortality kinetics were also studied using different imidacloprid doses. Acute toxicity allows the calculation of oral and contact hazard quotients (Q_{HO} and Q_{HC}) to predict the exposure of bees to pesticides [2]. In addition, the acute toxicity of insecticides allows the determination of a sublethal level. This sublethal level is important to study the chronic toxicity of agrochemicals and their adverse sublethal effects than can induce a deleterious impact on honeybee populations. Herein we report imidacloprid toxicity to *A. mellifera* with apparently unusual dose–response characteristics that have to be taken into account in the risk assessment procedure of a product used both in seed dressing and in foliar treatments.

MATERIALS AND METHODS

Materials

The effects of technical grade (98% pure) imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) from Bayer AG (Leverkussen, Germany) were investigated from June to August 1998. For each subspecies, bee workers (*A. m. mellifera* and *A. m. caucasica*) were captured from honey and pollen combs in a same healthy queen-right colony for all bioassays; all drones were discarded. Immediately before treatment, bees were anesthetized with carbon dioxide and kept in cages (10.5 × 7.5 × 11.5 cm) in a temperature-controlled chamber at 25 ± 2°C with 60 ± 10% relative humidity. Bees were fed a 50% sucrose solution ad libitum [12].

Experimental conditions

In each experiment, three cages of 20 bees were used for each dose of treatment. Experiments were replicated at least

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three times. Repetitions were within 1 week. As recommended in European Plant Protection Organization guidelines, control mortality was less than 15% in all experiments.

Modes of treatment

Oral application. The honeybees were deprived of food for 2 h before administration of imidacloprid. Imidacloprid solutions were prepared in a 1% dimethylsulfoxide solution and then diluted 10-fold in the 50% (w/v) feeding sucrose solution. The final concentration of dimethylsulfoxide solution in the sucrose solutions of control and assay tests was 0.1% (v/v). The dosing solutions were prepared fresh for each test. Each bee received 10 μl of 50% sucrose solution (vehicle) containing graded doses of imidacloprid or the dosing vehicle alone (control). After consuming this solution, bees were fed 50% straight sucrose solution ad libitum. Mortality was recorded at 2, 4, 6, 10, 14, 20, 24, and 48 h.

Contact application. One microliter of insecticide solution in 100% dimethylsulfoxide solution (vehicle) was applied with a microsyringe on the dorsal thorax. After the application, all bees were fed 50% sucrose solution ad libitum. Control bees received 1 μl of the vehicle. Bee mortality was recorded 24 and 48 h after topical application.

Data analysis

Mortality data were corrected according to Abbott [13]. The LD50s were determined by curve fitting using log-probit analysis. One-way analysis of variance was used to evaluate differences between groups.

RESULTS

The toxicity of imidacloprid to bee workers was investigated with different application modes. In all toxicological studies, each dose included three cages of 20 individuals and each study was replicated three times. Both *A. m. mellifera* and *A. m. caucasica* exhibited neurotoxic symptoms such as trembling, tumbling, and lack of coordination within 24 h of imidacloprid exposure.

The contact toxicity of imidacloprid was studied initially. In *A. m. mellifera* and for all replicates of the study, 24- and 48-h mortality rates increased for doses of between 1 and 7 ng/bee and then decreased for doses ranging from 7 to 15 ng/bee (Fig. 1A). At doses higher than 15 ng/bee, the mortality rate increased in a dose-dependent manner. For *A. m. caucasica*, mortality observed at 1 ng/bee was greater than those obtained at 2.5 and 5 ng/bee and was close to that observed at 10 ng/bee (Fig. 1B). In both species, there was no significant difference between the mortality at 24 and 48 h. Surprisingly, we observed only slight differences of mortality between replicates, as shown by the small standard deviations obtained (Fig. 1).

Oral exposure resulted in differences in mortality between 24 and 48 h at intermediate doses. In *A. m. mellifera*, 24- and 48-h mortality rates increased with doses up to approximately 50 ng/bee (Fig. 2A). At higher doses, mortality was rather variable. This phenomenon was more pronounced with *A. m. caucasica* (Fig. 2B). At 24 h, doses of up to approximately 20 ng/bee caused a maximum mortality rate of about 90%, while effects of doses between 20 and 90 ng/bee were quite variable. For doses between 90 and 200 ng/bee, the mortality rate remained more or less stable and gradually rose at doses above 200 ng/bee. At 48 h, the mortality profile was different from that obtained at 24 h. There was only a slight decrease

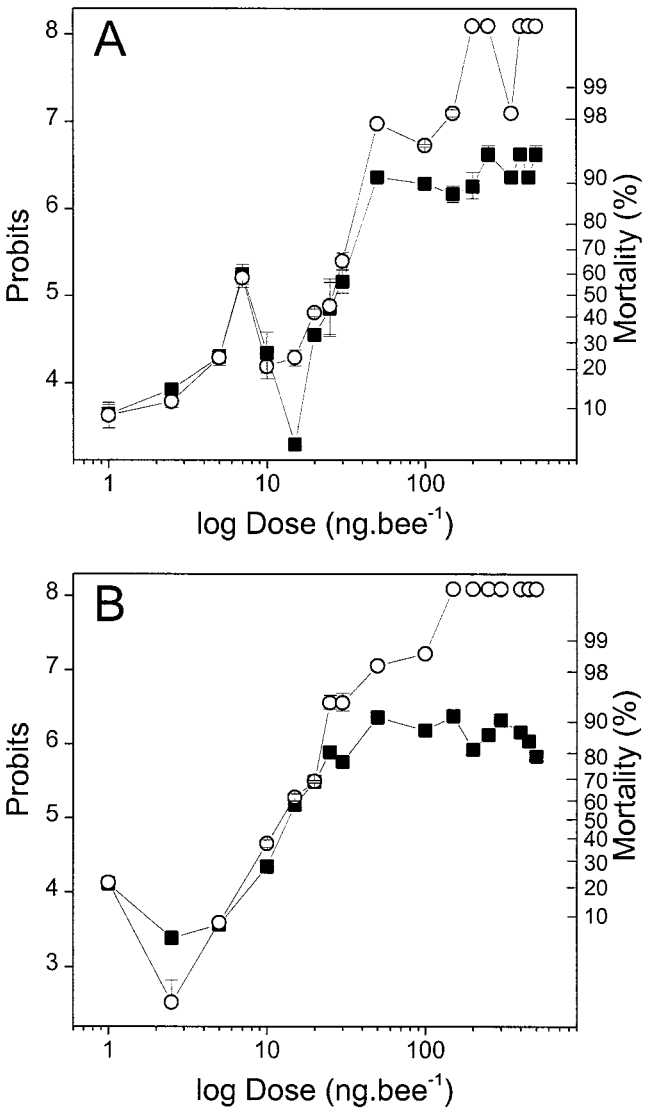

Fig. 1. Dose–response relation resulting from contact exposure to imidacloprid. (**A**) *Apis mellifera mellifera.* (**B**) *Apis mellifera caucasica.* Bee mortality observed 24 h (■) and 48 h (○) after contact application of different imidacloprid doses. Data represented the means ± SD of three experiments performed in triplicate. The absence of error bars corresponds to SD = 0.

in the 48-h mortality rate with doses between 20 and 90 ng/bee, and then the mortality gradually went up for both species as the doses of imidacloprid were increased. As with contact exposure, we also observed small variations of mortality between replicates.

The kinetics of mortality in both species were studied using the oral mode of intoxication because ingestion of contaminated nectar following treatment by seed dressing is the main exposure route. In *A. m. mellifera*, the maximum mortality level was reached within the first 10 h at 1 ng/bee (Fig. 3A). For doses ranging from 5 to 50 ng/bee and for all replicates of the study, the mortality kinetics were similar during the first 14 h but, at 50 ng/bee, mortality rates subsequently increased. At 200 ng/bee, mortality appeared to be delayed since it was lower than that obtained with doses of 5, 10, and 50 ng/bee during the initial observation period but continually increased after 24 h. Delayed mortality was also observed in *A. m. caucasica* (Fig. 3B). The mortality delay was more pronounced

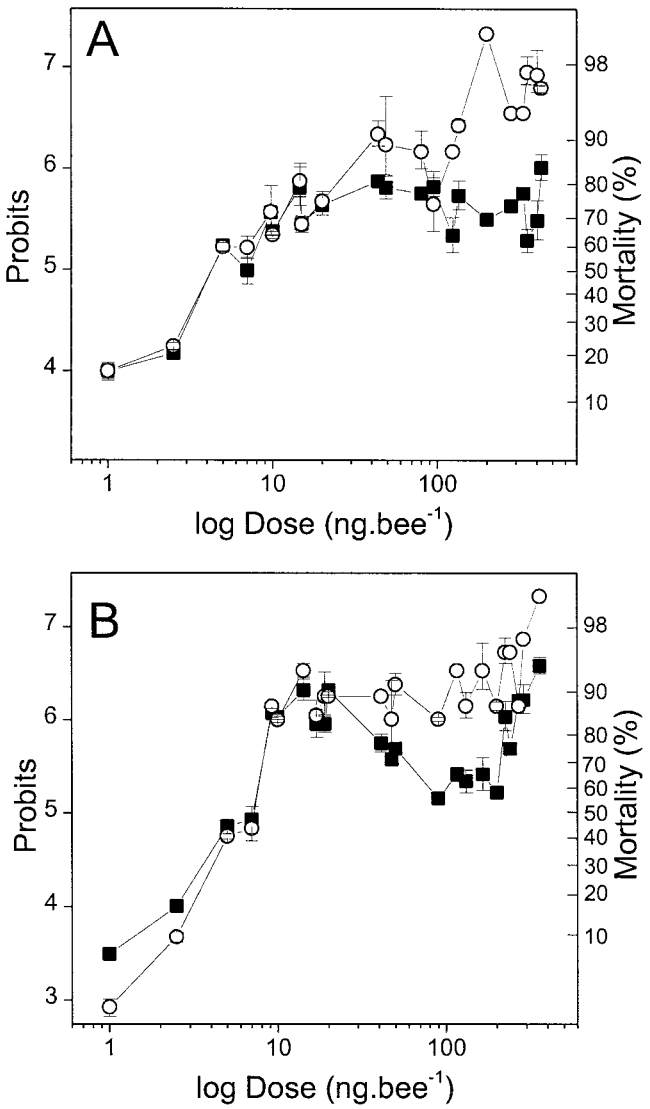

Fig. 2. Dose–response relation resulting from oral exposure to imidacloprid. (**A**) *Apis mellifera mellifera.* (**B**) *Apis mellifera caucasica.* Bee mortality observed 24 h (■) and 48 h (○) after oral application of different imidacloprid doses. Data represented the means ± SD of three experiments performed in triplicate. The absence of error bars corresponds to SD = 0.

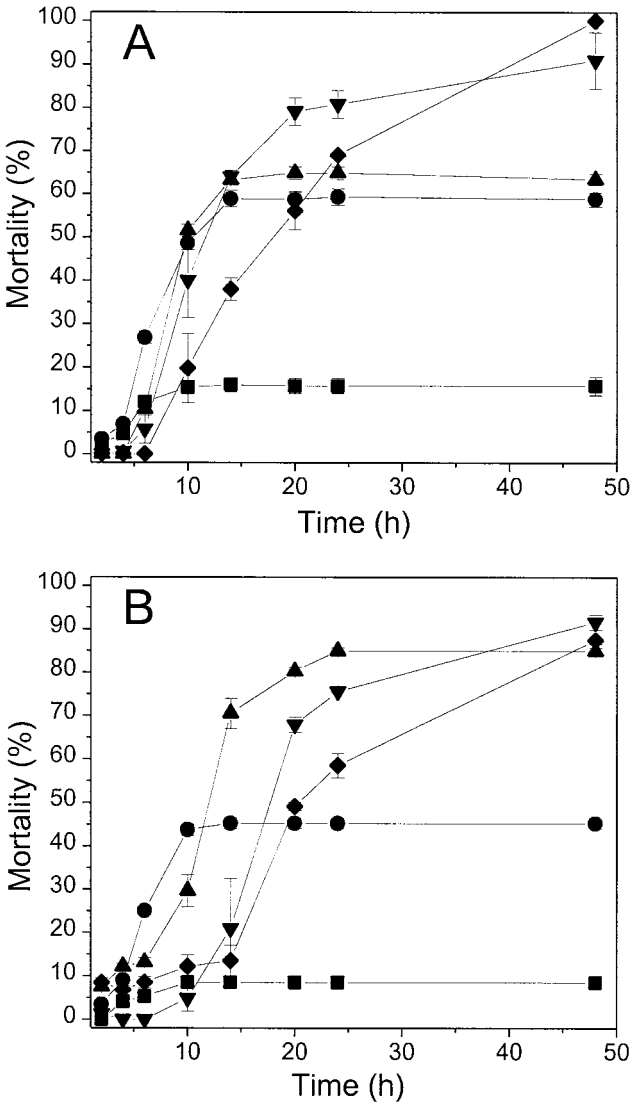

Fig. 3. Mortality kinetics after oral application of imidacloprid in *Apis mellifera.* (**A**) *A. m. mellifera.* (**B**) *A. m. caucasica.* Bee mortality was monitored after ingestion of imidacloprid at the doses of 1 (■), 5 (●), 10 (▲), 50 (▼), and 200 ng/bee (♦). Data represented the means ± SD of three experiments performed in triplicate. The absence of error bars corresponds to SD = 0.

than in *A. m. mellifera* and systematically increased with the doses throughout the tests. Similar kinetics were obtained in all replicates. Thus, the small variations between replicates in the kinetic studies confirm the slight differences of mortality observed in the dose–effect experiments.

The LD50 values of imidacloprid in honeybee species obtained with contact and oral tests are summarized in Table 1. The LD50 estimates of imidacloprid in *A. mellifera* were very low. In *A. m. mellifera,* the LD50 means at 24 and 48 h were approximately 4.5 and 24 ng/bee for oral and contact appli-

Table 1. The LD50 values of imidacloprid in *Apis mellifera mellifera* and *Apis mellifera caucasica.* The LD50 values were obtained from the experiments carried out with different imidacloprid doses either after oral or contact application. The LD50 values were calculated by log-probit analysis. In *A. m. mellifera,* for contact application, LD50 values were determined from the two ascending parts of the dose–effect curve. Values represented means of three experiments performed in triplicate. Values in brackets represented 95% confidence limits. Values in italics corresponded to LD50 values calculated at very low doses from the ascending part of the dose–response curve

	Oral LD50 (ng/bee)		Contact LD50 (ng/bee)	
	24 h	48 h	24 h	48 h
A. m. mellifera	5.4 [5.2–1.6]	4.8 [4.5–5.1]	*6.7 [5.2–8.2]* 23.8 [22.3–25.3]	*6.7 [4.4–9.0]* 24.3 [22.0–26.6]
A. m. caucasica	6.6 [5.1–8.1]	6.5 [4.7–8.3]	15.1 [11.9–18.3]	12.8 [9.7–15.9]

cations, respectively. In *A. m. caucasica,* the LD50 means at 24 and 48 h were approximately 6.5 and 14 ng/bee for oral and contact applications, respectively. Analysis of variance tests ($p < 0.05$) indicated a significant difference of sensitivity to imidacloprid between *A. m. mellifera* and *A. m. caucasica* for contact application at 24 h but not at 48 h.

DISCUSSION

Contact or oral intoxication by imidacloprid induces rapidly neurotoxic symptoms such as movement coordination problems, trembling, and tumbling. Similar behavior after imidacloprid application was described in a Coleoptera *Diaprepes abbreviatus* [14]. The biological activity of the Heteroptera *Podisus maculiventris* after different imidacloprid applications has also been investigated [15]. After 24 h, all modes of exposure to imidacloprid caused neurotoxic symptoms in most of individuals.

For a given species, imidacloprid toxicity changes with the route of exposure. In *P. maculiventris,* toxicity decreases in the order of topical exposure > ingestion > residual contact. In *A. mellifera,* imidacloprid, unlike most insecticides, is more toxic via oral route than by the contact mode. The toxicity of organophosphorus insecticides, such as chlorpyrifos, is four times higher by contact application than by oral application (contact LD50 = 59 ng/bee, oral LD50 = 250 ng/bee). Similarly, contact application of bifenthrin pyrethroid is seven times more potent than oral application (contact LD50 = 15 ng/bee, oral LD50 = 100 ng/bee).

The LD50 values of imidacloprid obtained in *A. mellifera* (LD50 ranging from 4 to 24 ng/bee) are low compared with other families of insecticides that have different modes of action. Three of the most toxic insecticides, the organophosphorus compound triazophos (contact LD50 = 55 ng/bee) and the pyrethroids cyhalothrin and deltamethrin (contact LD50 = 27 ng/bee, LD50 = 51 ng/bee), are all less harmful to honeybees than imidacloprid [16]. For these insecticides, the highest toxicity is obtained by contact treatment but not by oral route, as is the case for imidacloprid. Thus, imidacloprid is one of the most potent insecticides to honeybees and should not be applied during the flowering period [11]. Applications by seed dressing and granules could mitigate hazard to honeybees.

Imidacloprid is very selective toward insect species. Imidacloprid has a low insecticidal activity toward *Heliothis virescens* and *Spodoptera Littoralis,* two polyphagous pests (at 48 h, contact LD50 = 350 ng/mg of insect for *H. virescens* and LD50 = 650 ng/mg of insect for *S. Littoralis*) [17]. Hence, for this species, imidacloprid is not potent enough to efficiently control cotton insect pest populations. On the other hand, imidacloprid is extremely effective against sucking insects such as *Myzus persicae* (48-h oral LD50 = 3 pg/mg insect) [18]. Thus, honeybees have an intermediate sensitivity to imidacloprid (48-h oral LD50 = 50 pg/mg insect) compared with these pest species. The difference in sensitivity between the honeybee and sucking insects is an important feature because field application rates that are toxic *to M. persicae* may not pose a hazard to bee life.

One of the most surprising characteristics of imidacloprid toxicity is the unusual mortality profile observed with the two application modes, i.e., honeybee mortality rates increase with low doses of imidacloprid. At intermediate doses, toxicity falls off but then increases at high doses. After contact application 24- and 48-h mortality rates are similar, whereas with oral application, the mortality rate is higher at 48 h than at 24 h. Another interesting observation is that kinetics of toxicity show that mortality is delayed as dose increases. These particular features of imidacloprid toxicity suggest that several metabolic pathways might be involved in the imidacloprid toxicity. With low imidacloprid doses, the few toxic metabolites produced together with the parent compound might be responsible for the high mortality during the first 10 h. Medium doses of imidacloprid may trigger an induction of detoxifying enzymes that reduces honeybee mortality. With higher doses, the increase of mortality could be due either to a high amount of toxic metabolites or to the saturation of the pesticide-metabolizing enzymes. This is supported by the fact that honeybees, like other insects, possess inducible enzymes and can metabolize pesticides [19]. Induction of cytochrome P-450 has been reported in honeybees after 2 d of exposure to fluvalinate pyrethroid, which demonstrates that induction of pesticide-metabolizing enzymes can occur rapidly [20]. In polyphagous insects, small quantities of plant substances suffice to induce mixed function oxidases within 30 min following initiation of feeding [21]. Hence, it is not possible to rule out the possibility of a rapid metabolic activation of imidacloprid as already demonstrated for organophosphorus compounds [22]. Considering the high affinity binding of imidacloprid to bee brain membranes, it would be interesting to correlate the toxicity of metabolites to their relative affinity for the imidacloprid receptor [23].

Imidacloprid is the insecticide that has the highest acute toxicity to the honeybee *A. mellifera.* Its dose–response characteristics in the honeybee are unusual for insecticides and neurotoxic compounds, and this exemplifies well the necessity for conducting in-depth studies on pesticides from new chemical families. Specific protocols may be necessary to accurately assess the intrinsic toxicity of new pesticides and to better evaluate their toxicological profile and potential impact on honeybee colonies.

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