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Toxicity of two neonicotinoid insecticides via the food chain for larvae of the two spot ladybird *Adalia bipunctata*

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Abstract: The toxicity by ingestion of aphids contaminated by imidacloprid and thiacloprid was assessed in the laboratory for the larvae of the two spot ladybird *Adalia bipunctata*. Observed mortality was recorded during the larval and pupal development as well as the time needed to reach adult stage. Adults were then assessed for any adverse effects on reproduction.

Aphids used for the first set of experiment were placed in plastic petri dishes and directly treated with the insecticides at the recommended rate using a Spray-Tower apparatus. High mortality was rapidly observed and 100% mortality of the larvae fed with contaminated aphids was obtained after only 3 days of feeding with imidacloprid and 4 days with thiacloprid. For the second set of experiments, dwarf bean plants infested with aphids were treated outside with the insecticides using a knapsack sprayer connected to a sprayer ramp with flat-fan nozzle, as used in open field. Thiacloprid did not lead to significant mortalities with only 5% corrected mortality. Imidacloprid was more toxic, with 39% corrected mortality. No effect on adult development and fertility was observed.

For the last set of experiments, aphids were reared on dwarf bean units treated by irrigation with the two insecticides. This system was used to avoid direct contact between the aphids and the insecticides and assess the toxicity by ingestion of aphids only contaminated by feeding on plants treated with systemic insecticides. The doses applied to the units were determined, on basis of preliminary experiments, to limit aphid development of 50%, 75% and 90% after one week compared to control. Thiacloprid had no significant effects on larval survival, development time and adult fertility. Imidacloprid has significant effects at the highest rate tested on larval survival and development time with a corrected mortality of 25.7% and 20.7 days to reach adult stage instead of 19.6 days for the control. The two other rates had no significant effects.

These results showed that ingestion of contaminated prey could have toxic effects on ladybird larvae, but the effects are clearly related on how the food was contaminated.

Key words: *Adalia bipunctata*, imidacloprid, thiacloprid, ingestion

Introduction

Ladybirds are an important group of aphid and scale insects predators in several agricultural ecosystems. A lot of studies have been performed to assess the toxicity of pesticides on these beneficial insects and a majority of the insecticides tested, mainly pyrethrinoids, neonicotinoids and organophosporous compounds, were highly toxic (Hellpap, 1982; Hassan *et al.*, 1987, 1988, 1991, 1994; Sterk *et al.*, 1997; Olzak, 1999; Jamieson *et al.*, 2005; Hautier *et al.*, 2006; Jansen *et al.*, 2008; Katsarou *et al.*, 2009). Most of these results have been obtained in the laboratory with contact toxicity test, according to the IOBC standards.

The standardization of the methods to assess the toxicity of the pesticides on beneficial organisms is one of the outcome of the IOBC Working group Pesticides and Beneficial Organisms. However, if most of the products were acting mainly by contact when the methods have been developed in the 70's, the mode of action of a lot of the new compounds is different and the first tier testing based on contact toxicity test could perhaps underestimate the toxicity of such products.

One specific case is the group of systemic insecticides. As they are acting by ingestion on aphids and other sucking insects, the possible contamination of ladybirds by oral uptake, when they are feeding on these contaminated prey, is not taken into consideration with the first IOBC standard tests. As several authors have reported severe toxic effects of different insecticides when tested by oral uptake with contaminated food (De Cock *et al*., 1996, Olzak, 1999), there is a need to specifically address this possible way of contamination.

The aim of this study is to assess the effects for the larvae of the two-spotted ladybird *Adalia bipunctata* L. (Col.; Coccinellidae) of the ingestion of aphids contaminated with the neonicotinoid insecticides thiacloprid and imidacloprid. These products were selected for their possible high toxicity for ladybird and their relative long persistence in the environment that could increase the possible risk for ladybirds, e.g. preying on aphids feeding on plants contaminated with insecticide residues. *A. bipunctata* was selected as an indicator species for ladybirds, as this species was shown to be more sensitive to insecticide than larger species (Jansen & Hautier, 2006). Three different ways of contamination of the aphids were used, one that can be considered as a standard worst case for laboratory tests and two other ones that were closer to the field conditions.

Material and methods

Test organisms

Adult ladybirds from the laboratory rearing were kept by groups of 15-20 in plastic cages and were fed with honeybee pollen and aphids offered on cut plants (mainly *Acyrtosiphon pisum* (Harris) plus *Myzus persicae* (Sulzer), produced on French beans and sweet pepper, respectively). Synchronized patch of eggs laid on piece of crumpled paper were harvested daily. The rearing was done in a climatic chamber (20 ± 2°C, 60-90% RH, 7-10.000 lux with a 16/8 day/night photoperiod).

Products

The formulation Calypso (Bayer®, 480g/l, SC, thiacloprid) and Confidor 200SL (Bayer®, 200g/l, SL, imidacloprid) were used at their recommended field rate (0.25l/ha for Calypso – 120g/ha thiacloprid) and 0.25l/ha for Confidor – 50g/ha imidacloprid). Drinking water was used for the control groups.

Food contamination

The aphids delivered to the larvae were contaminated by 3 different methods. For the first test (direct treatment), aphids were harvested from the plants of the mass rearing, placed in large petri dish lids and sprayed with water (control) or the insecticides with a Burgerjon spray tower (Burgerjon, 1956) at 200l ± 10% spray mixture per ha. The aphids were left to dry 1 to 2 hours and a part of them was directly used to feed the larvae. The other part was kept at 2-8°C to be used 1 or 2 days later. This operation was repeated every 2-3 days to have all the time a stock of freshly treated aphids.

For the second test (treatment on plants), the principles were similar, except that the aphids were left on the plants and the whole units (plants with aphids) were treated with the insecticides with the help of a boom sprayer with Azo 110 flat fan nozzles at a rate of 400l ± 10% spray mixture per ha. The plants and the aphids were left to dry 1 to 2 hours and then the aphids were harvested and used to feed the larvae or kept at 2-8°C to be used 1 or 2 days later. These operations were renewed every 2-3 days to produce freshly contaminated aphids.

For the third tests, the aphids were contaminated by being confined on plants treated with the insecticides by irrigation. A first screening was carried out to determine the relationship between the doses of product added to the plant by irrigation and the development of the aphid population in the conditions of the experiment. For this, the insecticides were added by irrigation to the plant rearing containers at 5 concentrations (5 containers per concentration and 5 control). The containers were made of 50x35cm plastic trays with 75 French bean seeds seeded in vermiculite. The trays were pierced of several holes and placed in bigger trays filled with water to humidify the substrate. Water was added ad lid the first days to start the germination process during the first 6 days and then the exceeding water was retired and the substrate was left to dry 1 day in order to be able to absorb the insecticide. The insecticides were then added to the plastic trays in one liter of water. 1 day after product administration, 600mg of pea aphids was added to each unit and the units kept at 20 ± 2°C. After 7 days, the aphids were harvested and weighted. A linear regression was performed to estimate the doses of each insecticide that reduced the aphid population growth to 50%, 75% and 90% compared to the control. The aphids used to feed the ladybird larvae were then produced following the same procedure, with the doses of insecticides estimated earlier. New rearing containers were produced every 2-3 days in order to have a continuous stock of aphids ready to be used.

Toxicity tests
For the assessment of the toxicity, 2-3 day old larvae were isolated in exposure units made of a glass plate surrounded by a plastic cylinder (h: 3cm, ∅: 5cm) treated with Fluon GP1 to prevent the larvae from escaping. The larvae were fed daily with contaminated aphids till pupation. The pupae were isolated in plastic petri dishes and the adult emergence was checked. The pupal mortality was added to the larval mortality and the time required for the larvae to reach the adult stage was calculated. Adults of a same treatment were first pooled in rearing cages as described for the mass rearing and kept together at least one week after first egg laying. Then, the females were isolated in plastic petri dishes with aphids as food and transferred every 24h into new petri dishes. The number of eggs laid per day and the emergence of these eggs were assessed on 6 successive 24h-period. Adult ladybirds were only fed with untreated food during the pre-fertility and the fertility phase. The experimental conditions were similar than those described for the ladybird rearing.

Test design and statistical analysis
For each experiment, the toxicity was assessed on 4 x 10 larvae per product and for control. The mean development time was calculated for each replicate and the fertility was assessed on all living female ladybird. The mean mortalities, the development time to reach adult stage, the total number of eggs and viable eggs produced per females per day were analysis with the help of a one-way ANOVA test followed by a Tukey test ($p = 0.05$) for pairs comparisons. The percentages were transformed before analysis (arcsin transformation). Observed mortalities were corrected according to Abbott formula (Abbott, 1925).

Results

The results of the two first experiments (direct spray and aphids treated on plants) are listed in Table 1. The aphids directly sprayed with both insecticides with a Burgerjon spray tower were highly toxic for the ladybird larvae. 100% mortality was obtained after only 3 days of feeding for imidacloprid and four days with thiacloprid.

With the aphids sprayed on plants, the toxicity was reduced compared to the first experiments with 5.5% corrected with thiacloprid and 39.0% with imidacloprid. No impact was observed on the development time required for the larvae to reach the adult stages. Thiacloprid had no effects on the fertility performance of the adults, compared to control. With imidacloprid, due to the natural and accidental mortality of several adults before the start of the fertility assessments, the reproductive performance was unfortunately only assessed on one female and the statistical analysis was not possible with this product. The number of viable eggs produced by this surviving female seemed to be much lower than in the control, but the fertility performance of female ladybirds can be highly variable and even in the control, females that laid no or only a few viable eggs are not uncommon.

Table 1. Effects of the ingestion of aphids sprayed with insecticides on the larvae of the 2-spot ladybird. Survival rate, development time to reach adult stage, total and viable egg production by female and number of females assessed (n).

	Mortality		Development time (days) ± sd	Fertility		
	Observed (± sd)	Corrected		Eggs/female/ day ± sd	Viable eggs/ female/day (± sd)	n
Aphids contaminated by direct spray						
Control	10.0% ± 8.2a	-	21.2 ± 0.5	*Not assessed*		
Imidacloprid	100.0%b	100.0%	-			
Thiacloprid	100.0%b	100.0%	-			
Aphids harvested on sprayed plants						
Control	17.5%± 5.0a	-	20.9 ± 0.8a	27.6 ± 6.5a	13.5 ± 6.8a	14
Imidacloprid	50.0% ± 8.2b	39.0%	21.7 ± 0.3a	17.8	1.00	1*
Thiacloprid	22.5% ± 12.6a	5.5%	20.9 ± 0.4a	26.9 ± 7.2a	17.0 ± 8.4a	11

ANOVA and Tukey test (p = 0.05), Results followed by the same letter are not statistically different.
* only one replicate due to adult natural and accidental mortality before the fertility assessments

The results of the tests performed by feeding ladybird larvae with aphids produced on plants treated with the insecticides by irrigation are listed in Table 2. To reduce the aphid population growth of 50%, 75% and 90% compared to the control within one week, 3µl, 12µl and 25µl of Calypso had to be added to one plant rearing container of 75 French bean seeds, on basis of the preliminary dose-range trial. With Confidor, the amount of products was much lower, with 0.21µl, 0.32µl and 0.39µl to reduce aphid population growth within one week of 50%, 75% and 90% compared to the control.

With aphid reared on plants treated by irrigation with thiacloprid, no effects significantly different to the control were observed, indicating that even the doses of the insecticides that reduced up to 90% the aphid population growth had no impact. With imidacloprid, a corrected mortality of 25.7% was obtained at the highest tested rate, as well as an increase of the time needed for the larvae feed with the contaminated aphids to reach adult life stage, with 20.8 days instead of 19.9. These two differences were significantly different to the control. No other significant difference between control and imidacloprid-fed larvae groups was observed.

Table 2. Toxicity of insecticides for larvae of the 2-spot ladybird by ingestion of aphids reared on plants treated by irrigation with insecticides. Survival rate, development time to reach adult stage, total and viable egg production by female and number of females assessed (n).

	Mortality (%)		Development time (days) ± sd	Fertility		
	Observed (± sd)	Corrected		Eggs/female/ day ± sd	Viable eggs/ female/day (± sd)	n
Control	12.5% ± 5.0a		19.9 ± 0.4a	20.0 ± 7.1ab	14.8 ± 5.6a	12
Imidacloprid LD_{50}	27.5% ± 9.6ab	17.1	20.4 ± 0.2ab	24.3 ± 5.3ab	17.4 ± 4.2a	14
Imidacloprid LD_{75}	32.5% ± 12.6ab	22.9	20.0 ± 0.4ab	19.7 ± 8.2a	14.0 ± 6.7a	12
Imidacloprid LD_{90}	35.0% ± 12.9b	25.7	20.8 ± 0.1b	27.5 ± 4.4b	17.7 ± 4.9a	9
Control	22.5% ± 9.6a	-	22.4 ± 0.8a	18.1 ± 7.9a	8.7 ± 7.1a	13
Thiacloprid LD_{50}	27.5% ± 5.0a	6.5	21.0 ± 0.7a	19.2 ± 9.8a	8.2 ± 7.5a	11
Thiacloprid LD_{75}	27.5% ± 15.0a	6.5	21.7 ± 0.4a	17.9 ± 10.1a	10.1 ± 7.3a	9
Thiacloprid LD_{90}	30.0% ± 8.2a	9.7	21.8 ± 0.6a	19.6 ± 8.1a	10.0 ± 5.6a	8

ANOVA and Tukey test ($p = 0.05$), results followed by the same letter are not statistically different.

Discussion

The results obtained in this study showed that the ingestion of aphid contaminated with insecticides by ladybird larvae can have significant effects on their survival. However, the importance of effects, for the two neonicotinoid products tested, was clearly dependant on the way of contamination of the aphids selected. Results were drastically different if the products were directly applied on the aphids placed on a flat surface than when applied at the same rates on plants infested with aphids, with a reduction of 100% corrected mortality to 39.0% for imidacloprid and 100.0% to only 5.5% for thiacloprid. These differences could be explained by the distribution of aphids on the plants, the vertical dilution of pesticides sprays and the interception of the sprays by the foliage, leading to a lower concentration of the pesticide residues on the aphids. Thus, when the food is directly sprayed in a 2-D dimension with a spray tower, the toxicity is probably overestimated compared to a spray situation close to the field conditions and this spray scenario can only be used as a worst case.

The two insecticides tested can be applied as foliar spray in several crops, but the number of application is limited to 1 or 2 during the growing season. Therefore, the effects of the contamination of aphids by sprays on plants, even in the case of imidacloprid with 39.0% corrected mortality, will be limited to the shorts periods when the products were used. Furthermore, in this study, the aphids used to feed the ladybirds were continuously contaminated while in the field, the ladybird would probably feed on treated aphids during only a few days, as the insecticide treatments at the full rate will rapidly eliminate living aphids. Thus, the mortality due to ingestion of insecticides will probably be lower than those obtained in this study, even if high ladybird mortality will probably occurred as a consequence of larvae starvation.

The situation is different in the case of aphids contaminated by feeding on plants treated with the systemic insecticides. When they are applied at the recommended rate, these two insecticides are effective to control the aphids and therefore, there is no risk of ingestion of the product by the ladybirds, as no aphid colony can develop on these plants soon after application. However, from this starting dose, a decrease of the insecticide concentration in the plant will occur by different way, by example simply by the dilution of the product due to the plant growth or the degradation of the products (physico-chemical mechanisms, plant metabolism or plant sequestration). There is also some contamination of the non-target plants at residual doses, as plants in field borders can be contaminated by the spray drifts of foliar applications, or when untreated crops are succeeding a treated one in the same field. Thus, even if the risk for ladybirds when the insecticides were applied at the full rate is very low, there is always one moment when the doses will be reduced and allow the establishment and development of aphid colonies, and these aphid colonies contaminated with sublethal doses of insecticides could be at risk for ladybirds.

The results obtained in this study showed that the expected effects at these rates were limited, with a maximum of 25.7% corrected mortality with imidacloprid at the rate that reduced the aphid population development of 90% compared to the control. No other effects, except a short increase of the time needed for larvae to reach adult stage, were observed, indicating that the risk will be limited for one ladybird generation. However, these apparent low effects have to be correlated with the occurrence of such exposure scenario, as a repetition in the time or in the space of low but significant mortalities at each ladybird generation could have long-term detrimental effects on these beneficial organisms. Imidacloprid is particularly at risk for several reasons. Firstly, it is known to be relatively persistent, with field DT_{50} and DT_{90} of respectively 190 and 717 days (Anonymous, 2011). According to the very low rates needed to slow down aphid development, the risk of exposure of ladybirds to doses tested in this study would probably be found several years after the application of the product, with the risk to have a possible contamination of several ladybird successive generations. Secondly, as imidacloprid is known to be one of the most used insecticide in the world (Bonmatin *et al*., 2005), the risk to have a large contamination of the agro-ecosystem, in space and during the time, is high. Furthermore, several parameters could increase the risk, as the possible contamination of flower pollen and nectar (Chauzat *et al*., 2006), used as food resources by adult ladybirds, and the possible resistance of several aphid strains to imidacloprid, with an increase of the sublethal rates, that could be ingested by the ladybirds eating living aphids. Thus, the apparent low effects of imidacloprid by ingestion for ladybird larvae is a positive results for the protection of these beneficials, but the incidence and the repetition of this exposure scenario, that is actually unknown but suspected to be high in the case of an intensive use of this compound on successive crops, has also to be taken into consideration.

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