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Effects of four insecticides on the two-spotted ladybird *Adalia bipunctata* using a "Microcosm" test design

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Abstract: The effects of four insecticides, the two aphid-feeding inhibitors pymetrozine and flonicamide, the systemic insecticide thiacloprid and the contact insecticide pirimicarb were assessed on larvae of the two spotted ladybird *A. bipunctata* with the help of an extended lab-test. The larvae were held on French bean seedlings infested with aphids in units surrounded by a treated metal frame in order to prevent both ladybird larvae and aphids to escape. 2-3 day old larvae were introduced on the aphid infested plants and left to distribute on the vegetation canopy several hours before treatment. The units were then treated with the insecticide with the help of a knapsack sprayer with a flat-fan nozzle. Units were kept 8-9 subsequent days in the laboratory without food addition and were dismantled when first larvae started to pupate in the control. The larvae and pupae were counted and surviving larvae were kept individually in plastic petri dishes. They were fed with aphids till pupation. Development time, fecundity and fertility of the adults were then assessed. This method combines different aspects of toxicity, with direct toxicity by spray, contact toxicity with treated plants, toxicity by ingestion of contaminated aphid and by starvation due to the elimination or reduction in food availability by the insecticides.

One of the four insecticides tested, pymetrozine, was harmless with only 12% larval mortality. Flonicamide, thiacloprid and pirimicarb led to 65%, 80% and 84% corrected mortality, respectively. Thiacloprid, pirimicarb and flonicamide significantly increased the development time to reach adult stages. Total egg production by surviving female was not affected by the four tested product but flonicamide significantly affected emergence with 35% of egg hatching compared to 70% in the control and reduced by more than 50% the mean viable egg production by female, compared to control.

Key words: *Adalia bipunctata*, microcosm study, flonicamide, pymetrozine, thiacloprid, pirimicarb, starvation

Introduction

Since the beginning of the 70's, the IOBC Working Group "Pesticides and Beneficial Organisms" has developed specific testing schemes based on laboratory standard test methods, semi-field and field tests. The WG has used these routinely to assess the effects of more than 200 pesticides on a set of 15 to 25 beneficial species (Franz et al., 1980; Hassan et al., 1983, 1987, 1988, 1991, 1994, Sterk et al., 1999). Due to difficulties to perform large field tests, most of the work was performed under laboratory conditions, with the help of standardized methods. Field tests are indeed difficult to implement and time consuming and did not provide all the time valuable results, because of heterogeneity of data, lack of repeatability of experiments and difficulties in interpretation of complex results.

Though there was a very good correlation between laboratory and field results for products harmless in the laboratory according to IOBC standard methods (Vogt et al., 1994), it

was not always possible in the laboratory to assess all the parameters and interactions that led to side-effects on beneficial in field conditions, especially with the first steps of the testing scheme.

The major lack of these tests was the way of exposure in the laboratory for the mobile stage of the beneficials, limited to residual contact with a treated surface. As beneficials are most of the time very active organisms in search for food or hosts to parasitize, this way of contamination was considered as the most important in term of toxicity, especially with products acting by contact, as a majority of the pesticide of the 70's were. However, contamination by ingestion (uptake of contaminated food) or inhalation could be underestimated with these experiments and were only assessed at higher tier testing, during semi-field or field trials. Furthermore, impact of pesticides on interactions between beneficials and prey/host, as possible effect due to starvation, were rarely assessed in the laboratory.

Since several years, the pesticide market has evolved a lot. Several contact insecticides are no longer registered and have been replaced by new systemic insecticides, as neonicotinoids products or, for aphid control, by aphid-feeding blockers, as pymetrozine and flonicamide. With these categories of new products, there is a risk that IOBC standard test methods in the laboratory are no longer valid and lead to false results and risk assessment. Thus, new test methods, or an adaptation of actual methods, are needed to take into account this possible way of contamination, especially by contamination of food with systemic products.

Bigler and Waldburger (1988) have developed a microcosm type semi-field test for *Chrysoperla carnea* larvae using broad bean plants in larger units as exposure units and assessed with this method the toxicity of several products which were harmful in the laboratory (Bigler and Waldburger, 1994). This method was adapted to be used with the larvae of the seven-spotted ladybird *Coccinella septempunctata* (Schmuck et al. 1997) and was also adjusted to investigate effects of bait sprays products on larvae of the green lacewing and the seven-spotted ladybird under greenhouse or semi-field conditions (Vogt et Köppler, 2008). These methods allowed testing products that had a very specific mode of action in conditions very close to the practice but with the advantage of laboratory trials in terms of repeatability and interpretation of results. Methods that combine realistic exposure mode and simplicity of laboratory tests compared to field or semi-field tests are perhaps the solution to test the side-effects of products with a specific mode of action and for which other ways of contamination than contact with a treated surface can be important.

The aim of this work was firstly to adapt the method to the two-spotted ladybird *Adalia bipunctata* and secondly to use these methods to assess the toxicity of 4 insecticides: the two aphid-feeding blocker insecticides pymetrozine and flonicamide, the neonicotinoid systemic insecticide thiacloprid and pirimicarb. Compared to the previous methods developed for *C. carnea*, the methods were adapted to be closer to field conditions, with a direct spray of the test organisms distributed on the plants and no renewal of the food after treatments, to assess the possible effects of starvation. Pirimicarb, flonicamide and pymetrozine were considered as selective for this ladybird species (Hautier et al., 2006; Jansen et Hautier, 2007; Jansen et al., 2008) with classical methods in the laboratory while thiacloprid was highly toxic for ladybirds (Anonymous, 2004; Jamieson et al., 2005).

Material and methods

Exposure units

An exposure unit consisted of a group of 20-25 French bean seedlings (*Vicia fabae*) seeded in the central part (approximately 25x35cm) of a 35x55cm plastic tray. The surface was covered

with a fine layer of sand and the whole part surrounded by a metal frame (height: 10-12cm) treated with Fluon GP1 to prevent the insects placed inside the units to escape. The plants were seeded in the central part to avoid possible escape of insects by direct contact of plants and the metal frame.

Test products and product application

Four insecticides used to control aphids in open fields and orchards were tested at their maximum recommended field rate. Details of products, active ingredient identity and content, formulation type and tested rates are given in table 1. Products were diluted in water soon before being applied. Applications were made outdoor with a knapsack sprayer connected to a 2m wide sprayer ramp with 4 Teejet flat fan nozzle (XR series, 110, 50cm spacing). Units were placed in the centre of a 10m x 2m area and then treated. Pressure and speed of the ramp was adapted to deliver an application volume of 400l/ha ± 10%. Pesticide residues were left to dry outside (under rainfall protection if needed) for one or two hours before the units were brought back in the laboratory.

Table 1: List of insecticides tested. commercial name, active ingredient identity (a.i.) and content, formulation type and tested rate.

Commercial name	Active ingredient	Formulation and a.i. content	tested rate (a.i./ha)
Calypso	thiacloprid	SC, 480 g/l	120g
Pirimor	pirimicarb	WG, 50%	125g
Plenum	pymetrozine	WG, 50%	150g
Teppeki	flonicamid	WG, 50%	80g

Test design

Products were tested in groups of 2, in the same time as a water treated control. For the initial part of the test, there were 4 replicates by object, each replicate being made of one group of plants and 15 ladybird larvae. Replicates were randomly distributed in the climatic chamber. For the fertility assessment, egg production was assessed on all identified surviving females of each group.

Conduct of the trials

When the plants were 2-4cm high, 600mg of pea aphids (*Acyrtosiphon pisum*) were added to each unit. This amount was calculated from previous similar experiments to provide food in excess in the control without affecting the plants before the end of the exposure phase. 48h later, 15 2-3 day old *A. bipunctata* larvae were released into each unit. Aphids and larvae were provided from the mass rearing of the laboratory. Larvae were left to distribute into the vegetation for 2-4 hours and then the units were transferred outside for product application. After treatment, units were brought back in the laboratory and kept till the end of the exposure phase without addition of food. When first larvae started to pupate in the control, the units were dismantled and larvae and pupae harvested and counted in each unit. Mortality was calculated on basis of living larvae and pupae found, considering larvae not found as dead. Mortality was then corrected according to Abott (1925). Surviving larvae were kept individually in petri dishes and fed with aphids harvested in the exposure units, if possible, or with aphids from the mass rearing if no aphids exposed to test product were available, till pupation. Development time to adult stage was recorded.

When adults emerged, they were transferred into plastic breeding containers (15x15x25cm) and fed with cut French bean plants infested with pea aphids and honeybee pollen. Pieces of crumpled paper were placed in the rearing containers to stimulate oviposition. There was one breeding container per object. Food and paper was changed 3x per week. Date of first egg laying was noted and the fertility assessment was initiated the second week of egg laying. For this, females were individually confined in plastic petri dishes and fed with living aphids and pollen. Females were transferred to new petri dishes every 24h and the number of eggs laid was assessed on 6 successive 24h period. Eggs were counted and larval emergence was checked. Eggs that did not hatch 7 days after egg laying were considered as non viable. Total number of eggs/female/24h, hatching rate and number of viable eggs/female/24h were recorded.

Efficacy trials on aphids

In order to determine food availability in the exposure units, groups of 10 to 12 plants grown in plastic containers and infested with 300mg of aphids were treated with the test product with the same protocol than the exposure units with ladybirds. There were 3 sets of 4 replicates per product and control. The first set was dismantled 2 days after treatment, the second one 5 days and the last one 8 days. When they were dismantled, living aphids were harvested and weighed. A subsample of about 100mg of aphids was taken from each replicates to count the aphids and transform the weights into aphid numbers in each replicate.

Test conditions

All experiments, except product application, were performed at 22-25°C, 50-95% RH. Light (7000 to 10000 lux) was provided by sodium lamp (Son-T Agro) on basis of a 16/8 day/night photoperiod.

Statistical analysis

Observed mortality, development time to adult, total egg production, hatching rate and viable egg production were analyzed with the help of a one-way Anova test (Minitab Software). Percentages (mortality, hatching rate) were transformed into arcsin before analysis. In case of significant differences detected by the Anova, results were compared with the help of a Tukey test at p=0.05 level.

Results and discussion

Effects of insecticides on aphid development in similar conditions as during the test with ladybirds are illustrated by figure 1. In the control, aphid populations was growing in a exponential way with 4.5x more aphid after 8 days than at the beginning. According to their effects, the different insecticides can be split into two groups. Pirmicarb and flonicamid had both a strong and rapid impact on aphid populations with nearly no aphids found on the plants 2 days after treatments. Pymetrozine and thiacloprid, even if their mode of action is totally different, had similar impact on aphid populations, with an inhibition of aphid development more than a curative action in laboratory conditions. Even if at day 8, aphid population were reduced of more than 80% compared to control, they were at the same level as initially. These results were indicating that under the test conditions with ladybirds, pymetrozine and thiacloprid did not have a rapid and strong impact on aphid population and that food resources were available for ladybirds, even if they were reduced compared to the control. With pirimicarb and flonicamide, food resources were clearly limited in time and possible effects of

starvation could occur, even if ladybirds were able to feed on dead aphids for one or two days, as confirmed by visual observations. Flonicamide had also a different effect on the pea aphid than pymetrozine, while these two products are most of time showed as similar products, with the same mode of action.

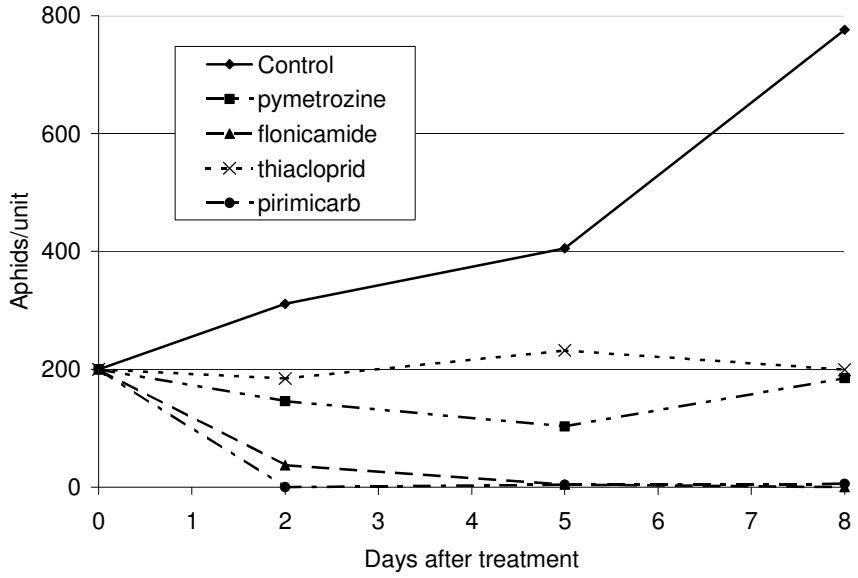

Figure1. Evolution of aphid population on French bean plants after treatment with insecticides

Effects of tested insecticides on *A. bipunctata* on plants are reported in table 2 (set 1, pymetrozine and thiacloprid) and in table 3 (set 2, pirimicarb and flonicamide). Mean control mortality reached 22.5% for the two sets of experiments and was in the range of previous experiments carried out with the same species and test design. Pymetrozine was the only insecticide that did not lead to mortalities different from control, with only 12.0% corrected mortality. Flonicamide, thiacloprid and pirimicarb caused 65.1%, 80.0% and 83.7% corrected mortality, respectively.

Ladybird larvae of the control groups needed 22.6 and 20.3 days to reach adult stage in set 1 and set 2, respectively. The second set of experiment was carried out with a mean temperature 1 to 2°C higher than the first set, thus results of products from different set cannot be directly compared each other. Development time to adult stage was longer in a significant way than in the control for thiacloprid, flonicamide and pirimicarb, with up to 2.5 days more to reach adult stage with thiacloprid and pirimicarb. Reasons of these differences are unknown but could be consequence of direct effect of the product, by contact or by ingestion, or indirect effects by starvation of the larvae, at least for pirimicarb. The consequence in term of biological control of these longer development period will probably be limited if the product is only used one time in the season in a given landscape and only affect one ladybird generation. In case of repeated applications at different moment of the season, this could affect successive generations, the effects in terms of number of generation per year, population level at the beginning of winter and long-term effects in terms of biological control are unknown.

Viable egg production of females obtained from larvae that survived the exposure phase was not different from control for pymetrozine, thiacloprid and pirimicarb. With flonicamide, though the total number of eggs/female/day was similar as in the corresponding control, the hatching rate was significantly reduced and the number of viable eggs produced per female was reduced by more than 50% compared to the control.

Table 2: Effects of pymetrozine and thiacloprid on larvae of *A. bipunctata* on plants. Observed and corrected mortality, development time to adult stage, egg production, hatching rate and viable egg production/female/24h.

	Control	pymetrozine	thiacloprid
Larval mortality (observed) ± sd	16.7% ± 15.3%a	26.7% ± 9.4%a	83.3% ± 7.5%b
Larval mortality (corrected)	-	12.0 %	80.0 %
Development time to adult stage (days) ± sd	22.59 ± 0.46a	23.58 ± 0.64a	25.33 ± 0.76b
Total egg production ± sd	22.80 ± 7.04a	22.03 ± 4.18a	19.50 ± 11.81a
Hatching rate ± sd	63.8% ± 11.9%a	64.1% ± 22.4%a	65.6% ± 10.6%a
Viable egg production ± sd	15.04 ± 4.20a	13.62 ± 5.42a	12.17 ± 6.84a

Anova test (one-way) followed by Tukey multiple comparison at p=0.05 level. Arcsin transformation for percentage before analysis. Numbers followed by the same letter are not different in a significant way.

Table 3: Effects of pirimicarb and flonicamide on larvae of *A. bipunctata* on plants. Observed and corrected mortality, development time to adult stage, egg production, hatching rate and viable egg production/female/24h.

	Control	pirimicarb	flonicamide
Larval mortality (observed) ± sd	28.3% ± 7.3% a	88.3% ± 7.3% b	75.0% ± 5.5% b
Larval mortality (corrected)	-	83.7 %	65.1 %
Development time to adult stage (days) ± sd	20.31 ± 0.31a	23.17 ± 0.47c	21.56 ± 0.61b
Total egg production ± sd	16.83 ± 7.12a	13.94 ± 7.13a	16.27 ± 6.00a
Hatching rate ± sd	69.9% ± 17.2%a	82.4% ± 10.5%a	34.9% ± 11.9%b
Viable egg production ± sd	11.56 ± 4.63a	11.00 ± 2.25a	5.47 ± 1.84b

Anova test (one-way) followed by Tukey multiple comparison at p=0.05 level. Arcsin transformation for percentage before analysis. Numbers followed by the same letter are not different in a significant way.

On basis of glass plates and extended laboratory tests on plants carried out with the same product on ladybirds, harmlessness of pymetrozine was expected. Harmful effects of pirimicarb and flonicamide were however unexpected as these products were found harmless for ladybirds in the laboratory (Hautier et al., 2006, Jansen et Hautier, 2007, Jansen et al., 2008).

Pirimicarb was generally considered as a selective aphicide with no effects on aphid predators in the field except for hoverflies (Hellpapp, 1982, Jansen, 2001). However, test methods in the laboratory did not include possible starvation of the larvae after aphicide treatment and with the field tests, the ladybird populations were only assessed a few days after treatments. Thus, the starvation of ladybird larvae surviving the treatment, as shown by results of efficacy trials (Table 1) could be the explanation of such high toxicity. These type of effects were never considered in the laboratory or in semi-field and this omission could underestimate the toxicity of several compounds selective by contact but that could have long-term detrimental effects on prey/predator balance in agricultural ecosystems. However, negative impacts of these effects in the field could be balanced by several parameters. Firstly a possible recolonisation of the treated fields can occur and offer new aphids for the ladybird, as the persistence of pirimicarb in the field is very limited. Secondly, the experiments in the laboratory were carried out with 2-3day old larvae and it could perhaps be possible for older larvae to complete their development to reach adult stage without consequence. Results of fertility assessment with pirimicarb showed that adult obtained from surviving larvae were able to produce the same number of viable eggs than control female. Thirdly, even if *A. bipunctata* larvae are specific aphid predator, they can also fed on other prey than aphids, as ladybird eggs or other ladybird larvae of the same species (cannibalism) or of another species (heterospecific predation). These parameters could finally limit the impact of pirimicarb on ladybird even if the toxicity observed in this study, in conditions very close to the field, is particularly high.

In comparison, pymetrozine appears as a very promising insecticide for aphid control. The toxicity by contact was similar than with pirimicarb but in addition, the relative slow action of the insecticide on aphid populations allow young ladybird larvae to feed several days on living aphids and to reach adult stage. Thus, if the aim of the treatment is to suppress aphids and to maintain aphid predator populations in the ecosystem as high as possible, pymetrozine is a more valuable product than pirimicarb.

With Flonicamide, the high toxicity observed could have the same origin than for pirimicarb, with a starvation of the ladybird soon after treatment, as indicated by the trials carried out on aphids only. As for pirimicarb, several parameters could limit the real impact in the field, while significant effects on fertility were also observed with 52% viable eggs less produced by females compared to control. This reduction was the direct consequence of a reduction in the hatching rate of the eggs, indicating possible effects of flonicamide on ladybird mating.

With thiacloprid, as effects on prey availability was similar than with pymetrozine, the mortality cannot be attributed to starvation but is probably the consequence of direct effects, by direct spray, contact with treated plants and/or, contaminated food uptake.

In conclusions, the methods proposed in the study allow to reflect a part of the complexity of possible side-effects of pesticides in the field with standardized methods in the laboratory. Direct spray of test organisms distributed in the vegetation, contact with a treated surface, ingestion of contaminated food and possible effects due to reduction of food availability were assessed with the same experimental protocol. Unexpected high mortality of products that were considered as selective according to standard IOBC test before highlight

the need for these kind methods that combined both realistic exposure mode and possible standardization and repeatability in the laboratory. This is particularly true for products with specific mode of actions, as systemic fungicides and insecticides.

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