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Possible synergistic effects of fungicide-insecticide mixtures on beneficial arthropods

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Abstract: The effects of different mixtures containing one fungicide and one insecticide, some of them previously known to have synergistic effects on bees, on adults of the parasitic wasp *Aphidius rhopalosiphi*, protonymphs of the predatory mite *Typhlodromus pyri* and larvae of the ladybird *Adalia bipunctata* were assessed in the laboratory. LD50 tests on glass plates using standard methods were used for the two first species while LD50 was assessed on ladybird larvae following topical application of 0.5 µl product droplets. The mixtures prochloraz + lambdacyhalothrine, epoxyconazole + thiacloprid, pymetrozine + fluazinam and taufluvalinate + boscalid, as well as each individual compound, were assessed in one or two sets of five to eight sequential dilutions. The proportions of insecticides and fungicides were based on normal agricultural practice, keeping the same ratio throughout the dilutions.

The results showed that the mixtures prochloraz + lambda-cyhalothrine and epoxyconazole + thiacloprid had a strong synergistic effect on the three species, with an LD50 2.5 to 11 times lower than that of the insecticide alone, while the fungicide tested separately had no toxicity at all. The LD50 of the two other mixtures was no different from the effects of the insecticide alone.

These results suggest that the toxicity of several insecticides for beneficial arthropods often applied in mixtures with fungicides could be underestimated if they are assessed individually, as is currently done. Although only a limited number of associations have been tested so far, these results raise questions about the relevance of risk assessment of single products for beneficial arthropods and, by extension, for all non-target species.

Key words: synergistic effects, tank-mixes, insecticides, fungicides

Introduction

Plant protection products are continuously assessed for their possible impact on non-target organisms. Some of these assessments are conducted before the products are put on the market, mainly for regulatory purposes, while others are performed when they are available to end users, in order to determine specific characteristics such as the possibility of using them in IPM. Although the tests performed by the industry, development services, scientists and groups of producers are numerous and help to characterize the compatibility of plant protection products with beneficial arthropods, most of the assessments are based on single products, tested individually.

However, this does not reflect the reality of plant protection use, in that many farmers do not hesitate to mix products in sprayer tanks. Some mixtures, such as combinations of different herbicides or fungicides, are used deliberately in order to increase the spectrum of activity in one application. Some of these mixtures are recommended by the manufacturers or development services, and the expected benefits in term of efficacy are well known. However, many tank mixtures, such as those consisting of one insecticide and one fungicide, are created solely in order to apply two products that should normally be applied independently in a single operation, in order to save time, water and energy. Where they are possible, these mixtures are very common and could cause problems if unexpected effects occurred.

Several pieces of research into bee toxicity have drawn attention to the synergistic effects of mixtures of certain insecticides and fungicides. A mixture of an imidazole fungicide, prochloraz, and a pyrethroid insecticide, deltamethrin, was found to be highly toxic for the honeybee *Apis mellifera* L. (Hym.; Apidae), whereas the products were considered to be safe when applied separately at the doses tested in the mixture (Colin and Belzunces, 1992). These unexpected effects were also obtained for a set of nine ergosterol biosynthesis inhibitor fungicides belonging to the imidazoles and triazoles family, when combined with the pyrethroid insecticide lambda-cyhalothrine. The LR50 values for honeybees obtained by topical application were 3.3 to 16.2 times lower for the mixtures than for the insecticide alone, whereas the toxicity of the fungicide was negligible at the doses assessed in the mixtures (Pilling and Jepson, 1993). Similar results were obtained for combinations of certain fungicides used on oilseed rape and the pyrethroid insecticides lambda-cyhalothrine and alpha-cypermethrin, whereas combinations with fungicides with other modes of action than those of triazoles and imidazoles, such as chlorothalonil, iprodione and carbendazim, showed no synergistic effects for honeybees (Thompson and Wilkins, 2003).

These synergistic effects were also detected for honeybees, bumblebees and wild solitary bees for combinations of triazole fungicides and neonicotinoid insecticides, with synergy found between fenbuconazole and acetamiprid on *A. mellifera* and the solitary bee *Osmia cornifrons* (Radoszkowski) (Hym., Apidae) (Biddinger *et al*., 2013) and for the combination propiconazole + clothianidin on *A. mellifera*, *Bombus terrestris* and the solitary bee *Osmia bicornis* (L.) (Hym., Apidae) (Sgolastra *et al*., 2016). These results showed that the synergy was not limited to the pyrethroid insecticides only.

There are no clear indications of possible synergistic effects of fungicide-insecticide mixtures on non-target arthropods other than bees in the literature. The aim of this work was to investigate these possible effects on beneficial organisms. Three species belonging to distinct taxa – a hymenopteran, a coleopteran and a phytoseid mite – were used, and four fungicide-insecticide combinations were tested. Two of these were previously known to have synergistic effects on bees (pyrethroid insecticide + imidazole and neonicotinoid insecticide + triazole), while the other two corresponded to mixtures widely used in Belgium (an “oilseed rape” mixture consisting of a pyrethroid insecticide and an SDHI fungicide applied during flowering, and a “potato” mixture consisting of a late blight fungicide and an aphicide) whose synergistic effects had never previously been tested.

Material and methods

Tests with A. rhopalosiphi

The insects used for the test had been mass-reared in the laboratory. Male and female parasitic wasps aged 0-48 hours were used. The test methods followed the first part (exposure of adults to treated glass plates) of the standard test developed for this species on glass plates, which is widely used for registration (Mead-Briggs *et al*., 2000), except for the number of insects and replicates. For each dose of individual product or mixture, one unit containing 20 wasps (with a male/female ratio of between 8/12 and 12/8) was used. Mortality was recorded after 48 hours of exposure. The experiments were performed at 20 ± 2 °C and 60-90% RH, with indirect lighting (400W Son-T agro sodium) providing 1000 to 1500 lux on the basis of a 16/8 day/night photoperiod.

Tests with T. pyri
The mites used for the test had been mass-reared in the laboratory. Protononymphs aged 24 hours were used. The test methods followed the first part (exposure of adults to treated glass plates) of the "Open cell" standard developed for this species on glass plates, which is widely used for registration (Blümel *et al*., 2000), except for the number of insects and replicates. For each dose of individual product or in mixture, one unit containing 25 protonymphs was used. Mortality was recorded after 7 days of exposure. Control units were treated with water. The experiments were performed at 25 ± 2 °C and 60-90% RH, with 400W Son-T agro sodium lighting (8000 to 10,000 lux) on the basis of a 16/8 day/night photoperiod.

Tests with A. bipunctata
The products were assessed by topical application. The larvae had been mass-reared in the laboratory. For each dose and control, a group of 20 larvae was considered. The products were diluted in water with added soap (Triton X 100, 0.5% v/v). One 0.5 µl droplet of the product solution, or of water and soap for the control, were placed on the thorax of larvae aged 4 to 5 days, placed individually in small petri dishes. The larvae were fed with aphids and mortality was recorded after 48 hours. Trials with younger larvae (2 to 3 days old) were initially planned, but the size of the larvae made the correct application of the droplet impossible and larger (and older) larvae were needed. The tests were performed at 20 ± 2 °C and 60-90% RH, with 400W Son-T agro sodium lighting (8000 to 10,000 lux) on the basis of a 16/8 day/night photoperiod.

Products and mixtures
Four mixtures of one insecticide and one fungicide were assessed on each species, as well as each of the individual compounds. The mixtures were based on possible and common practices in Belgium and the insecticide/fungicide ratio was always identical and based on the recommended field rates for each individual product. Two of the mixtures (pyrethroid insecticide + EBI fungicide and neonicotinoid insecticide + EBI fungicide) were already known to have synergistic effects on bees. The other two corresponded to mixtures commonly used on oilseed rape (Cantus + Mavrik 2F) and potato (Shirlan + Plenum).

The list of products, mixtures and ratios is given in Table 1. The products were always prepared to obtain the tested doses diluted in 200 l of water. For the residual glass plate toxicity tests, the product was applied with the help of a Burgerjon spray tower. For the topical application, a Burkard hand microapplicator syringe was used.

The tests were performed in different sets. To start with, the fungicides and insecticides were assessed alone with a set of 4 to 6 dilutions in a large range (5- to 10 times), with the maximum tested fungicide rate usually corresponding to the field rate. According to the results of these first tests, a second set was performed with a more limited range of dilution (2 to 5 times), in order to obtain data that would enable LR50 values to be calculated. For this final set, all dilutions with the insecticide and the insecticide-fungicide mixtures were assessed, whereas the number of tests performed in the set of doses of fungicide alone was sometimes reduced when it was clear from the first results that no mortalities were expected at the rates tested. In some cases, a third set of dilutions was performed to obtain more complete data for LR50 assessment with a high level of accuracy.

Table 1. Information on products assessed alone and in tank mixtures on the three beneficial species.

Trade name	Active substance	Use	Chemical class	Concentration	Recommended field rate
Sportak	Prochloraz	Fungicide	Imidazole (EBI)	450 g/l	450 g a.s./ha
Karate Zeon	Lambda-cyhalothrine	Insecticide	Pyrethrinoid	100 g/l	5 g a.s./ha
Mixture proportion: 50 ml Karate Zeon + 1.0 l Sportak in 200 litres of water/ha					
Rubric	Epoxyconazole	Fungicide	Triazole (EBI)	125g/l	125 g a.s./ha
Biscaia	Thiacloprid	Insecticide	Neonicotinoid	240 g/l	72 g a.s/ha
Mixture proportion: 300 ml Biscaya + 1.0 l Rubric in 200 litres of water/ha					
Cantus	Boscalid	Fungicide	SDHI	50% w/w	250 g a.s/ha
Mavrik 2F	Tau-fluvalinate	Insecticide	Pyrethrinoid	240 g/l	48 g a.s/ha
Mixture proportion: 250 ml Mavrik 2F + 500g Cantus in 200 litres of water/ha					
Shirlan	Fluazinam	Fungicide	Pyridinamine	500 g/l	150 g a.s./ha
Plenum	Pymetrozine	Insecticide	Pyridine	500 g/kg	75 g a.s./ha
Mixture proportion: 150 g Plenum + 300 ml Shirlan in 200 litres of water/ha					

Statistical analysis

The LR50 values of the products tested alone and the mixtures were obtained via Probit analysis, using log10 distribution. The products alone and their mixtures were compared by interpooling the 95% IC limits of the LR50 values determined, considering the LR50 as different if the IC did not overlap. Only products and mixtures tested at the same time on the same populations of insects were compared, with the exception of some fungicides that clearly showed no effects at the maximum field rate during the first sets of dilutions. To keep the tables and figures simple, the doses of mixtures shown are always those of the most toxic compounds (usually the insecticide).

Results

The results of the tests are given in Table 2 (*A. rhopalosiphi* glass plate test), Table 3 (*T. pyri* glass plate test) and Table 4 (*A. bipunctata* topical application). The mean control mortality was around 10% for all the tests, with a maximum of 15.0%.

The LR50 values differed widely between products and species. In most cases, the fungicides were not toxic at all, with nearly no observed mortality in laboratory at doses corresponding to the recommended field rate. As most of the insecticides were highly toxic and the fungicides would have needed to be diluted to between 100 and 10,000 times their recommended field rate in order to be assessed in the same ratio as in the initial tank mixtures, the mortality caused by these products was considered to be nil and was disregarded in the comparison of product and mixtures. The only comparisons made therefore related to individual insecticides and insecticide-fungicide mixtures. The only exception was the use of the fungicide Shirlan on *A. bipunctata*, where the recommended field rate gave mortalities close to 50%, which meant that an LR50 value could be estimated. However, as this fungicide was associated with Plenum, which gave no mortality at doses up to the field rate, for this specific combination the comparison was made between the fungicide alone and the fungicide associated with the insecticide.

Table 2. Residual toxicity on glass plates of insecticides and fungicides applied alone and in tank mixtures on adults of the parasitic wasp *A. rhopalosiphi*. LR50 values, 95% IC and comparison of data.

	LR50 (IC 95%)	95% IC (low)	95% IC (high)
Rubric (Epoxyconazole)	> 1.0 l/ha	Not available	Not available
Biscaya (thiacloprid)	0.1484 ml/ha	0.0963 ml/ha	0.2283 ml/ha
Biscaya + Rubric	0.0499 ml/ha	0.0316 ml/ha	0.0822 ml/ha
Ratio	***3.0x***	***	
Cantus	> 500 g/ha	Not available	Not available
Mavrik	20.23 ml/ha	13.81 ml/ha	88.74 ml/ha
Mavrik + Cantus	20.89 ml/ha	12.16 ml/ha	84.1 ml/ha
Ratio	***0.97x***	***NS***	
Shirlan	> 300 ml/ha		
Plenum	> 150 g/ha		
Shirlan + Plenum	> 300 ml + 150g		
Ratio	Not available		
Sportak	> 1.0 l/ha	Not available	Not available
Karate	1.541ml/ha	0.947 ml/ha	2.670 ml/ha
Karate + Sportak	0.136ml/ha	0.086 ml/ha	0.204 ml/ha
Ratio	***11.33x***	***	

LR50 determined by Probit analysis (Log distribution) and IC 95% overlapping. NS = overlapping and LR50 not different, ***, no IC overlapping and LR50 different.

The mixtures of EBI fungicide with pyrethroid or neonicotinoid insecticide (prochloraz + lambda-cyhalothrine and epoxyconazole + thiacloprid) showed clear synergistic effects, with LR50 values between 2.46 and 11.33 times lower than those of the insecticide alone, whereas the fungicide was not toxic at all. These effects were always detected on all three species tested. The two other combinations (fluazinam + pymetrozine and tau-fluvalinate + boscalid) did not show synergistic effects on any of the species tested, with LR50 values between 0.69 and 1.63 times the LR50 value of the insecticide and overlapping of the LR50 95% IC limits.

Table 3. Residual toxicity on glass plates of insecticides and fungicides applied alone and in tank mixtures on protonymphs of the predatory mite *T. pyri*. LR50 values, 95% IC and comparison of data.

	LR50 (IC 95%)	95% IC (low)	95% IC (high)
Rubric (Epoxyconazole)	> 1.0 l/ha	Not available	Not available
Biscaya (thiacloprid)	14.14 ml/ha	10.47 ml/ha	18.77 ml/ha
Biscaya + Rubric	2.15 ml/ha	0.27ml/ha	1.67 ml/ha
Ratio	***6.57x***	***	
Cantus	> 500 g/ha	Not available	Not available
Mavrik	0.3239 ml/ha	0.1921 ml/ha	0.5353 ml/ha
Mavrik + Cantus	0.1985 ml/ha	0.1328 ml/ha	0.2811 ml/ha
Ratio	***1.63x***	***NS***	
Shirlan	> 300 ml/ha		
Plenum	> 150 g/ha		
Shirlan + Plenum	> 300 ml + 150g		
Ratio	Not available		
Sportak	> 50 ml/ha	Not available	Not available
Karate	13.23 µl/ha	11.01 µl/ha	15.96 µl/ha
Karate + Sportak	5.59 µl/ha	4.49 µl/ha	6.87 µl/ha
Ratio	***2.46x***	***	

LR50 determined by Probit analysis (Log distribution) and IC 95% overlapping. NS = overlapping and LR50 not different, ***, no IC overlapping and LR50 different.

Table 4. Residual toxicity by topical application (0.5 µl droplet) of insecticides and fungicides applied alone and in tank mixtures on larvae of the ladybird *A. bipunctata*. LR50 values, 95% IC and comparison of data.

	LR50 (IC 95%)	95% IC (low)	95% IC (high)
Rubric (Epoxyconazole)	> 1.0 l/ha	Not available	Not available
Biscaya (thiacloprid)	10.942 µl/ha	7.953 µl/ha	13.839 µl/ha
Biscaya + Rubric	3.5225 µl/ha	1.9625 µl/ha	6.8628 µl/ha
Ratio	***3.10x***	***	
Cantus	> 500 g/ha	Not available	Not available
Mavrik	1.237 ml/ha	0.7811 ml/ha	2.1308 ml/ha
Mavrik + Cantus	1.394 ml/ha	0.8542 ml/ha	2.447 ml/ha
Ratio	***0.89x***	***NS***	
Shirlan	244.03 ml/ha	57.9 ml/ha	530.3 ml/ha
Plenum	> 150 g/ha		
Shirlan + Plenum	351.6 ml/ha	247.1 ml/ha	540.7 ml/ha
Ratio	***0.69x***	***NS***	
Sportak	> 300 ml/ha	Not available	Not available
Karate	70.87 µl/ha	31.31 µl/ha	149.7 µl/ha
Karate + Sportak	14.36 µl/ha	5.375 µl/ha	29.9 µl/ha
Ratio	***4.9x***	***	

LR50 determined by Probit analysis (Log distribution) and IC95% overlapping. NS = overlapping and LR50 not different, ***, no IC overlapping and LR50 different.

Discussion

The results obtained clearly show that mixtures combining an EBI-type fungicide with a pyrethrinoid- or neonicotinoid-type insecticide, which are known to have a synergistic effect on different species of honeybees and bumblebees (Colin and Belzunces, 1992; Thompson and Wilkins, 2003; Biddinger *et al*., 2013; Sgolastra *et al*., 2016), had the same type of effects on three different species of beneficial arthropods belonging to three distinct families and somewhat remote from one another from a systematic point of view: a mite from the Phytoseiidae family, a parasitic hymenopteran and a coleopteran. These results indicate that the phenomenon is not unique to pollinating insects and probably occurs in a greater number of arthropod species.

When synergistic effects had been demonstrated statistically, the ratios between the LR50 of the mixture and of the insecticide were usually between 3 and 6 times, with a maximum of 11.3 times. These values are comparable to those obtained on the honeybee through topical application, with ratios between 3.3 and 16.2 times (Pilling and Jepson, 1993). The absolute LR50 values and their ratios are of course indissociable from the methods used to obtain them. In this case, the methods used were initial toxicity tests (residual toxicity on glass for *Aphidius* and *Typhlodromus*, direct application for ladybirds), carried out under conditions far removed from the actual conditions of use of the products. These methods are basically used to demonstrate the potential toxicity of a product, and the values obtained during these initial tests are purely indicative. The actual effects are often subsequently found to be less when the tests are carried out with methods closer to the reality in the field, in particular in terms of doses, exposure routes, application techniques and substrates. This means that it is likely that the mixtures for which synergistic effects have been demonstrated in the laboratory will not be three to six times more toxic than the insecticide alone, but that the addition of an EBI-type fungicide to a pyrethrinoid or neonicotinoid insecticide does potentially increase the toxicity of these insecticides on beneficial arthropods. The true extent of this increase, however, must be measured under less artificial conditions than the initial toxicity tests.

Two of the four mixtures, including families of fungicides and insecticides commonly used and not yet tested together on a beneficial insect species, showed no synergistic effects, with LR50 individual product/mixture ratios between 0.69 and 1.63 times. At present, only combinations of imidazole or triazoles with pyrethronoid or neonicotinoid cause problems for beneficial arthropods. However, by no means all possible combinations of fungicides and insecticides have been tested for their synergistic effects on beneficial arthropods, and more detailed studies will be required to demonstrate the presence or absence of synergistic effects for the main classes of fungicides and insecticides.

Since the observed synergies have been clearly demonstrated on several very different species, it is possible that a battery of tests carried out on a single species may be sufficient, at least on an exploratory basis. This could be a species for which a method exists which is easy to implement, to enable as many mixtures as possible to be tested with the given resources.

Besides the problem of the increased toxicity of the insecticide to beneficial arthropods, the synergy that arises when a fungicide and an insecticide are applied raises an important question. Is this synergy confined to cases where the two products are applied together, or does it occur in all situations where the insect is exposed to both products, even when applied separately? Examples of such situations would be when a fungicide and an insecticide are applied at an interval of a few days on the same crop, with significant residues of the first product still remaining when the second product is applied; or when mobile insects such as bees and adult ladybirds are exposed to products applied at the same time to two neighbouring fields and visited successively by the insect.

These questions raise some limitations in evaluating the product-specific risk without taking current practices into account. They give some cause for concern, as it is logical to assume that the increase in an insecticide's toxicity through synergy is probably not limited to bees and beneficial insects, but affects a large number of other non-target species, including humans.

Although the synergies currently known relate only to a limited number of substances, not all of the many possible combinations have been tested and it is a matter of urgency that this phenomenon should be explored in greater depth and the uncertainties reduced.

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