Pest Manag Sci 59:279–286 (online: 2003)
DOI: 10.1002/ps.626

Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (*Apis mellifera* L, Hymenoptera)

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Abstract: It had been found earlier that the chloronicotinyl insecticide thiacloprid (as the 480 g litre^{-1} SC Calypso®) poses a favourably low toxicity hazard to the honeybee, *Apis mellifera* L. As with pyrethroids, the metabolization of chloronicotinyl compounds involves monooxygenases, which are known to be inhibited by some ergosterol biosynthesis inhibitor (EBI) fungicides potentially co-applied with these insecticides. The potential synergistic enhancement of the toxicity of thiacloprid to honeybees when co-applied with such fungicides was therefore studied under laboratory and semi-field conditions. Fungicides of other chemical classes were also examined for synergistic potential to reveal other metabolic interactions. In the laboratory, only a slight synergistic effect was observed with the anilinopyrimidine fungicide examined, while a significant enhancement of thiacloprid toxicity to honeybees was found with EBI fungicides. In three tunnel tests conducted under different environmental conditions to simulate field exposure, no increased mortality was observed when honeybees were directly sprayed with thiacloprid (Calypso) alone or in combination with the EBI fungicide tebuconazole (250 g litre^{-1} EW, Folicur®). There was also no synergized reduction in the foraging intensity on the treated crop. In general, the foraging intensity decreased after thiacloprid treatment but was restored within 24–48 h. The hive vitality was not affected by either thiacloprid or its tank mix with tebuconazole. Our results suggest that, at the recommended use rates, thiacloprid poses a negligible lethal risk to honeybees when applied either alone or in tank mixes with fungicides of various chemical classes.

Keywords: thiacloprid; *Apis mellifera*; synergism; azole; fungicides; tank mix

1 INTRODUCTION

Chloronicotinyls are a new group of insecticides which are highly effective against various pest species.[1–3] Some of these chloronicotinyl insecticides are highly toxic to the honeybee, *Apis mellifera* L, and are not allowed to be used while the target crop is flowering.[4,5] This restriction, however, is not required for the new chloronicotinyl insecticide thiacloprid, which is recommended for pest control in fruit orchards and poses a favourably low toxicity hazard to the honeybee.[3,6] The option to apply this insecticide shortly before or even during flowering allows the effective control of pest species that otherwise are hard to control. During farming practice, however, insecticides are frequently sprayed in combination with various fungicides. During the last decade, a synergistic enhancement of the toxicity of pyrethroids, when combined with EBI fungicides, has been reported for the honeybee.[7–14] The biochemical mechanism of this synergism was seen in an interaction of the fungicide with the cytochrome P-450 monooxygenase system, responsible for detoxifying pyrethroid insecticides.[11,15,16] Since monooxygenases are also involved in the metabolization of chloronicotinyls (hydroxylation of the imidazolidine and thiazolidine rings)[17,18] it was of great interest to examine whether or not the toxicity of thiacloprid to the honeybee is synergized by EBI fungicides that may be co-applied in tank mixes.

In this paper, the contact toxicity of thiacloprid (as the 480 g litre^{-1} SC, Calypso®) to honeybees alone and in combination with EBI fungicides was examined in standard laboratory assays.[19,20] Since non-EBI fungicides are co-applied frequently with insecticides, some non-EBI fungicides were also tested for their synergizing potential to thiacloprid to reveal other possible metabolic interactions. Finally, the field relevance of the potentially synergized toxicity of

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(*Received 2 April 2002; revised version received 23 May 2002; accepted 29 August 2002*)

thiacloprid to honeybees by fungicides was examined in three tunnel cage tests.

2 MATERIAL AND METHODS

2.1 Test substances

The selection of fungicidal products was not restricted to those fungicides currently recommended for use in the main target crop of thiacloprid (fruit orchards) since the product portfolio is inevitably changing with time. Rather it was felt more important to include products representative of different fungicidal modes of action which might be used in tank mixes with thiacloprid. The selected fungicide products were prochloraz, tebuconazole, tolylfluanid, mancozeb, cyprodinil and azoxystrobin. The formulations used and the reported honeybee toxicities of the compounds are given in Table 1. Samples of the thiacloprid, tebuconazole and tolylfluanid formulations were obtained from the manufacturer (Bayer AG, D-51368 Leverkusen) with an analytical verification of the active ingredient content (>96% of the nominal content). Samples of the prochloraz, mancozeb, cyprodinil and azoxystrobin formulations were purchased from a local dealer. The chemical structures of the compounds examined are given in Fig 1.

2.2 Honeybees

All tests described were carried out using adult worker honeybees, *Apis mellifera* L (Hymenoptera: Apidae), taken from a single queen-right colony. These colonies had not been treated with antibiotics or varroacides within 4 weeks of the start of the study and were free from diseases.

For the contact toxicity tests, adult worker bees *Apis mellifera carnica* L were collected from the hive combs (avoiding the brood nest area) or from the flight board. Before treatment, honeybees were acclimatized to the test conditions for at least 2 h.

For tunnel cage tests 1 and 3, honeybees (*A mellifera carnica*) were obtained from a commercial beekeeper (Mr Josef Gilli, Reinartzstrasse 25, D-53925 Kall). Commercially managed beehives were disintegrated and the combs with the honeybees were rejoined within smaller bee hives containing approximately 3000 worker bees and three combs (two food combs and one brood comb) each. One sister queen in egg laying mode was added to each of these hive colonies within a separate and closed cage. On the next day, the colonies were assigned to one of the tunnel cages, by using a random list, and the queen cages were disclosed.

Bees (*Apis mellifera mellifera* L) for tunnel cage test 2 were provided by a professional queen-breeder (Martín Braunstein, Los Hornos, La Plata, Buenos Aires). From the parent hive three bee nuclei were generated with three brood frames, one honey frame and one empty frame (in total five frames). At test initiation each of the nuclei contained approximately 8000 adult bees and one young sister queen.

2.3 Laboratory toxicity tests

The contact toxicity of thiacloprid SC alone and co-applied with various fungicides was tested on *A mellifera carnica* following the EPPO test guideline No 170.[19] Test materials were diluted in water containing triethanolamine dodecylbenzenesulfonate (Adhäsit®) as a wetting agent. Doses were applied as 0.005-ml drops onto the ventral thorax of the bees anaesthetised with carbon dioxide. Controls were dosed with an aqueous Adhäsit solution (0.3 + 500 weight + volume; 0.005 ml). Three replicates of 10 bees were treated per dose level including controls. After treatment, bees were maintained in a controlled environment cabinet at 25 (±2) °C, 50–60% relative humidity in the dark, and fed with sucrose + water (0.5 + 1 weight + volume) ad libitum. Honeybees were examined for treatment-related behavioural effects 4 h after dosing. Further checks on behavioural effects and mortality were made at 24-h intervals over 4 days. Initial tests were carried out to establish the baseline honeybee toxicity data of thiacloprid SC and the examined 'indicator' fungicides when applied alone. Thiacloprid was then co-applied with one of the fungicides at ratios that were representative of possible tank mixes.

2.4 Tunnel tests

Three tunnel tests were conducted to examine the effects on honeybee behaviour and honeybee survival of either a single, a combined or a consecutive spray treatment with the chloronicotinyl insecticide thiacloprid and the EBI fungicide tebuconazole. Tebuconazole was chosen since the combination of this fungicide with thiacloprid revealed the relatively strongest toxicity enhancement of the latter to

Table 1. Fungicides examined for their synergizing potential under laboratory conditions and reported contact toxicity of the contained active ingredients to the honeybee

Test compound	*Formulation, trade name*	*Contact LD_{50} (μg AI per bee)*	*Reference*
Thiacloprid	480 g litre SC; Calypso®	20.6–82.1	6
Prochloraz	400 g litre EC; Sportak®	132.6	8
Tebuconazole	250 g $litre^{-1}$ EW; Folicur®	97–175.8	[a]
Tolylfluanid	500 g kg^{-1} WP; Euparen M®	>200.0	21
Mancozeb	750 g kg^{-1} WG; Dithane®	193.0	22
Cyprodinil	750 g kg^{-1} WG; Unix®	>101.0	22
Azoxystrobin	250 g $litre^{-1}$ SC; Amistar®	>200.0	22

[a] Bayer AG, unpublished data.

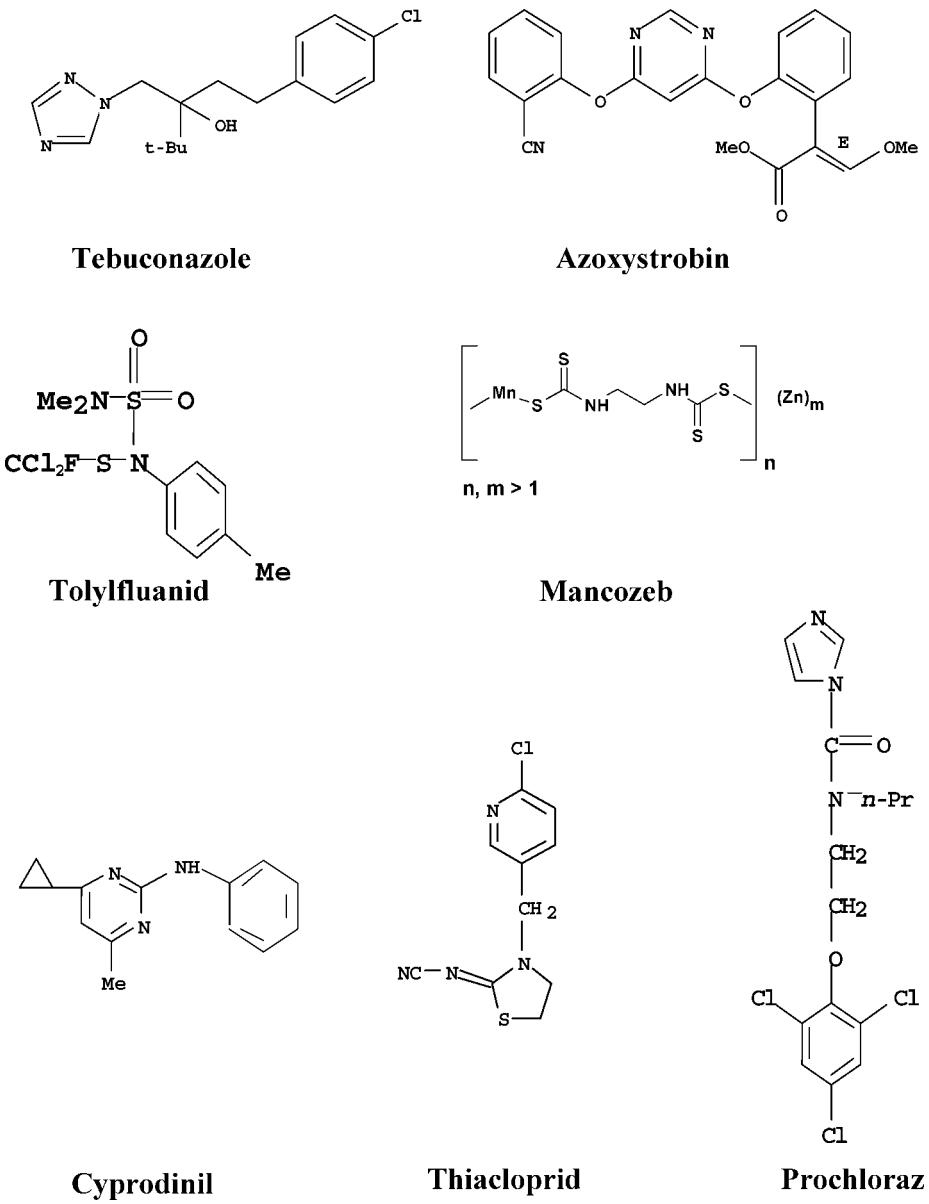

Figure 1. Chemical structures of thiacloprid and fungicides tested.

honeybees under laboratory test conditions. The tested concentrations of thiacloprid SC were 4 (first tunnel test) and 2.5 (second tunnel test) times higher than the field recommended dose (0.25 + 1000 by volume) in order to (1) account for those situations where orchards are treated with water volumes lower than 500 litres m^{-1} crown height and to (2) include the highest spray deposit per unit treatment area. Whereas an average of 40% of the applied rate is assumed to deposit within tree canopies,[23] 77–92% of the applied rate is reported to deposit in flowering oilseed rape[24] cultivations (BBCH stage 65). Accordingly, the applied rates of 144 (first tunnel test) and 96 g thiacloprid (second tunnel test) per hectare oilseed rape and *Phacelia tanacetifolia* Benth, respectively, cover well the range of spray deposit to be expected in tree canopies resulting from the highest proposed rate of 180 g thiacloprid ha^{-1} fruit orchard. In the third tunnel test, both rates/concentrations of thiacloprid were re-examined.

Tunnel tests 1 and 3 were conducted in the vicinity of Euskirchen-Billig (Germany), adjacent to the area Billiger Wald (approximately 10 km SW of Euskirchen, Germany). The second tunnel test was conducted at the experimental farm of the Agronomy Faculty of the National University of La Plata (57 km SW of Buenos Aires, Argentina). Study plots were drilled with either oilseed rape (8–11 kg seed per hectare) or *P tanacetifolia* (10 kg seed per hectare). Drilled seeds were not treated with either fungicides or insecticides, and there were no pesticidal treatments of the emerged crop throughout the study.

With onset of crop flowering, five study plots were confined within crop fields by gauze tunnels of $10 \times 5\,m^2$, 3 m high, 2 mm mesh size (test 1 and 3) and $17 \times 4\,m^2$, 2.9 m high, 1 mm mesh size (test 2), respectively. A bucket containing tap water was placed inside each tunnel as a water supply for the bees. Three to four days before treatment, the honeybee hives were put into the gauze tunnels and the tunnels were impartially assigned to one of the examined treatments (Table 2).

Spray treatments were performed during the flowering peak of the crop. The spray equipment was calibrated beforehand to ensure application of the desired spray volume (recorded deviations ± 5%). Treatments were done in the late morning when honeybees were actively foraging within the study plots, except treatments number 5 of test 2 and 3 (Table 2), which were performed after honeybees had ceased their foraging activity. After treatment, the honeybees were allowed to forage on the treated study plots until the end of flowering (ie 10, 9 and 7 days during tests 1, 2 and 3, respectively). Afterwards, the hives were individually labelled and returned to the home apiary where they could forage on other crops and wild plants as they wanted.

Weather conditions prevailing in the tunnel cages were recorded during treatment and over the entire test period. During the study period, while bee hives were maintained within the tunnel cages, the following testing endpoints were recorded:

2.4.1 Foraging activity and behaviour of the honeybees
All behavioural anomalies eg exaggerated motility, discoordinated movements (trembling, shaking, apathy) of the honeybees were recorded. Foraging intensity, ie the number of bees foraging on the treated plants was recorded daily (1–7 counts over 1–2 min) on impartially selected 1-m^2 sub-plots within the confined crop area. In addition, the number of bees arriving at the landing board and loaded with pollen were counted with the same frequency during test 2.

2.4.2 Mortality of honeybees
In front of the hive nuclei, linen sheets ($60 \times 50\,cm^2$, tests 1 and 3) or white plastic nets ($70 \times 100\,cm^2$, test 2) were placed on the ground to facilitate the recovery of dead bees. The number of dead bees was recorded each morning. For statistical analyses, mortality data of comparable treatment groups were pooled, ie water and tebuconazole treatments, thiacloprid-only treatments (low and high rate separately), and combined treatments of thiacloprid and tebuconazole. Since untransformed data were not normally distributed (Kolmogorov–Smirnov and Shapiro–Wilk test) they were log-transformed before further statistical analyses. After log-transformation, data were subjected to a one-way ANOVA analysis.

2.4.3 Vitality of the bee hives
Hive weight, pollen stores, colony strength and brood nest size of each nucleus were determined immediately before transferring the hive nuclei into the tunnel cages and after the exposure period. Colony strength was determined by estimating the percentage of the total comb area (three double-sided combs) covered by honeybees. The quantification of brood nest sizes and food stores (nectar and pollen) was made by estimating the percentage of comb cells containing either food or brood for each side of the three combs.

3 RESULTS

3.1 Contact toxicity of thiacloprid to honeybees applied alone and in combination with various fungicides under laboratory conditions

In the standard contact toxicity test according to EPPO 170, a 48-h LD_{50} of 0.013 mg AI per bee was calculated for thiacloprid 480 g $litre^{-1}$ SC. Discoordinated movements, staggering and apathy were observed for doses of 0.006 mg AI per bee or higher. Behavioural effects resulted in either death or recovery within 24–48 h, depending on dose. No effects were observed at doses of 0.003 mg AI per bee or lower.

At doses corresponding to recommended use rates, no lethal or sublethal effects were observed for thiacloprid SC or any of the fungicides examined (Table 3). Mortality assessments 48-h post-treatment also revealed no increased honeybee toxicity for thiacloprid when co-applied with mancozeb or azoxystrobin at doses corresponding to recommended use rates. After co-application of thiacloprid and tolylfluanide, seven out of 30 honeybees exhibited short-term discoordinated movements but recovered within 24 h. Similar short-term behavioural effects and a slightly increased mortality was recorded for a co-application of thiacloprid and cyprodinil. In contrast, the EBI fungicides prochloraz and tebuconazole strongly enhanced the toxicity of thiacloprid (Table 3). Discoordinated movements, apathy and death were recorded in 87 and 70% of the treated bees,

Table 2. Treatments applied to honeybees during the three tunnel cage studies[a]

Treatment No	AI ($g\,ha^{-1}$) Formulation[b] [dilution (v + v)]		
	Test 1	Test 2	Test 3
1	Tap water (= control)	Tap water (= control)	Tap water (= control)
2	Thiacloprid 144 [1 + 1000]	Thiacloprid 96 [0.67 + 1000]	Thiacloprid 96 [0.67 + 1000]
3	Thiacloprid 144 + tebuconazole 375	Tebuconazole 375 [5 + 1000]	Thiacloprid 144 [1 + 1000]
4	Thiacloprid 144; 3 days later, tebuconazole 375	Thiacloprid 96 + tebuconazole 375, applied on foraging honeybees	Thiacloprid 144 + tebuconazole 375, applied on foraging honeybees
5	Tebuconazole 375; 3 days later, thiacloprid 144	Thiacloprid 96 + tebuconazole 375, applied after bees ceased foraging	Thiacloprid 144 + tebuconazole, applied after bees ceased foraging

[a] All compounds and compound combinations were applied at 300 litre water per hectare.
[b] Thiacloprid 480 g $litre^{-1}$ SC (Calypso); tebuconazole 250 g $litre^{-1}$ EW (Folicur).

Test material	Dose applied (mg AI per bee)	Mortality (24h;%)	Mortality (48h;%)
Test Run I			
Control	—	0	0
Thiacloprid	0.001	0	0
	0.010	7	10
Prochloraz	0.001	0	0
	0.010	7	13
Thiacloprid + prochloraz	0.001 + 0.001	0	0
	0.010 + 0.010	80	87
Test Run II			
Control	—	0	0
Thiacloprid	0.002	3	3
Mancozeb	0.008	0	0
Thiacloprid + mancozeb	0.002 + 0.008	3	3
Tolylfluanid	0.011	0	0
Thiacloprid + tolylfluanid	0.002 + 0.011	10	13
Cyprodinil	0.008	0	0
Thiacloprid + cyprodinil	0.002 + 0.008	20	20
Azoxystrobin	0.003	3	3
Thiacloprid + azoxystrobin	0.002 + 0.003	3	3
Tebuconazole	0.003	0	0
Thiacloprid + tebuconazole	0.002 + 0.003	70	70

Table 3. Contact toxicity of thiacloprid applied alone and in combination with various fungicides, to the honeybee *Apis mellifera* in the laboratory

respectively. Surviving bees were free of symptoms within 48 h (prochloraz) and 24 h (tebuconazole).

3.2 Effects of thiacloprid alone and in combination with tebuconazole on honeybees under field-relevant exposure conditions

3.2.1 Weather conditions during the tests

Air temperatures prevailing within the tunnel cages during the foraging time of the honeybees were between 17 and 26 °C in tests 1 and 3. Air temperatures were lower during test 2 (mostly between 10 and 20 °C) which explains the overall lower foraging activity observed during this test (Fig 2). Post-treatment precipitations of >2 mm were recorded during test 1 (32 mm between 24 and 72 h after treatment) and test 2 (35 mm between 48 and 120 h after treatment).

3.2.2 Mortality of honeybees

For statistical analyses, mortality data of comparable treatment groups were pooled, ie water and tebuconazole treatments, thiacloprid only treatments (low and high rate separately), and combined treatments of thiacloprid and tebuconazole. Mortality data and their statistical analyses are shown in Table 4.

Applications of thiacloprid 480 g litre^{-1} SC at 96 and 144 g AI ha^{-1} in flowering rape or *P tanacetifolia* plots did not result in significantly increased bee mortalities compared with either a water or a spray treatment with tebuconazole 250 g litre^{-1} EW at 375 g AI ha^{-1} (Table 4). Nor was any synergistically enhanced mortality of honeybees observed with a co-application of thiacloprid and tebuconazole at recommended use rates. No conspicious behavioural anomalies of honeybees, such as exaggerated motility or discoordinated movements (trembling, shaking, apathy), were observed in any of the treatments during the study.

3.2.3 Foraging activity

The foraging intensity of honeybees decreased transitorily in response to a treatment with thiacloprid (Fig 2(A–C)). This response occurred irrespective of whether thiacloprid was applied alone or in combination with tebuconazole. Foraging intensity returned rather quickly to pre-treatment levels and no longer differed from the controls 48 h post-treatment.

The transitory reduction of the foraging activity is reflected also by the records on the number of bees returning to the hive with pollen loads. This number was apparently lower than in the control over 24 –48 h following a treatment with thiacloprid applied alone or in combination with tebuconazole (Fig 3).

These results suggest that honeybees respond to thiacloprid 480 g litre^{-1} SC applied at 96 or 144 g AI ha^{-1} by a transitory reduction of flower visitation, but return to normal foraging activity within 24–48 h post-treatment. This transitory avoidance of treated flowers was apparently not increased by a co-application of tebuconazole (Fig 2(A–C)).

3.2.4 Hive vitality

Hive weight development, colony strength, food stores and brood nest sizes appeared unaffected by either treatment. There were some incidental differences between individual hives, but no systematic differences were detected between treatments. For example, a lower brood nest size was observed for the thiacloprid treatment group during the first experiment but was not recorded in the same test run for the combination treatments with tebuconazole (co-application or subsequent treatments). In tunnel test 2, a

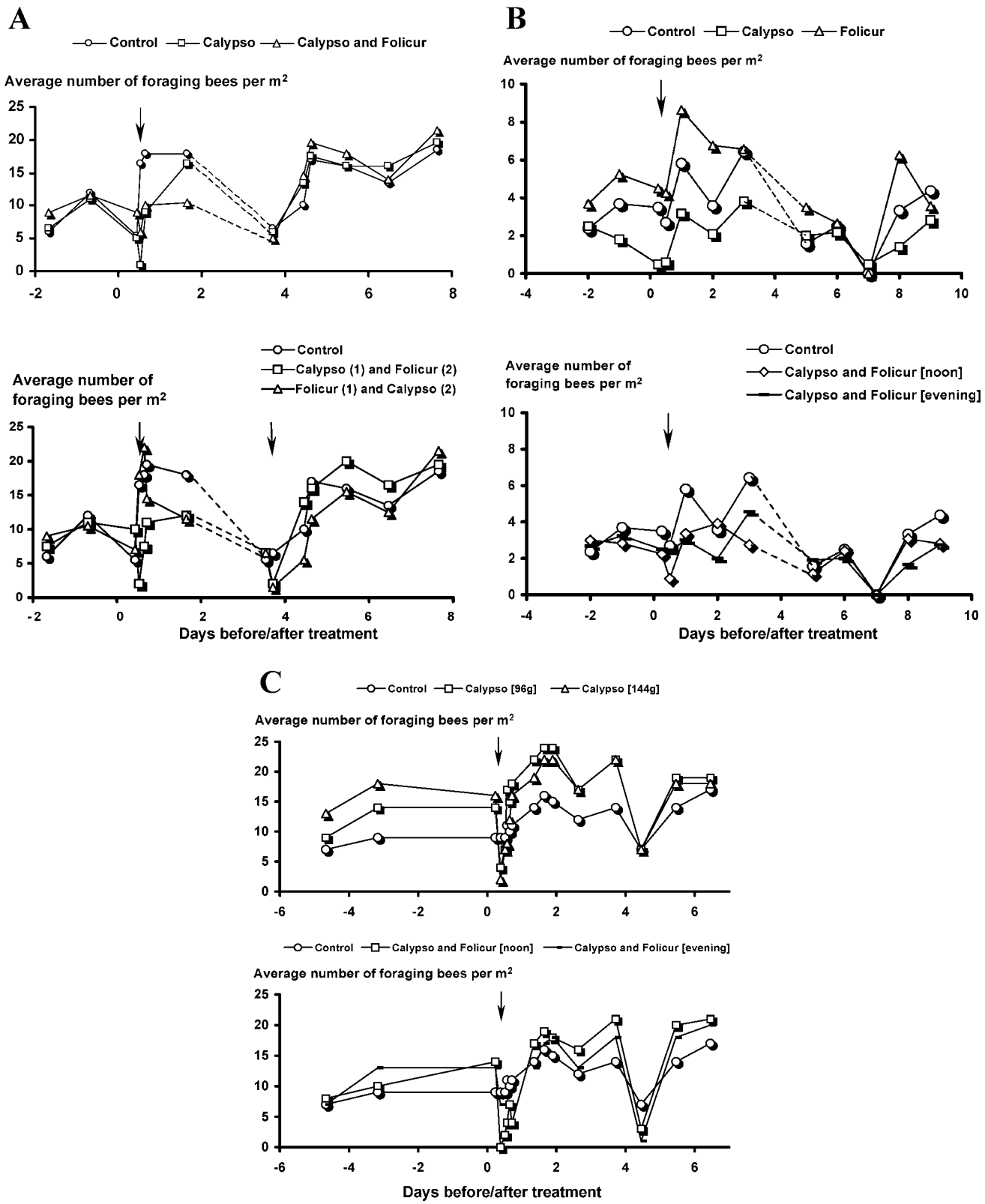

Figure 2. Foraging intensity recorded during the tunnel cage tests (A=test 1, B=test 2, C=test 3) as related to treatment. The number of foraging honeybees was counted within randomly chosen 1-m^2 subplots within the flowering crop. The figures give averages of up to seven countings per day. Arrows indicate treatment applications. Numbers given in Fig 2(A) after the test material in parentheses indicate the treatment sequence (1=first treatment, 2=second treatment). Numbers given in Fig 2(C) after the test material in parentheses indicate the applied quantity (g) of test material per hectare.

smaller brood nest was recorded for tebuconazole alone but not for the thiacloprid or the combined treatments with thiacloprid and tebuconazole. In test 3, brood nest sizes of all treatment groups were within ±25% of the corresponding control value and hives exposed to higher (144 g AI ha^{-1}) or later (evening treatment) thiacloprid treatments revealed even slightly better breeding performances than the control

Treatment group	Number of dead bees per day (± SD)[b]		
	Pre-treatment	Treatment day	Post-treatment
Water/tebuconazole ($n=5$)	29.4 (±37.8)[a]	7.4 (±8.8)[a]	11.2 (±12.2)[a]
Thiacloprid [96 g AI ha^{-1}] ($n=3$)	9.7 (±4.5)[a]	4.7 (±4.0)[a]	3.0 (±1.0)[a]
Thiacloprid [144 g AI ha^{-1}] ($n=5$)	13.8 (±6.9)[a]	9.8 (±11.9)[a]	5.8 (±2.5)[a]
Thiacloprid + tebuconazole ($n=3$)	17.3 (±14.0)[a]	11.0 (±3.5)[a]	5.7 (±2.1)[a]

Table 4. Honeybee mortalities recorded during the tunnel cage studies as related to treatment (Calypso = SC 480, Folicur = EW 250)[a]

[a] Treated bee hives contained between 3000 and 8000 honeybees.
[b] Numbers followed by the same letter within a row are statistically not significantly different ($P > 0.05$; ANOVA).

hives. Therefore, it appears reasonable to assume that the recorded differences in hive vitality parameters between the treatment and the control groups reflect variabilities inherent in the test system, and are incidental rather than treatment-related.

4 DISCUSSION

In modern agriculture insecticides and fungicides are frequently co-applied in a tank mix. The application of tank mixes may pose a risk to honeybees by a synergistic enhancement of bee toxicity by the co-applied compounds. In the UK incidents with honeybees have been reported following co-application of some pyrethroid insecticides and EBI fungicides to flowering oilseed rape.[25,26] It has been reported that EBI fungicides interact with microsomal monooxygenases which are involved in the oxidative metabolization (detoxification) of pyrethroids.[11,15,16] Since monooxygenases also are involved in the metabolization of chloronicotinyl insecticides (hydroxylation of the imidazolidine and thiazolidine rings, respectively),[17,18] it was of particular interest to know whether or not EBI fungicides might synergize the bee toxicity of these compounds. Since fungicides of other chemical classes are frequently co-applied with insecticides in tank mixes, fungicidal products representative for different chemical classes were also examined to reveal interactions between thiacloprid and co-applied fungicides with effects other than on the mono-oxygenase system.

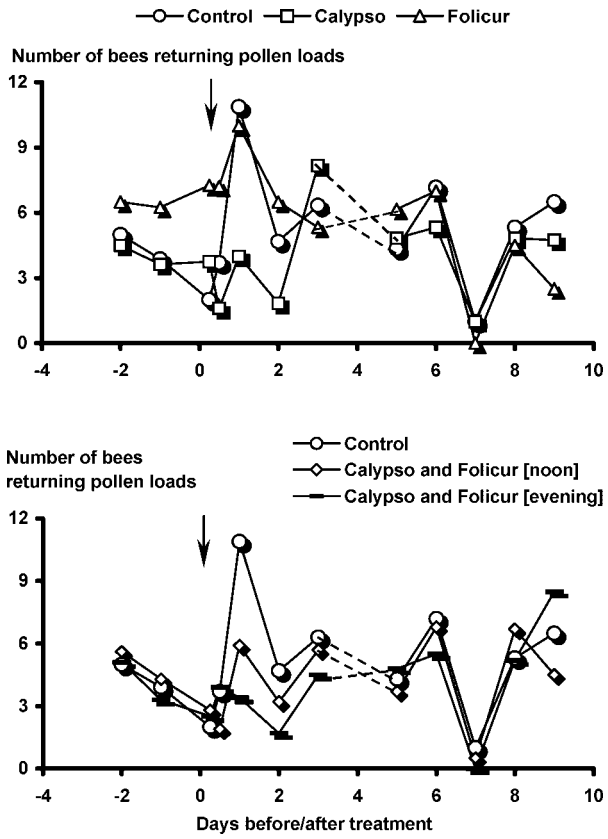

Figure 3. Number of honeybees which were recorded to return with pollen loads to the hive during the tunnel cage test 2. The figures give averages of up to seven countings per day. Arrow indicates treatment applications.

In the laboratory tests, a 48-h LD_{50} of 0.013 mg thiacloprid per honeybee was calculated after topical application of thiacloprid 480 g litre^{-1} SC. No treatment-related effect was found for doses of 0.003 mg AI per bee or lower. These values are in agreement with previously reported contact LD_{50} values of 0.021–0.082 mg thiacloprid per bee[6] and indicate a low toxicity hazard of this chloronicotinyl insecticide to honeybees.[27] The co-application of thiacloprid with fungicides not inhibiting ergosterol biosynthesis did not significantly enhance its honeybee toxicity. In the laboratory, a slightly increased mortality compared with control treatments was observed only with cyprodinil, an anilinopyrimidine fungicide. In contrast, a strong enhancement of the toxicity of thiacloprid to honeybees was recorded in the laboratory tests when this chloronicotinyl insecticide was co-applied with EBI fungicides (eg tebuconazole or prochloraz, see Table 3).

For testing the field relevance of the observed synergistic enhancement of the toxicity of thiacloprid to honeybees by EBI fungicides under laboratory conditions, three tunnel cage tests were performed. In these studies, the insecticide *versus* fungicide ratio was increased in favour of the fungicide when compared with the laboratory study. More recently, it has been reported that the synergistic enhancement of pyrethroid toxicity by EBI fungicides to honeybees can be influenced by the ratio of fungicidal *versus* insecticidal activity of a tank mix; the higher the fungicidal proportion, the stronger the synergistic enhancement.[28] If comparable relations are assumed for chloronicotinyl and EBI fungicide tank mixes, the likelihood of detecting potential adverse effects to honeybees would

have been higher for the tunnel cage than for the laboratory studies, ie the tunnel cage studies would reflect more of a worst-case scenario.

The three tunnel cage tests revealed comparable findings. At typical use rates, a co-application of thiacloprid with the EBI fungicide tebuconazole did not show any synergistic enhancement of bee mortality under field relevant exposure conditions. In response to a thiacloprid spray treatment, honeybees did transitorily reduce foraging intensity. A co-application of tebuconazole did not appear to enhance synergistically the reduction in foraging intensity.

On the hive level, no adverse effects were recorded for either thiacloprid alone or a thiacloprid and tebuconazole tank mix treatment. These data suggest that a synergistic enhancement of honeybee toxicity, as observed in laboratory tests, did not predict a potentially synergistic enhancement of such products in tank mixes under field exposure conditions. A comparable finding was recently reported for a tank mix of the pyrethroid tau-fluvalinate and the EBI fungicide difenoconazole.[29]

ACKNOWLEDGEMENTS

The authors wish to acknowledge the valuable comments of W Pflüger and F-J Placke on an earlier version of this manuscript and to thank Josef Gilli, Dolores Martínez Ginés and Micaela Buteler for their efficient assistance during the field trials.

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