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Original Article

EFFECTS OF EXPOSURE OF HONEY BEE COLONIES TO NEONICOTINOID SEED-TREATED MAIZE CROPS

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Abstract

The effects to honeybee colonies (*Apis mellifera* L.) during and after exposure to flowering maize (*Zea mays* L.), grown from seeds coated with clothianidin and imidacloprid was assessed in field-realistic conditions. The experimental maize crops were adjacent to the other flowering agriculture plants. Honey bee colonies were placed in three differently protected maize fields throughout the blooming period, and thereafter they were transferred to a stationary apiary. Samples of pollen loads, bee bread, and adult bees were collected and analyzed for neonicotinoid residues. To ensure high specificity and sensitivity of detection of the analyzed pesticides, a modified QuEChERS extraction method and liquid chromatography coupled with tandem mass spectrometry were used. Clothianidin was detected only in the samples of pollen loads. Their residue levels ranged from 10.0 to 41.0 ng/g (average 27.0 ng/g). Imidacloprid was found in no investigated sample. No negative effects of neonicotinoid seed-treated maize on the development and long-term survival of honey bee colonies were observed. The low proportion of *Zea mays* pollen in total bee-collected pollen during the maize flowering period was noted. The findings suggest that maize plants are less attractive forage for honey bees than phacelia (*Phacelia tanacetifolia* Benth.), buckwheat (*Fagopyrum* Mill.), white clover (*Trifolium repens* L.), goldenrod (*Solidago* L.), and vegetation from Brassicaceae family.
The results indicate a possibility of reducing the risk of bees being exposed to the toxic effect of insecticidal dusts dispersed during maize sowing by seeding, in the areas surrounding maize crops, plants that bloom later in the year.

Keywords: honey bees, maize, neonicotinoid insecticides, pollen, residues, seed treatment, short- and long-term assessment.

INTRODUCTION

Pesticides applied to crops are considered a significant factor in the decline of pollinators, of which honey bees are the most important species. This hypothesis is partly confirmed by monitoring of honey bee colonies which shows that bee-collected plant material (e.g., nectar, pollen) and bee products such as honey, bee bread, and beeswax contain residues of many pesticides (Genersch et al., 2010; Johnson et al., 2010; Mullin et al., 2010; Chauzat et al., 2011;

Johnson et al., 2013). In order to fill the many gaps in the current knowledge regarding links between bee mortality and pesticides in general, and neonicotinoid insecticides in particular, the risk assessment of plant protection products on bees requires continuation.

Neonicotinoid insecticides are widely used in the coat to protect the bee favorable field crops such as winter and spring oilseed rape (*Brassica napus* L.), maize (*Zea mays* L.), and sunflower (*Helianthus annuus* L.). Neonicotinoid seed treatment is a relatively new, modern technology, which confers many advantages: it is very efficient, dosages needed are low, it provides long-lasting protection, and it requires a relatively limited number of insecticide sprays (EFSA, 2012). However, neonicotinoids such as imidacloprid, clothianidin, and thiamethoxam are extremely toxic to bees with lethal and sublethal effects depending on the level of exposure. The lethal dose (LD_{50}) (the dose at which half of the exposed bees die) for clothianidin is 2.8 - 3.7 ng per bee for oral ingestion; for thiamethoxam, it is 4 - 5 ng/bee; and for imidacloprid, it is 3.7 ng/bee (Iwasa et al., 2004; Decourtye and Devillers, 2010; Laurino et al., 2011). Moreover, systemic neonicotinoids may persist for a long time in plant tissues, plant parts, or soil. Therefore, contaminated stores of nectar and pollen gathered by honey bees in combs pose a threat to their health during beekeeping season but also during wintering. In the last few years, many researchers suggested that sublethal doses of some insecticides may also lead to such a disruption to the normal functioning of bees that it constitutes a hazard at the colony level. In laboratory and semi-field conditions, the chronic lethal toxicity and sublethal effects of neonicotinoids on reproduction and behavior in individual bees have been observed (Decourtye et al., 2001; Suchail et al., 2001; Decourtye et al., 2003; Decourtye et al., 2005; El Hasani et al., 2008; Aliouane et al., 2009; Mommaerts et al., 2010; Gregorc and Ellis, 2011; Henry et al., 2012; Schneider et al., 2012). However, these results do not confirm the field studies in which bees were exposed to food contaminated with neonicotinoids at realistic field concentrations that caused no increase in bee mortality or loss of wintering bee colonies (Cutler and Scott-Dupree, 2007; Pohorecka et al., 2012).

So far, one field crop clearly linked with a significant spring loss of bee foraging, is maize (*Zea mays* L.), treated with clothianidin. It was revealed that the sowing of treated maize seed with the use of pneumatic drilling machine pose an additional, considerable routes of pesticide exposure for honey bees. Drift of insecticidal dusts of coated maize seed during sowing results in contamination of the surrounding areas with flowering bee forage plants and may cause high bee mortality (Greatti et al., 2003; Greatti et al., 2006; Bortolotti et al., 2009; Girolami et al., 2009; Pistorius et al., 2009; Marzaro et al., 2011; Georgiadis et al., 2012; Sgolastra et al., 2012). In North America, where at least 94% of the 36 million hectares planted with maize is treated with clothianidin or thiametoxam, those crops are considered a major source of neonicotinoid exposure for bees living near agricultural fields (Krupke et al., 2012). However, it remains unclear whether neonicotinoid seed-treated maize has side-effects on honey bees when bee-attracting plants in the adjacent areas do not bloom during the time of maize sowing.

Over recent years, in Poland, the area of maize cultivation has increased extensively, reaching almost 1 million ha in 2012. In the climatic conditions of Poland, maize plants bloom in July and can at that time be one of the significant pollen sources for honey bees.

Field studies were carried out to investigate the effects to honey bee colonies during and after exposure to flowering maize crops treated with neonicotinoids. Methods involved determining the level of neonicotinoid residues in bee-collected pollen and bee bread; exploring the use of maize forage by bee colonies; and evaluating the short- and long-term impact of possible residues on the health status of bee colonies.

MATERIAL AND METHODS

Maize crops

The field studies were conducted on maize crops, varieties LG 32.32, Alvito, and Kosmo in collaboration with the Department of Experi-

mental Agriculture Institute of Soil Science and Plant Cultivation. All formulations applied for maize pest control were registered in Poland and used according to label recommendations. In 2011, two maize fields (with an area of 36 ha for field A and 6 ha for field B) were protected with imidacloprid (formulation GAUCHO 600 FS in a dose of 83.3 mL per 50,000 seeds) and clothianidin (MODESTO 480 FS in a dose of 156 mL per 50,000 seeds) as seed dressing, respectively. In 2012, maize was planted on 30 ha (field C) and the COURASE 350 FS (a.i. imidacloprid) applied at a dose of 150 mL per seed unit (50,000 seeds) for seed treatment. The maize seeds sown in every area were also protected with fungicide MAXIM XL 035 FS (a.i. fludioxonil, metalaxyl), and all crops were sprayed with herbicides one or two months before their blooming period. The maize flourished from 12 July to 2 August 2011 and from 9 July to 30 July 2012. The experimental fields were adjacent to other flowering agriculture crops.

Bee colonies

Apis mellifera carnica and *Apis mellifera caucasica* colonies were established in Wielkopolski hives (frame size 360 mm × 260 mm). In early July, 15 colonies were transferred to the close vicinity of each flowering maize plantation for a period of approximately three weeks, and thereafter colonies were moved to a fall apiary. In each group, five hives equipped with pollen traps were designated only for pollen load collection. The 10 colonies from control groups (one in 2011 and one in 2012) were placed in an area where no maize plants grew.

Before colonies were relocated in the maize fields, they were inspected, and the biological and health status of each colony was estimated. Samples of worker bees were taken from the periphery combs of the brood nest. Laboratory analyses were performed for the presence of pathogens and parasites (i.e., *Varroa destructor*, *Nosema* spp.) and for chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), deformed wing virus (DWV), and Israeli acute paralysis virus (IAPV), using methods reported in Pohorecka et al. (2011). Each colony's population size was estimated every 3 to 4 weeks until the end of the beekeeping season. For this purpose, the number of combs covered by bees was counted, and the brood area was measured. Bee mortality was monitored during the whole experiment by counting of the number of dead adults on hive bottom boards and in 1×1 m white trays set up on the ground in front of the hive entrances. In September 2011 and 2012, colonies from experimental and control groups were prepared for overwintering. Each colony received approximately 20 L of sugar solution. *Varroa destructor* treatment was performed with amitraz (the formulations: Biowar 500 and Apiwarol, Biowet, Puławy, Poland) and 3.5% oxalic acid sugar solution. The status of the overwintered bee colonies (adult bees and brood population) was estimated in April 2012.

Collection of samples

The pollen samples were collected several times during the maize flowering. The pollen loads that the bees had collected within 3 to 4 days were taken separately from the pollen traps of each of the five colonies intended for this purpose and weighted. Samples of bee bread (approximately 10×10 cm pieces of combs) and adult bees (workers from brood chambers) were taken at the same time, after the maize blooming. Samples of pollen and bee bread were split into two parts (subsamples), one intended for the pollen analysis and one for the residue analysis. All collected samples were frozen and stored at a low temperature of about -20°C.

Palynological analysis

Analyses of pollen loads and bee bread were performed separately for each colony and harvest date using the method described in Pohorecka et al. (2012).

Residue analysis

To ensure high specificity and sensitivity of detection of the analyzed insecticides (imidacloprid, clothianidin, thiamethoxam, acetamiprid, and thiacloprid), a modified QuEChERS extraction method and liquid chromatography coupled with tandem mass spectrometry were used. Reagents and materials, sample preparation, LC-MS analysis, and validation parameters

are reported in Pohorecka et al. (2012). Limits of detection (LOD) of imidacloprid, clothianidin, thiamethoxam, acetamiprid, and thiacloprid for pollen samples were respectively 0.8, 1.0, 0.3, 0.2, and 0.4 ng/g, and limits of quantification (LOQ) amounted 3.0, 3.0, 1.5, 1.0, and 2.0 ng/g. LOD of these compounds for bee samples amounted respectively 0.5, 2.0, 1.0, 0.3, and 0.1ng/g, and LOQ were respectively 2.0, 6.0, 3.0, 1.0, and 0.5 ng/g.

Statistical analysis

All statistical analyses were carried out using Statistica 8 software. The means were tested using the ANOVA test, and multiple comparisons with the Tukey test. Comparisons of parameters for non-parametric groups were conducted using the Mann–Whitney U-test or the Kruskal–Wallis test with a significance level of α = 0.05. Spearman's rank correlation was used to assess the relationship between variables. For all analyses, p-value <0.05 was considered significant.

RESULTS

Residue analysis

Of the two neonicotinoid insecticides used for protecting maize crops, only the clothianidin was found in the examined material. This compound was detected in all samples (n = 20) of pollen loads. Clothianidin contamination of pollen ranged from 10.0 to 41.0 ng/g and on average was 27.0 ng/g (± 10.0). The levels of clothianidin residues in pollen load samples depended on the percentage of *Z. mays* pollen in the total mass of the samples (Spearman rank order correlation Rs = 0.73). Clothianidin was not identified in any bee bread or bee sample. Although acetamiprid and thiacloprid have not been used to control maize pests, their residues were detected in a great number of pollen samples and in the single samples of bees. The residue levels of these substances, however, were very low in the majority of samples (Tab.1).

Honey bee colony assessment

Health status and population size

In both years, the honey bee colonies in the field study were healthy, as confirmed by laboratory analysis. The level of *V. destructor* infestation was very low and on average amounted to 0.1 and 0.05 mites per 100 bees in samples from colonies placed in maize fields treated with imidacloprid (group A) or clothianidin (group B), respectively. No parasites were detected in the samples of bees from colonies localized in maize crops treated with imidacloprid in 2012 (group C). A similar level of *Varroa* mites (0.1 and 0.2 parasites per 100 bees) was found in bee samples of the control groups. Spores of *Nosema* spp. were detected only in eight among all examined bee samples and level of infestation did not exceed 1 million spores per bee in any of them. In the majority of samples, no viruses were detected, except for a few samples in which the DWV was found.

Mortality rate was monitored during the whole beekeeping season. In 2011, from the placement of colonies in the maize fields on 12 July to 18 October, in group A, an average of 141.4 dead bees (from 67 to 259) per colony was found; these values were 113.2 (from 77 to 176) in group B and 132.4 (from 51 to 200) in the control group. Therefore, the numbers of dead bees on the bottom boards and trays were very low, and groups did not differ significantly ($H = 2.31$, $n = 30$, $p = 0.31$). In 2012, colonies were evaluated for a shorter period, from 9 July to 31 August. At the time, very low mortality was also noted in both group C and the control groups, at 22 and 30 dead bees per colony, respectively. In 2011 and 2012, from the placement of the colonies on the maize fields until wintering, the colonies developed normally in all groups. Numbers of combs covered by bees and the brood areas assessed during each inspection demonstrated that the colonies were in good condition. Population of adult and brood were typical for the time of season and did not differ significantly from control groups (Tab. 2 and 3).

Decreased bee populations observed in August and September were the result of natural processes of bee colony structural changes

Table 1.

Incidence of neonicotinoids not used for the protection of maize (percentage of positive samples) and level of their concentration (ng/g) in analyzed samples of bees and pollen collected by bees during the 3 weeks of maize blooming

Origin of the samples	Type of the samples	n[5]	Acetamiprid		Thiacloprid	
			mean ± SD[6]	positive (%)	mean ± SD	positive (%)
Group A[1]	Pollen loads	20	1.7 ± 1.2	100	1.7 ± 3.0	45
	Bee bread	10	2.5 ± 0.8	100	0.4 ± 0.6	60
	Bees	10	0.1 ± 0.0	10	nd	0
Group B[2]	Pollen loads	20	7.1 ± 12.6	70	nd	0
	Bee bread	10	10.4 ± 6.2	100	nd	0
	Bees	10	0.2 ± 0.3	30	nd	0
Group C[3]	Pollen loads	30	nd[7]	0	3.2 ± 4.5	60
	Bee bread	10	nd	0	4.2 ± 3.5	90
	Bees	10	nd	0	nd	0
Control[4]	Pollen loads	20	0.8 ± 1.1	50	0.4 ± 0.9	25
	Bee bread	20	1.7 ± 3.4	15	1.4 ± 5.2	60
	Bees	20	nd	0	nd	0

[1,3] samples collected from colonies located in maize treated with imidacloprid (field A and C)
[2] samples collected from colonies located in maize treated with clothianidin (field B)
[4] both control group
[5] number of the samples
[6] standard deviation
[7] not detected

Table 2.

Population size of honey bee colonies (2011)

Measure-ment date	Number of combs covered by bees (mean ± SD[4])			Brood area (dm2) (mean ± SD)		
	Group A[1]	Group B[2]	Control[3]	Group A	Group B	Control
12.07.11	20.0 ± 0.0	20.0 ± 0.0	20.0 ± 0.0	53.9 ± 7.3	54.3 ± 15.6	56.7 ± 9.8
02.08.11	17.3 ± 1.9	17.1 ± 0.8	17.9 ± 0.9	53.5 ± 8.4	51.1 ± 8.1	55.3 ± 8.7
23.08.11	16.0 ± 2.1	14.7 ± 0.8	16.1 ± 1.2	47.2 ± 10.0	51.1 ± 11.1	49.0 ± 7.2
13.09.11	8.8 ± 0.9	8.5 ± 0.7	9.0 ± 0.0	4.2^{ab*} ± 3.3	6.8^{b} ± 4.4	2.9^{a} ± 3.6
03.10.11	8.8 ± 1.1	8.2 ± 0.7	8.8 ± 0.4	7.3^{b} ± 4.3	7.5^{b} ± 3.8	3.9^{a} ± 2.2
25.10.11	7.8 ± 0.9	7.5 ± 1.0	7.9 ± 0.7	0.0	0.0	0.0

[1] colonies located in maize treated with imidacloprid (field A)
[2] colonies located in maize treated with clothianidin (field B)
[3] control group
[4] standard deviation
* different letters (a, b) indicate significant differences between the means ($p<0.05$).

occurring in the late summer in Polish climatic conditions. The development of the colonies after overwintering was assessed during the spring inspections conducted from April 2012. All colonies overwintered properly. The number of bees that had died during winter was low and similar in all groups. In each hive from groups A and B, and control, 6.0, 6.5, and 6.2 combs were on average covered by worker bees, respectively.

The pollen harvest

During the 3 weeks of maize blooming, colonies located on 36-ha (field A) and 30-ha (field C) maize crops protected with imidacloprid on average collected 1,150 g (± 435.9) and 1,057 g (± 254.9) of pollen loads, respectively. Colonies located in the 6-ha maize crops treated with clothianidin (field B) in pollen traps on average gathered 310 g (± 159.7) of pollen. The average area of pollen produced as bee bread in combs by each colony was 14.1 dm^2 (± 2.8), 12.3 dm^2 (± 6.4), and 4.4 dm^2 (± 3.6) for colonies placed in A, C, and B crops, respectively. However, the content rate of *Z. mays* pollen grains in the total of pollen loads and bee bread was slight. In both years the colonies gathered extremely low amounts of Z. *mays* from crops treated with imidacloprid (Tab. 4).

In all samples of pollen loads and bee bread collected by colonies while placed in the three maize crops, predominate pollen types were of Brassicaceae, *Fagopyrum*, *Cichorium* type, *Solidago* type, *Trifolium repens*, *Rubus* type, *Centaurea cyanus*, Asteraceae, and *Achillea* type.

In subsequent years, the honey bee colonies from the control groups on average collected 394 g (± 156.1) and 585 g (± 214.9) of pollen. The pollen originated mainly from Brassicaceae, *Phacelia*, *Fagopyrum*, *Trifolium repens*, *Centaurea cyanus*, *Sinapis*, *Rubus* type, *Solidago* type, *Cichorium* type, *Achillea* type, *and Trifolium pratense*.

Table 3.

Population size of honey bee colonies (2012)

Measurement date	Number of combs covered by bees (mean ± SD[3])		Brood area (in dm^2) (mean ± SD)	
	Group C[1]	Control[2]	Group C	Control
09.07.12	20.0 ± 0.0	20.0 ± 0.0	61.6 ±11.5	57.3 ± 5.3
31.07.12	20.0 ± 1.9	20.0 ± 0.9	55.2 ± 10.2	55.8 ± 17.3
23.08.12	8.2 ± 0.8	8.2 ± 1.2	56.1 ± 5.3	67.0 ± 18.4

[1] colonies located in maize treated with imidacloprid (field C)
[2] control group
[3] standard deviation

Table 4.

Average content of *Zea mays* pollen grains in samples of pollen collected by honey bee colonies placed in the maize crops (2011, 2012)

Origin of samples/type of samples	Content of *Z. mays* pollen grains in samples of pollen (%)	
	Pollen loads from traps (mean ± SD[4])	Bee bread from combs (mean ± SD)
Group A[1]	1.8 ± 2.7	2.2 ± 2.2
Group B[2]	26.3 ± 15.4	11.9 ± 15.3
Group C[3]	1.3 ± 2.2	0.6 ± 0.5

[1,3] colonies located in maize treated with imidacloprid (field A and C)
[2] colonies located in maize treated with clothianidin (field B)
[4] standard deviation

DISCUSSION

In the present study, we used realistic conditions to monitor honey bee exposure to imidacloprid and clothianidin seed-treated maize. Seeds were coated at the recommended commercial rate for Poland, and honey bee colonies were placed at the edge of large maize areas throughout the blooming period to ensure maximum exposure. The study was carried out in an agricultural region and alternative forage was available to workers at the same time.

It turned out, that the bees foraged poorly on the maize crops. In total mass of pollen (both from traps and combs) collected by bees placed in maize treated with imidacloprid, the rate of *Z. mays* pollen grain did not exceed 3%. Perhaps for this reason, in none of the investigated samples was imidacloprid detected.

The residues of neonicotenoids have been extensively studied in recent years. And yet, bee-collected pollen contaminated with imidacloprid was found only in the two studies, however, reported residues caused no negative impact on honey bees colonies (Nguyen et al. 2009; Chauzat et al. 2011).

Though the surface of maize crops protected with clothianidin was significantly lower, the proportion of *Z. mays* grains in pollen loads was 15 times higher, and it was 6 times higher in bee bread. Clothianidin residues were detected in all pollen load samples collected form traps. We found no clothianidin in any sample of bee bread pollen stored by bees in combs. Possibly, the reason was the two-fold decreased content of maize pollen grains in bee bread compared to pollen. The level of contamination of pollen was lower than the acute oral and contact LD_{50} values. The average concentration of clothianidin was 27 ng/g, which is higher than the residue levels reported by Krupke et al. (2012), who found contamination of bee-collected pollen samples from maize treated with clothianidin at 13.9 ng/g.

Additionally, in 50% of pollen and bee bread samples, residues of acetamiprid and thiacloprid were detected; however, residue levels were lower than the acute oral and contact LD_{50} values. Nevertheless, it indicates that these neonicotinoids are widely used to protect other crops that are a source of nectar or pollen for bees as well.

Our results demonstrated that the level of clothianidin content in pollen collected by bees had no noticeable effect on the status of bee colonies during beekeeping season. Overall, we found no differences between colonies from imidacloprid- or clothianidin-treated crops and control groups. In addition, assessment of colonies in the spring revealed no differences in bee populations, overwinter colony survival, or overall colony health in the compared groups.

The field studies examining the effects on honey bee colonies of clothianidin treated oilseed rape detected about 10 times lower clothianidin levels in both pollen and nectar and also found no significant short- and long-term influence on development and size of the bee populations (Cutler and Scott-Dupree, 2007; Pohorecka et al., 2012).

In our field trials the pollen collected by bee colonies (all groups) originated from cultivated (e.g., *Phacelia*, *Fagopyrum*, *Trifolium repens*) and wild (e.g., *Solidago* spp., *Achillea* spp.) plants. However in the experiment of Krupke et al. (2012) experiment, the content of maize pollen in the loads amounted 50%, which proves that the maize crops might be an attractive pollen source for honey bees.

These findings suggest that honey bee colonies might accumulate greater amount of maize pollen in areas of intensive cultivation of maize monocultures without alternative bee preferred, flowering crops. This may pose potentially higher risk of exposure of honey bees to the toxic effect of neonicotinoids.

CONCLUSIONS

Clothianidin used in the coat to protect of maize crops resulted in contamination the bee-collected pollen at a level not exceeding oral and contact lethal dose for bees.

The very low proportion of maize pollen in total bee-collected pollen during the maize flowering period could prevent detection of imidacloprid residues.

Exposure to clothianidin seed coated maize presents negligible risk to honey bee colonies.
Maize plants are a less attractive forage for honey bees than phacelia (*Phacelia* Juss.), buckwheat (*Fagopyrum* Mill.), white clover (*Trifolium repens* L.), goldenrod (*Solidago* L.), and vegetation from Brassicaceae family.
Potential side-effects of exposure of honey bee colonies to flowering maize crops treated with neonicotinoid as seed dressing might be higher in areas intensively planted with maize monocultures without alternative bee preferred blooming crops in the adjacent fields.
The results indicate a possibility of reducing the risk of bees being exposed to the toxic effect of insecticidal dusts dispersed during maize sowing by seeding, in the areas surrounding maize crops, plants that bloom later in the year.

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