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Original Research

Effects of Dietary Transgenic *Bacillus thuringiensis* Maize Pollen on Hive Worker Honeybees

Z. Lipiński[2]*, M. Farjan[1], K. Żółtowska[1], B. Polaczek[3]**

[1]University of Warmia and Mazury, Faculty of Biology, Division of Biochemistry,
Oczapowskiego 1A, 10-719 Olsztyn, Poland
[2]Wengris 8, 10-735 Olsztyn, Poland
[3]Free University of Berlin, Institute of Zoology, Königin-Luise-Str. 1-3, 14195 Berlin, Germany

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Abstract

To assess potential impacts of transgenic insect-resistant (MON 863 x MON 810) Bt maize-pollen consumption on hive honeybees, two-, three-, four- and five-day-old workers were fed during five days with a mixture of honey and sugar (Control, Group I), honey with non-transgenic Limagrain maize pollen (Group II) and honey with *Bacillus thuringiensis* maize pollen (Group III). The consumption rate, weight of bees, protein content, antioxidant enzyme activities and total antioxidant status were estimated. There were no significant differences in any of the above parameters among the groups of bees fed on the three diets. Only trehalose and maltose levels were higher in bees fed a pollen-free control diet compared to bees fed either transgenic or non-transgenic maize pollen. Our data indicate that the transgenic pollen had no adverse impact on studied markers of young hive honeybees.

Keywords: *Bacillus thuringiensis*, honeybee, transgenic maize pollen, antioxidant status

Introduction

Pest-associated losses of total agricultural production are estimated at 14% [1]. In addition to direct losses caused by insects, there are additional costs in the form of pesticides used for pest control. The application of pesticides leaves residues in the food and results in environmental pollution. This has necessitated the search for new tools to enhance plant resistance to insect pests. With the advent of genetic engineering it is possible to insert genes from bacteria such as *Bacillus thuringiensis* (Bt), which encode proteinaceuos toxins (Cry) effective against lepidopteran, dipteran or coleopteran insects [1]. A recent increase in the global area planted in genetically modified (GM) crops has been accompanied by more research about their threat to the environment [1-4].

*e-mail: lipinski@sprint.com.pl
**e-mail: k.zoltowska@uwm.edu.pl

A collection of important studies deals with how GM plants potentially affect the European honeybee, *Apis mellifera* [5, 6]. At least one-third of the world's agricultural crops need to be pollinated by insects and other animals [7]. The honeybee is of paramount importance as a pollinator in the natural environment and agriculture, and pollinates 70-84% of all flowering plants in the European Union [8]. Most studies on the effects of transgenic crops on honey bees have used purified transgene products, i.e. proteins [2, 9].

Pollen is the main source of protein for honeybees and it is stored in large amounts in the hive [10]. Because some Bt toxins that target lepidopteran insects are present in pollen it is important to determine whether intact pollen from transgenic plants has a negative effect on honeybees. Bt maize is a transgenic crop grown commercially on millions of hectares throughout the world [11]. Admittedly, maize pollen is normally only an extra source of feed for

bees, but as maize cultivation has become more widespread, it could become a major source. Therefore it has become very important to study how GM maize pollen affects the health of honeybees.

Research conducted to date has dealt mainly with how Bt toxins, pure or present in pollen, influence honeybees' survivability [5, 6, 12, 13]. However, there are not enough dates concerning biochemical aspects of the presence of Cry proteins [4].

Essential markers of the physiological condition of insects are disorders within the antioxidant system and carbohydrate metabolism. Problems with these processes can consequently lead to weakening of honeybees, and eventually to developmental disorders and even colony death [14, 15].

We have thus examined how GM maize pollen present in food of honeybees affects the activity of antioxidant enzymes, total antioxidant status and sugars - maltose and trehalose. Furthermore, we have chosen to study the pollen of MON863 x MON810 maize, which is a hybrid obtained from a conventional crossing of two lines of GM maize - MON863 and MON810. These maize hybrids contain *cry3Bb1* and *cry1Ab* genes, thus making plants resistant to coleopteran and lepidopteran pests [5].

Materials and Methods

Maize pollen was gathered in July 2007 from experimental crops:

1) GM MON863 x MON810 near Berlin, Germany, containing transgenic proteins: Cry3Bb1 and Cry1Ab, and
2) an unmodified strain, Limagrain LG 22.43 near Olsztyn, Poland.

Limagrain LG 22.43 is the variety most commonly used in this area. The pollen was passed through two sieves (pore size 1.0 mm and 0.55 mm) to remove debris. Until the experiment the sieved pollen was stored in a sealed glass jar in the dark at -18ºC. Both types of maize pollen were used to prepare the experimental groups' feed mixture of rapeseed honey and pollen (non-transgenic for Group II and transgenic Bt for Group III) in 3:1 ratio. The honeybees of the control group (Group I) received a honey-sugar mixture (3:1). The honey was collected from non-transgenic rapeseed.

Table 1. Weight of worker bees, plus protein, trehalose and maltose contents in their bodies.

Age of bees in the group (days)	Group		
	I (control)	II (on diet with non-transgenic pollen)	III (on diet with transgenic pollen)
Weight of bees [mg]			
7	153.42 ± 24.77*	146.12 ± 9.88	141.96 ± 8.36
8	145.75 ± 11.18	144.52 ± 11.85	154.69 ± 14.04
9	161.56 ± 5.10	160.31 ± 16.63	157.34 ± 13.71
10	158.19 ± 18.01	161.78 ± 6.49	152.90 ± 9.75
Protein content [mg/g tissue]			
7	2.97 ± 0.49	2.68 ± 0.57	2.63 ± 0.40
8	2.86 ± 0.46	2.67 ± 0.37	2.31 ± 0.58
9	2.43 ± 0.23	2.21 ± 0.32	2.51 ± 0.52
10	2.66 ± 0.52	2.36 ± 0.34	2.93 ± 0.62
Trehalose content [mg/g tissue]			
7	0.889 ± 0.027^{a}	0.483 ± 0.001^{b}	0.459 ± 0.145^{b}
8	0.750 ± 0.065^{a}	0.457 ± 0.114^{b}	0.452 ± 0.008^{b}
9	0.899 ± 0.018^{a}	0.463 ± 0.058^{b}	0.533 ± 0.060^{b}
10	0.876 ± 0.071^{a}	0.528 ± 0.118^{b}	0.378 ± 0.001^{c}
Maltose content [mg/g tissue]			
7	0.243 ± 0.075^{a}	0.176 ± 0.031^{b}	0.147 ± 0.039^{b}
8	0.189 ± 0.081^{a}	0.156 ± 0.039^{b}	0.112 ± 0.029^{b}
9	0.451 ± 0.035^{a,c}	0.148 ± 0.028^{b}	0.166 ± 0.003^{b}
10	0.295 ± 0.067^{a}	0.117 ± 0.035^{b}	0.092 ± 0.008^{b}

* Mean ± SD, different letters indicate significant differences between means.

Apis mellifera carnica nursery worker bees were reared from capped brood combs with the thermostat set at temp 34.5ºC. All combs originated from one colony. The newly-emerged workers were collected and color-tagged every 24 hours on four consecutive days and placed back in their mother hive. The day after the last tagging the tagged bees were collected again. All the bees were starved for two hours before initiation of the experiment, so they were equal in terms of their gut contents. They were then assigned to respective 2-, 3-, 4- and 5-day-old groups, and five of each age were weighed and randomly placed in plastic queen shipment cages (990 x 400 x 100 mm). Each experimental group kept in one cage contained 20 bees and appropriate food and a piece of wet sponge to supply water. The experiment was carried out in six replicates of each experimental group. Bees fed on diets for five days, during which time the cages were kept in the dark at room temperature (28ºC) to avoid excessive melting of supplied food. The bees had access to water and food *ad libitum*.

After five days all bees were frozen to death at -20ºC. The cages without the bees were then weighted to determine food consumption. Bees in the same age (7, 8, 9 or 10 days old) from one cage were collected to form one analytical sample. Their legs and wings were removed. Each sample was weighed and homogenized with 0.9% NaCl (1g tissue with 10 ml NaCl). The homogenates were centrifuged at 800 x g for 15 min at 4ºC. The supernatant was used for biochemical analysis.

The activity of superoxide dismutase (SOD) was determined according to the method by Beauchamp and Fridovich [16], glutathione transferase (GT) by Rice and Evans [17], and peroxidase (POX) by Chance and Maehly [18]. Total antioxidant status (TAS) was determined according to the method by Re [19]. Protein content was measured by Bradford's [20] method. High pressure liquid chromatography (HPLC) was used to determine trehalose and maltose content. The separation was carried out on Rezex RMN Carbohydrate Na^+ column (30 x 0.78 cm) at the flow rate of deionized water of 0.4 ml per minute, in a Shimatzu chromatograph with a refractometric detector. The results were expressed in mg of sugar per g of fresh tissue matter.

Table 2. The antioxidant system markers of bees.

Age of bees in the group (days)	Group		
	I Control	II (on diet with non-transgenic pollen)	III (on diet with transgenic pollen)
Total Antioxidant Status [Trolox equivalents/g tissue]			
7	18.63 ± 3.04*	20.19 ± 2.10	17.74 ± 1.60
8	18.71 ± 4.48	19.43 ± 2.62	18.20 ± 4.05
9	18.71 ± 2.29	19.02 ± 5.14	18.24 ± 4.13
10	26.44 ± 3.37	20.59 ± 3.20	23.39 ± 2.50
Peroxidase activity [mU/mg protein]			
7	12.7 ± 4.46	10.7 ± 2.56	10.2 ± 5.45
8	11.3 ± 5.76	12.7 ± 1.79	12.4 ± 2.41
9	13.0 ± 2.62	20.2 ± 3.20	13.6 ± 2.49
10	7.7 ± 3.69	16.9 ± 4.38	14.5 ± 2.84
Superoxide dismutase activity [% inhibition/mg protein]			
7	25.74 ± 5.71	26.78 ± 5.44	30.95 ± 9.29
8	22.25 ± 3.73	26.98 ± 8.42	25.93 ± 4.06
9	25.15 ± 2.77	27.97 ± 4.88	31.99 ± 6.83
10	26.34 ± 5.84	26.64 ± 6.98	26.11 ± 8.76
S-glutathione transferase activity [U/mg protein]			
7	1.71 ± 0.64	2.94 ± 0.62	2.52 ± 0.982
8	2.53 ± 0.40	2.85 ± 0.53	2.59 ± 0.356
9	2.25 ± 0.40	2.83 ± 0.96	2.52 ± 0.302
10	2.17 ± 0.45	3.35 ± 0.76	2.41 ± 0.531

* Mean ± SD

ANOVA was applied and the Tukey test was used to determine significant differences between group means, where $p < 0.05$ was considered significant.

Results

Under the experimental conditions of our study the transgenic insect-resistant MON 863 x MON 810 maize pollen had no significant impact on young worker honeybees. There were no statistically significant differences in SOD, POX and GT activities among all age groups fed on the respective three kinds of food (Table 1) There was also no significant difference between the two groups of that consumed pollen in terms of protein, trehalose and maltose content, body weight as well as mass of eaten food (Table 2). Only three differences were observed in this study and these differences were between the control group fed only honey/sugar (Group I) and the two pollen-fed groups (II, III). There were higher levels of maltose and trehalose in bees fed the honey/sugar control diet (Table 2), probably as a result of the high level of saccharose in the control diet. A third difference based on observation was a lack of coprophagia in worker honeybees fed with both kinds of maize pollen.

Discussion

Worker bees need to get pollen from plants throughout their lifetime. Nurse worker bees eat more pollen than adult workers, with a peak in pollen intake on day nine after emerging [3]. Higher consumption of pollen translates into a larger dose of Bt toxin taken in by honeybees. This is also why these hive bees are a good model for studying the affect of GM plant pollen on the health of beneficial insects. Still the question remains on how the pollen of the transgenic maize influences the physiological process and behaviour of honeybees.

We have shown that under our experimental conditions, the transgenic maize MON 863 (*cry3Bb1* gene) x MON 810 (*cry1Ab* gene) pollen had no adverse impact on young caged worker bees. We observed that the body weight, rate of food consumption, protein and sugar contents, the activity of antioxidative enzymes and total anioxidant status were very similar in the groups of bees fed transgenic versus non-transgenic maize pollen (Tables 1, 2). This suggests that transgenic and non-transgenic maize pollen were of similar nutritional value to hive bees. The correctness of this assumption confirms the lack of coprophagia in bees fed with Bt-pollen and non- Bt-pollen, in contrast to bees fed only with a honey-sugar mixture. It means that the control group bees suffer from lack of lipids, proteins, carbohydrates and vitamins.

Our observations are congruent with the report of Schur et al. [21], who showed that Bt maize pollen containing high levels of Cry1Ab protein did not adversely affect bee survival, foraging frequency, behavior and brood development during the seven-day period of pollen shed. In 2003 Hanley et al. [9] studied larvae and pupae fed with pollen of maize modified with Cr1Ab and Cr1F toxins. They showed no changes in the amount of proteins in hemolymph and weight of honeybees' brood fed with transgenic pollen in comparison with non-transgenic pollen group. In addition, no toxicity was also noted in honey bee adults fed purified Cry1Ab lepidopteran active toxin [5]. The purified Cry3B toxin (coleopteran active) given in a sugar syrup at concentrations of 0.066% or 0.332% had no effect on larval survival or pupal dry weight [22].

Our results concerning the antioxidant status of honeybees fed with GM pollen are in agreement with observations made by Liu et al. [4], who also did not find any differences in the activity of superoxide dismutase between worker bees fed with diets containing the non-transgenic or transgenic cotton pollen.

In conclusion, under our experimental conditions, the pollen of transgenic maize had no direct adverse impacts on caged worker bees. This result is consistent with earlier reports that the pollen of transgenic Bt maize is safe for worker bees. However, our experimental diets were different from real diets of honey bees in the field. Therefore, our experimental results cannot thoroughly demonstrate the safety of the Bt maize to honey hive bees. Thus, future experiments are needed to evaluate the direct and indirect, especially long-term, impacts of the pollen of transgenic Bt maize on honeybees.

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