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The effect of Cry1AB insecticidal protein on the incidence of entomopathogenic fungi infecting aphids on *Bt* maize

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Abstract

There are substantial discrepancies in risk assessments of transgenic maize in Europe. The aim of the study was to determine the quantitative changes in the proportion of fungi-infected aphids and possible differences in the species composition of entomopathogenic fungi on conventional and genetically modified maize, expressing Cry1Ab protein from *Bacillus thuringiensis* (*Bt*). The experiments were performed in Lower Silesia, Poland, in 2008–2010. Three cultivars were used, i.e. DKC 3421 YG (*Bt*), isogenic non-*Bt* DKC 3420, and the conventional 'Bosman' as a reference. Aphids infected with fungi were counted *in situ* on 18 plants per plot every two weeks through each of the growing seasons. Additionally, twice a year dead aphids were also collected (60 aphids per treatment) and used for fungi identification. Cry1Ab insecticidal protein had no effect on the incidence of entomopathogenic fungi infecting aphids on *Bt* maize. The number of fungi-infected aphids and their time of occurrence were similar on the three compared cultivars. The same three or four species of entomopathogenic fungi-infected aphids were found in each treatment. Predominating in all the treatments were *Pandora neoaphidis*, *Entomophthora planchoniana* and *Conidiobolus thromboides.*

Key words: Cry1Ab protein, genetically modified crop, mycosed aphids, pest, transgenic maize.

Introduction

Bacillus thuringensis (*Bt*) maize expressing the insecticidal protein Cry1Ab very effectively controls the primary target pest, the European corn borer (*Ostrinia nubilalis* Hübner) (Lepidoptera: Crambidae) (Bereś, 2010). In plant protection, this prevents from causing damage throughout the season and economic loss, thus eliminating the need for extensive monitoring and application of corn borer insecticides. Aphids (Hemiptera: Aphididae) are another group feeding on maize. However, they occur irregularly and are not a problem every season (Faria et al., 2007). Adults and nymphs suck sap and produce honeydew. High densities can cause plants to turn yellow and appear unproductive. Yield loss may occur on water-stressed plants (Honek, 1994). Up to now, four aphid species have been identified on maize in Poland, i.e. *Rhopalosiphum padi* (Linnaeus, 1758), *Metopolophium dirhodum* (Walker, 1849), *Sitobion avenae* (Fabricius, 1775), and *Rhopalosiphum maidis* (Fitch, 1856) (Pieńkosz et al., 2005). A similar finding prevails in most European countries, sometimes with the changes of dominant species (Honek, 1994).

With the rapid increase in the commercial use of genetically modified (GM) plants, there is an increasing demand for information on their possible impact on non-target organisms. Of particular interest are the natural enemies of pests and antagonistic microorganisms, such as entomopathogenic fungi, which play an important role in pest regulation. Fungi can effectively reduce aphids (Ekesi et al., 2005). On maize, the infection of *M. dirhodum* and *R. padi* by pathogenic fungi reached 68.5% and 82.1%, respectively (Krawczyk et al., 2006). According to the same authors, apterae females and larvae were infected in the greatest number. The mummified aphids were mostly infected by *Pandora neoaphidis* (Remaudiere et Hennebert) Humber.

The *Bacillus thuringensis* delta-endotoxin protein Cry1Ab from GM maize is considered very selective in its action and should not affect non-target arthropods associated with maize. The fact that the bacterial protein is not transported in the plant phloem leaves aphids unaffected by its direct action. Many studies have shown that *Bt* maize has no effect on *R. padi*, *M. dirhodum* or *S. avenae* development (Dutton et al., 2002; Habuštova et al., 2014). Despite this, the newest data from the enzyme linked immunosorbent assay (ELISA) tests referring to Cry3Bb toxin produced to control western corn rootworm (*Diabrotica virgifera virgifera* LeConte) on maize indicate possibilities of *Bt* protein flow from the plant to predators via the aphids (Stephens et al., 2012). According to these authors, ladybird *Harmonia axyridis* feeding upon *Rhopalosiphum maidis* on Cry3Bb plants in the field may experience greater mortality and shorter life spans. Burgio et al. (2011) highlighted the potential for Cry1Ac endotoxin uptake by aphids feeding on transgenic oilseed rape plants. In the study by Lumbierres et al. (2004), it was found that young nymphs of *R. padi* were colonizing *Bt* transgenic maize at a higher density, especially when the plants started growing in a commercial field. Together these data suggest that non-target organisms can be affected indirectly by *Bt* maize via a secondary pest, maize leaf aphids. To date, no studies on the indirect impact of GM maize plants with Cry1Ab protein on entomopathogenic fungi-infected plant dwelling aphids have been performed, and, in the available literature, there is a deficiency of data concerning this problem.

The aim of the present study was to determine the quantitative changes in the proportion of fungi-infected aphids, and possible differences in the species composition of entomopathogenic fungi that kill these insects on conventional and *Bt* maize grown for grain.

Materials and methods

The experiments were performed in Budziszów (51°06′ N, 17°02′ E), near Wrocław, Lower Silesia, Poland, in 2008–2010. Three cultivars of maize were cultivated: 1) genetically modified (GM) plants MON 810, expressing Cry1Ab protein from *Bacillus thuringensis* (*Bt*) cv. DKC 3421 Yield Gard® ("Monsanto", USA), 2) an isogenic non-*Bt* cv. DKC 3420 ("Monsanto"), and 3) a conventional, reference cv. 'Bosman' (national cultivar 'Nasiona Kobierzyce'). All the three cultivars are described with FAO numbers 250–260. The field experiment was set up in complete randomized blocks with four replicates. The area of each experimental plot was 1.600 m^2 (40 × 40 m). An alley distance of 4.5 m was used between plots. The experiments were conducted on the same plots for three consecutive years; hence we could expect the growing amount of *Bt* endotoxin. No insecticides were used in this experiment. All the agrotechnologies applied in the maize field, including fertilizers, were identical on the entire area of the experiment.

The fungi-infected aphids were counted *in situ* on 18 plants per plot (72 per treatment), every two weeks from the beginning to the end of the maize growing season. If maize plants were found infested by aphids, the aphid colonies were visually examined to find individuals with external symptoms of fungal infection. Plants were chosen randomly at three points on the diagonal of each plot (six plants at each point). Additionally, twice a growing season the fungi-infected aphids were also collected for fungi identification. The first time was always in June, and the second one – if insects were available – at the beginning of October. Fifteen dead aphids were collected at random from each plot (one aphid from one plant diagonally across the plot), 60 aphids per treatment. Microscope slides of dead aphids were prepared. The taxonomic keys by Keller (1987, 1991) and Humber (1997) were used for the parasitic fungi identification.

Data were analyzed by *ANOVA* in the repeated measures procedure. In order to recognize the differences between the maize cultivars with respect to the number of aphids as well as fungi-infected aphids the collected data were analysed using Tukey's honest significant difference (post-hoc) test at a 5% level of significance. The data were normally distributed. To avoid the influence of seasonal trends statistical analysis was also carried out separately by one-way *ANOVA* ($P < 0.05$) for each data set.

Results

Number and quantity changes of fungi-infected aphids. In the three years of the study, in total 10461 aphids infected by entomopathogenic fungi were recorded on all the observed maize cultivars (Table 1). Clearly more fungi-infected aphids were found in 2008 (6929) compared to 2009 (2707) or to 2010 (825). Almost the same number of fungi-infected insects occurred on both *Bt* maize (4059) and the isogenic non-*Bt* cultivar (3866), whereas the incidence of infected aphids was somewhat lower on the conventional reference cv. 'Bosman' (2536).

Table 1. Total number of fungi-infected aphids recorded from maize plants in 2008–2010

Year	DKC 3421 YG	DKC 3420	'Bosman'	Total
2008	2776	2792	1361	6929
2009	945	854	908	2707
2010	338	220	267	825
Total	4059	3866	2536	10461

In 2008, aphids infected by entomopathogenic fungi were found only on the three observation dates, i.e. at the end of June, in mid-July, and at the end of July (Fig. 1). In the first observation, 356 dead aphids were recorded on the reference cv. 'Bosman', 716 insects on cv. DKC 3420, and 822 individuals on the *Bt* cv. DKC 3421 Yield Gard. The maximum number of mycosed aphids occurred during the second observation. Again, the lowest number of infected insects was observed on the cv. 'Bosman' (753). On the *Bt* cultivar 1261 aphids were mycosed, while on the isogenic non-*Bt* cultivar – 1222 aphids. At maximum aphids' infection the maize plants were at the BBCH stages 59–61. In the third count, 252 aphids were found infected on the conventional cultivar, 688 on *Bt* maize, and 854 on the isogenic non-*Bt* cultivar. No statistically significant differences were found between the number of fungi-infected aphids noted on the three cultivars ($F = 0.9182$, df = 2, $p = 0.4127$).

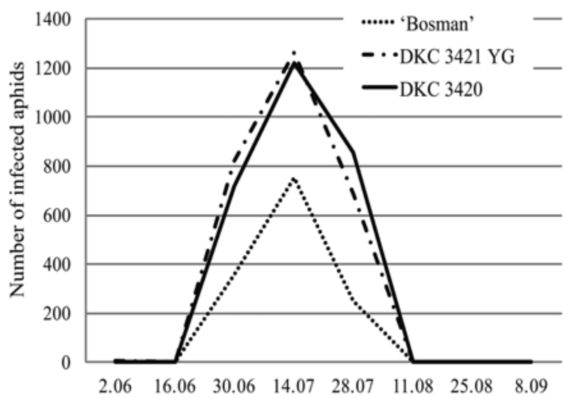

Figure 1. Number of aphids infected by entomopathogenic fungi recorded from maize plants in 2008

In 2009, fungi-infected aphids were recorded on maize from the end of June to mid-September (Fig. 2). The maximum number of mycoses was found on all three cultivars before the 10th day of July, when plants were at the BBCH stage 53. At the maximum 613 dead insects were collected on the cv. 'Bosman', 653 on the cv. DKC 3420, and 657 on the cv. DKC 3421 YG. The fungi-infected aphids were still numerous in the second half of July and at the beginning of August. Later, i.e. in the second half of August and in the first half of September, only single dead individuals occurred on the maize. As in the previous year, no significant differences were found between aphids infected with fungi on analysed cultivars ($F = 0.005642$, df = 2, $p = 0.9943$).

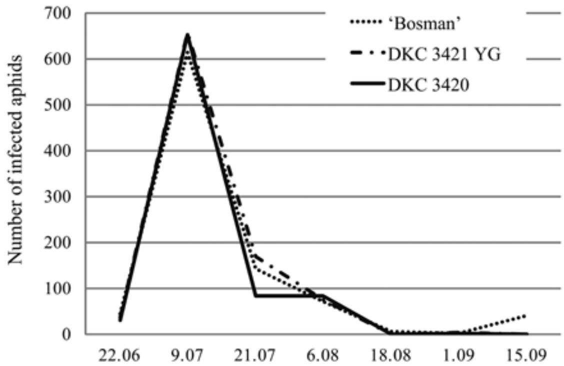

Figure 2. Number of aphids infected by entomopathogenic fungi recorded from maize plants in 2009

In 2010, mycosed aphids were recorded on maize plants from the last ten days of June to the beginning of September (Fig. 3). On each cultivar, the highest number of infected insects occurred, as in 2009, before the 10th day of July, on plants at BBCH stage 53. At the time of the maximum incidence of infections, almost similar numbers of mycoses were found on the conventional ('Bosman') cultivar (90), on the cv. DKC 3421 YG (93), and on the isogenic non-*Bt* maize (105). Decreasing numbers of fungi-infected aphids were observed later in the season. The last, single individuals were found in the first days of September. Also, in that last year no significant differences were found between number of fungi-infected aphids among the three maize cultivars ($F = 0.02440$, df = 2, $p = 0.9759$).

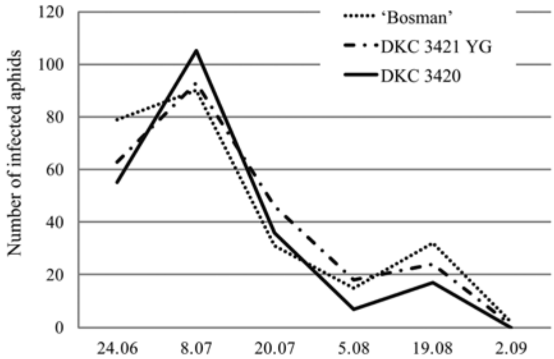

Figure 3. Number of aphids infected by entomopathogenic fungi recorded from maize plants in 2010

Spectrum of fungal species. In 2008, two species of entomopathogenic fungi were identified from the dead aphid mummy material collected in July on cvs DKC 3421 YG and 'Bosman' and three species on cv. DKC 3420 (Table 2). The most numerous species on all the three cultivars was *P. neoaphidis*. This species infected from 66.1% (cv. DKC 3420) to 70.8% (cv. 'Bosman') of all mycosed aphids. Less abundant was *Entomophthora planchoniana*. Mortality caused by this latter fungus ranged from 26.8% (cv. DKC 3420) to 33.3% (cv. DKC 3421 YG). *Conidiobolus thromboides* was identified only in individual dead insects collected from cv. DKC 3420 plants. As shown in Table 2, not all collected (60 individuals) aphids were infected by entomopathogenic fungi, but the factors responsible for the other individuals' mortality were not found. No aphids infected by entomopathogenic fungi were observed on maize in October 2008.

Table 2. Species composition of entomopathogenic fungi in 2008 (July)

Species	DKC3421 YG		DKC 3420		'Bosman'		Total	
	n*	%	n	%	n	%	n	%
Pandora neoaphidis (Remaudiere et Hennebert) Humber	36	66.7	37	66.1	34	70.8	107	67.7
Entomophthora planchoniana Cornu	18	33.3	15	26.8	14	29.2	47	29.7
Conidiobolus thromboides Dreschler	0	0	4	7.1	0	0	4	2.6
Total	54	100.0	56	100.0	48	100.0	158	100.0

* – number of fungi-infected aphids out of 60 collected

Four species of entomopathogenic fungi were identified in mummies collected from DKC 3421 YG plants in July 2009, while three species were found in those from cv. DKC 3420 and from cv. 'Bosman' (Table 3). In a few cases mixed infections were also found. As in the previous year the dominant species on all maize cultivars was *P. neoaphidis*, and this caused mortality ranging between 62.3% (cv. DKC 3420) and 77.1% (cv. DKC 3421 YG) of all the collected aphids. Less numerous were infections caused by *C. thromboides*. In the DKC 3420, 'Bosman' and DKC 3421 YG cultivars 26.4%, 25.9% and 14.0% of the collected aphids were infected by this fungus, respectively. *E. planchoniana* was found infecting only single aphids on all cultivars that year. *Neozygites fresenii* was identified only from a single aphid, collected from *Bt* maize (cv. DKC 3421 YG). Mixed infections of *P. neoaphidis* and *C. thromboides* were observed only on DKC 3421 YG and DKC 3420 cultivars.

In October 2009, on all maize cultivars almost all of the collected aphids were infected by entomopathogenic fungi (Table 4). Three fungal species were observed, and mixed infections were identified in the insect material from each experimental treatment. In this period the dominant enthomopathogen was *E. planchoniana*. The highest mortality of aphids caused by this fungus was found on cv. DKC 3421 YG plants (57.9%). On cv. 'Bosman' the aphid mortality reached 51.7%, while on the cv. DKC 3420 it only amounted to 40.7%. A high

Table 3. Species composition of entomopathogenic fungi in 2009 (July)

Species	DKC3421 YG		DKC 3420		'Bosman'		Total	
	n	%	n	%	n	%	n	%
Pandora neoaphidis (Remaudiere et Hennebert) Humber	44	77.2	33	62.3	37	68.5	114	69.5
Entomophthora planchoniana Cornu	2	3.5	2	3.8	3	5.6	7	4.3
Conidiobolus thromboides Dreschler	8	14.0	14	26.4	14	25.9	36	21.9
Neozygites fresenii (Nowakowski) Remaudiere et Keller	1	1.8	0	0	0	0	1	0.6
Mixed infection *C. thromboides* + *P. neoaphidis*	2	3.5	4	7.5	0	0	6	3.7
Total	57	100.0	53	100.0	54	100.0	164	100.0

percentage (40.7%) of aphids on the isogenic non-*Bt* cultivar (DKC 3420) was infected by *N. fresenii*. Fewer aphids were infected by the same species on cv. 'Bosman' (25.9%), and on *Bt* maize (17.5%). *P. neoaphidis* and *C. thromboides*, which had occurred in greater numbers in samples collected in July 2009, caused only single infections in October that year. Conversely, on all the observed cultivars mixed infections of *E. planchoniana* and *N. fresenii* were common in October. Mortality caused by these infections ranged from 16.9% (cv. DKC 3420) to 22.8% (cv. DKC 3421 YG).

Table 4. Species composition of entomopathogenic fungi in 2009 (October)

Species	DKC3421 YG		DKC 3420		'Bosman'		Total	
	n	%	n	%	n	%	n	%
Pandora neoaphidis (Remaudiere et Hennebert) Humber	1	1.8	1	1.7	0	0	2	1.1
Entomophthora planchoniana Cornu	33	57.9	24	40.7	30	51.7	87	50.0
Conidiobolus thromboides Dreschler	0	0	0	0	3	5.2	3	1.7
Neozygites fresenii (Nowakowski) Remaudiere et Keller	10	17.5	24	40.7	15	25.9	49	28.2
Mixed infection *E. planchoniana* + *N. fresenii*	13	22.8	10	16.9	10	17.2	33	19.0
Total	57	100.0	59	100.0	58	100.0	174	100.0

In July 2010, there were three (on cvs DKC 3421 YG and DKC 3420) or four (on cv. 'Bosman') species of entomopathogens identified in the mycosed aphids (Table 5). As in October of the previous year *E. planchoniana* was the most abundant on all cultivars. The level of infection caused by this species was very high, and it was similar on each cultivar, oscillating between 76.5% of aphids mycosed on cv. DKC 3421 YG, to 80.8% on cv. DKC 3420. The second important species, identified in samples collected in July was *P. neoaphidis*, causing likewise, mortality similar on all the cultivars, ranging from 18.2% on the cv. 'Bosman' to 21.6% on the cv. DKC 3421 YG. Another two species: *C. thromboides* and *N. fresenii*, were also found in mycosed aphids, but only single aphids were infected by these species. Similar to 2008, in October 2010, no aphid mycoses were observed on maize.

Table 5. Species composition of entomopathogenic fungi in 2010 (July)

Species	DKC3421 YG		DKC 3420		'Bosman'		Total	
	n	%	n	%	n	%	n	%
Pandora neoaphidis (Remaudiere et Hennebert) Humber	11	21.6	10	19.2	10	18.2	31	19.6
Entomophthora planchoniana Cornu	39	76.5	42	80.8	43	78.2	124	78.5
Conidiobolus thromboides Dreschler	0	0	0	0	1	1.8	1	0.6
Neozygites fresenii (Nowakowski) Remaudiere et Keller	1	1.9	0	0	1	1.8	2	1.3
Total	51	100.0	52	100.0	55	100.0	158	100.0

Discussion

Pests in agroecosystems have a wide range of natural enemies, including predators, parasitoids and pathogens. Fungal entomopathogens are often used for classical biological control, but not very much is known about them when occurring naturally in agricultural habitats (Meyling, Hajek, 2010). The most widely recognized fungal pathogens that infect aphids come from the order of Entomophthorales. This order includes six species of fungi commonly recorded from pest and non-pest aphids worldwide: *Pandora neoaphidis*, *Conidiobolus obscurus*, *Entomophthora planchoniana*, *Neozygites fresenii*, *Zoophthora phalloides* and *Z. radicans* (Powell, Pell, 2007). Several Hypocreales (Ascomycota) genera, such as *Beauveria*, *Verticillium* and *Paecilomyces*, are also known to infect aphids (Milner, 1997). Four species of entomopathogenic fungi have already been identified from mycosed aphids collected from maize in Poland, and these are *P. neoaphidis*, *C. thromboides*, *E. planchoniana* and *N. fresenii* (Krawczyk et al., 2006). The first species occurred most abundantly. The same four species were found infecting aphids on all the maize cultivars used in our present experiment. *P. neoaphidis* was the most numerous species in July of 2008 and 2009, while *E. planchoniana* dominated in October 2009 and in July 2010. Mycoses caused by aphid-pathogenic Entomophthorales, such as *Pandora neoaphidis*, play important roles in the natural control of aphids worldwide (Chen, Feng, 2005).

Fungi identified on aphids feeding on *Bt* maize are not well known, and in the literature there is a gap concerning this topic. In recent years, a lot of attention has been paid to investigating potential adverse effects of *Bt* plants on beneficial insects and other non-target organisms (Lővei, Arpaia, 2005; O'Callaghan et al., 2005). These organisms are exposed to insecticidal protein if they feed on transgenic plant tissue or consume organisms that have consumed the toxin. A large number of studies exploring risk assessments of GM maize with Cry1Ab protein on different non-target organisms, whether predatory or parasitic, indicate no effect (Naranjo, 2009; Twardowski et al., 2012; 2014). Aphids feed predominantly on phloem sap (Douglas, 2003), but they also provide food for many beneficial organisms. It seems reasonable to consider the influence of *Bt* maize on entomopathogens through their aphid hosts. The vast majority of studies indicate no effect of such varieties on aphids. In most cases laboratory investigations concluded that aphids could not be affected because the toxin is not transported in the phloem sap on which these insects feed (Raps et al., 2001; Pons et al., 2005). However, there are also a few reports relating different GM plants, mentioned in introduction, pointing to the prospective, indirect action on these herbivores and their natural enemies (Lumbierres et al., 2004; Burgio et al., 2011; Stephens et al., 2012). Open field studies are crucial for the evaluation of potential side effects of GM crops, and outcomes on aphids could cascade up to higher trophic levels, thus affecting the whole food web. In our other trials, no effect of *Bt* maize on bird cherry-oat aphid population development was recorded (unpublished). Similar results were achieved by Eckert et al. (2006), and Ramirez-Romero et al. (2008). Also, Habuštova et al. (2014) showed in field studies that the infestation of aphids, mainly *Rhopalosiphum padi* and *Metopolophium dirhodum*, was similar in both *Bt* and non-*Bt* plots. Because of the reflections above, and according to the results of our trials, we assume no effect of *Bt* maize on entomopathogens recorded on cereal aphids colonizing maize in the GM ecosystem. In the review form Lundgren et al. (2009) stated that there is evidence for both positive and negative indirect effects of Cry toxin on entomopathogens. In the literature some effects of *Bt* toxins on fungi in soil were better examined. For example Saxena and Stotzky (2001) indicated that the toxin released in root exudates of *Bt* maize or from the degradation of biomass of this genetically modified plant is not toxic to fungi, as well as other soil organisms. Some synergistic interactions between Cry toxin and entomopathogen fungi have also been found (Wraight, Ramos, 2005). Taken this latter knowledge into account, Lundgren et al. (2009) pointed out that hypothesis about positive or negative effects of *Bt* plants on fungi should be validated by looking on specific entomopathogen species or community.

Conclusions

1. The number of aphids infected by entomopathogenic fungi and their time of occurrence in the course of the growth season were very similar on the conventional maize cultivar 'Bosman', on the DKC 3421 Yield Gard *Bt*, and their isogenic line DKC 3420. No statistical differences between cultivars were found.

2. The same three or four entomopathogenic fungi-infected aphids were identified on the conventional maize, as well as on the transgenic *Bt* and on the isogenic non-*Bt* maize cultivars. *Pandora neoaphidis* (Remaudiere et Hennebert) Humber was the most abundant species in July of 2008 and 2009. *Entomophthora planchoniana* Cornu predominated in the enthomopathogens assemblage in October 2009 and in July 2010.

3. In the three years of the study no influence of the *Bt* maize on the number, quantity changes of fungi-infected aphids, or on the spectrum of fungal species was found.

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Insekticidinio baltymo Cry1AB įtaka entomopatogeninių grybų, infekuojančių amarus ant *Bt* kukurūzų, paplitimui

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Santrauka

Europoje transgeniniai kukurūzai yra vertinami nevienareikšmiškai. Tyrimų tikslas – nustatyti kiekybinius grybais infekuotų amarų santykio pokyčius ir galimus entomopatogeninių grybų rūšinės sudėties skirtumus ant tradicinių ir genetiškai modifikuotų kukurūzų, ekspresuojančių Cry1Ab baltymą iš *Bacillus thuringiensis* (*Bt*). Tyrimai atlikti 2008–2010 m. Žemutinėje Silezijoje, Lenkijoje. Tirtos trys kukurūzų veislės: DKC 3421 YG (*Bt*), izogeninė (ne *Bt*) DKC 3420 ir tradicinė 'Bosman' (kontrolinė). Vegetacijos sezonų metu kas dvi savaitės ant 18 augalų laukelyje *in situ* buvo suskaičiuoti užsikrėtę grybais amarai. Papildomai du kartus per metus buvo renkami negyvi amarai (60 amarų iš kiekvieno varianto), kurie naudoti grybams identifikuoti. Insekticidinis baltymas Cry1AB entomopatogeninių grybų, infekuojančių amarus ant *Bt* kukurūzų, paplitimui neturėjo įtakos. Grybais užsikrėtusių amarų kiekis ir jų pasirodymo laikas ant visų trijų tirtų veislių augalų buvo panašus. Tos pačios trys arba keturios entomopatogeniniais grybais užsikrėtusių amarų rūšys buvo nustatytos kiekviename variante. Visuose variantuose vyravo *Pandora neoaphidis*, *Entomophthora planchoniana* ir *Conidiobolus thromboides*.

Reikšminiai žodžiai: Cry1Ab baltymas, genetiškai modifikuoti augalai, grybais infekuoti amarai, kenkėjas, transgeniniai kukurūzai.