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Interaction between toxin crystals and vegetative insecticidal proteins of *Bacillus thuringiensis* in lepidopteran larvae

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Abstract Insecticides based on crystalline toxins of *Bacillus thuringiensis* are very good biological plant protection products. However, the spectrum of activity of some toxins is narrow or resistance among insects has been developed. We tested the insecticidal activity of crystals of the *B. thuringiensis* MPU B9 strain alone and supplemented with Vip3Aa proteins against important pests: *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), *Cydia pomonella* L. (Lepidoptera: Tortricidae) and *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae). The Cry toxins were more active for *D. pini* but less active against *S. exigua* and *C. pomonella* than Vip3Aa. Supplementation of Cry toxins by small amounts of vegetative insecticidal proteins demonstrated synergistic effect and significantly enhanced the toxicity of the insecticide. The results indicate the utility of Cry and Vip3Aa toxins mixtures to control populations of crops and forests insect pests.

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Introduction

Bacillus thuringiensis are Gram-positive bacteria producing several toxins with activity against protozoans, mites, nematodes, and insects. The lethality of *B. thuringiensis* towards a wide range of insects, including lepidopteran, dipteran and coleopteran larvae, is attributed largely to crystal proteins (Cry) produced during sporulation (Palma et al. 2014). In vegetative stage of growth other toxins are produced, named vegetative insecticidal proteins (Vips). Vip1 and Vip2 proteins act as binary toxins and show activity against Coleoptera, whereas Vip3 are active against Lepidoptera (Chakroun et al. 2016).

Some of the Cry toxins are applied as plant protection products because they are not toxic to vertebrates, rapidly degrade in the environment, and therefore do not accumulate in the soil, water or animal tissues. Due to the short half-life they do not exert a strong selective pressure in the environment, which might lead to the acquisition of resistance by insects. Production cost of these biological agents is relatively low. Another advantage is the lack of negative impact of these preparations on the soil fauna (Villaverde et al. 2014). Moreover, the use of biopesticides in crop protection leads to decreased levels of pesticide residues in foods and, as a result, to lower risk level for the consumer (Czaja et al. 2015). Therefore, the utilisation of bioinsecticides containing *B. thuringiensis* toxins is strictly in line with the

principles of integrated pest management, provided that they are effective and safe (Villaverde et al. 2014).

Presently there are over 400 *B. thuringiensis*-based formulations that have been registered in the market and most of them contain insecticidal proteins and viable spores, though the spores are inactivated in some products. Usually they are used as foliar sprays. An alternative method for delivering the toxins to the target insect is expression of the toxin-encoding genes in transgenic plants (George and Crickmore 2012). The use of *B. thuringiensis* (Bt) crops provides benefits in insect control, reduction of technical difficulties and costs associated with spraying the plants and reducing the amount of mycotoxins (e.g. fumonisin) in maize grain (Koch et al. 2015). However, continuous exposure of insects to toxins produced by genetically engineered plants contribute in decreasing susceptibility of pests population. Recently, reduced efficacy of Bt crops caused by field-evolved resistance has been reported for some populations of five of 13 major pest species examined, compared with resistant populations of only one pest species in 2005 (Tabashnik et al. 2013a). Also the strategy of using transgenic plants for pest management is often not possible (e.g. in forests) or is restricted by law.

In spite of the fair insecticide effect of crystalline proteins, some insects are not sensitive to their action. Various strategies are undertaken to increase the effectiveness of insecticides based on the *B. thuringiensis* toxins, including modifications such as toxin truncation, improvement of activation, domain swapping, site-direct mutagenesis, and peptide addition (Deist et al. 2014). Creation of mixtures containing different toxins isolated from a few strains of *B. thuringiensis* is another strategy of bioinsecticide development (Konecka et al. 2015).

Different types of Cry toxins may interact synergistically to increase the mortality of target insects. The mixtures should contain toxins having the lowest similarity and different mechanism of action, since then they have the highest activity. This can also broaden the biopreparation activity spectrum of target pests (Fernández-Luna et al. 2010; Tabashnik et al. 2013b). Insects resistant to one type of toxin, e.g. Cry1Ac, may be sensitive to other, e.g. Vip3 (Qian et al. 2015). However, interaction type between Bt proteins against crop pests cannot be easily predicted. Sometimes the same toxin combination in an insecticide acts synergistically, additively or antagonistically on different insect species, even from the same order (De Schrijver et al. 2015). Taking these factors into consideration, a special kind of pyramided transgenic Bt crops have been designed to raise activity and delay evolution of resistance in pests. This strategy proved to be optimal for genetically engineered crops (Carrière et al. 2015). The aim of the study was to evaluate the interaction effect of combinations of *B. thuringiensis* toxin crystals and vegetative insecticidal protein mixtures in important pests: *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), *Cydia pomonella* L. (Lepidoptera: Tortricidae) and *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae), and to determine mixtures with synergistic (and possibly the highest) activities of toxins, which could be used on non-transgenic crops and when spraying is the only possible method of insecticide application, e.g. in forest protection.

Materials and methods

Toxin isolation

B. thuringiensis MPU B9 strain isolated from the intestine tracts of *C. pomonella* (Konecka et al. 2007) was used as a crystal protein gene source. Crystals were isolated according to the method described by Guz et al. (2005) with some modifications. The strain MPU B9 was cultured on Brain Heart Infusion (BHI, Oxoid) with addition of bacteriological agar (15 g l^{-1}; Biocorp) at 30 °C, for 20 h. Subsequently, bacterial cells were spread on sporulation medium plates and cultured at 30 °C for another 4–5 days in order to attain sporulation. The degree of sporulating cells was estimated by staining a culture sample with amido black and carbol fuchsin, followed by examination under standard light microscope. Crystal-spore mixture was then collected from plates, washed in 1 M NaCl and twice in distilled water (each time centrifuged at $3000 \times g$ for 30 min), and finally suspended in 50 mM Tris- HCl pH 7.5 with 10 mM KCl. The suspension was then put on ice and sonicated with ten pulses, 10 s each (22 kHz frequency, 14 mm amplitude) using a UD-11 automatic ultrasound disintegrator. Next, in order to separate crystals from spores, the suspension was put on discontinuous sucrose gradient (67, 72, 79, 87%) and centrifuged ($13,000 \times g$, 50 min,

22 °C) Sorvall Evolution RC (Thermo Scientific) using swing bucket rotor. Layers containing crystals were collected and washed three times with sterile, demineralized water. The purity of crystals was estimated under light microscope, preceded with staining step performed according to the procedure mentioned above. Crystals suspended in sterile demineralized water were then frozen (-80 °C) and lyophilized in vacuum (133×10^{-3} mBar) for 24 h. Lyophilized samples were kept dry at 4 °C until use. For bioassays, a portion of lyophilizate was suspended in 50 mM Tris, pH 8.0 and the concentration (ng of dry weight per μl) was adjusted to desired values.

The composition of the proteins forming crystals was determined by mass spectrometry. Crystal proteins were solubilized and separated in SDS-PAGE. Bands corresponding with Cry toxins were cut out from the gel and enzymatically degraded to obtain oligopeptides. Separation of the peptides was done with liquid chromatography. The masses of peptide fragments were determined by Orbitrap Spectrometer (Thermo). Peptide fragments match was resolved in Mascot software (http://www.matrixscience.com).

Expression and isolation of Vip3Aa proteins was performed according to our previous work (Baranek et al. 2015). Briefly, *E. coli* strains with *vip3Aa*-bearing expression plasmids were cultured in LB broth with 100 μg ml^{-1} ampicillin, and subsequently induced with 1 mM IPTG. After incubation, the cells were centrifuged, frozen and disrupted using lysozyme treatment and sonication. Lysates were then centrifuged at $14,000 \times g$ for 20 min, filtered through 0.22 μm PVDF syringe filters, and dialyzed in 14 kDa MWCO tubes against 50 mM Tris, pH 8.0. The concentration of Vip proteins present in lysates was estimated densitometrically.

Insect toxicity bioassays

In order to estimate the toxicity of *B. thuringiensis* MPU B9 protein crystals, Vip3Aa58 and Vip3Aa59 proteins, as well as mixtures of these insecticidal factors, three insect pest species were employed: *S. exigua*, *C. pomonella*, and *D. pini*. Toxicity assays were carried out by using identical procedure as previously described (Baranek et al. 2015). Briefly, for LC$_{50}$ estimation of MPU B9 crystal toxins, lyophilized crystals were suspended in 50 mM Tris, pH 8.0, and administered on the surface of insect diet in six different concentrations (36 insects per concentration). Assays were performed in three repetitions. Differences between LC$_{50}$ values of different Bt toxins were considered statistically significant when fiducial limits did not overlap. For Cry-Vip interaction tests, toxin mixtures and separate mixture components were administered in different concentrations, with 24 insects ascribed to each. Two repetitions were done. All of the tests were carried out for ten days after which dead insects were counted. Mortality correction was done by using Abbot's formula (O'Callaghan et al. 2012).

Determination of interaction between crystalline and vegetative insecticidal proteins

We evaluated potential synergism or antagonism for each toxin combination. A synergistic effect is observed when the activity of some mixtures is greater than expected, based on the activities of the individual components. Antagonism is characterized by a significantly reduced observed toxicity compared to the expected toxicity of the mixture (Li and Bouwer 2014). We used the method as described previously by Tabashnik et al. (2013b) based on the assumption that the proportion of larvae surviving exposure to a combination of toxins is a product of the proportions of larvae that survive exposure to each of the toxins separately. For the mixture of two (Cry and Vip) toxins expected value of survival can be calculated using the formula: $S_{(cryvip)exp} = S_{(cry)obs} \times S_{(vip)obs}$, where $S_{(cryvip)exp}$ is the proportion of larvae expected to survive exposure to a combination of Cry and Vip toxins, $S_{(cry)obs}$ is the observed proportion of larvae that survived exposure to Cry toxin, and $S_{(vip)obs}$ is the observed proportion of larvae that survived exposure to Vip toxin. Survival correction was done by using Abbot's formula (O'Callaghan et al. 2012). The expected mortality (M) for larvae exposed to a combination of toxins was calculated using equation: $M_{exp} = (1 - S_{(cryvip)exp}) \times 100\%$.

For each of the combination of toxin concentrations tested, we used Fisher's exact test with two-tailed probability to determine if a significant difference ($p < 0.05$) occurred between the observed and expected number of dead and alive larvae. Interaction was considered synergistic when observed mortality was higher than expected and antagonistic when

observed mortality was lower than expected. When no relevant difference occurred, only additive effect between two toxins was assumed.

Results

Larvicidal activity of crystalline and vegetative toxins

Results of mass spectrometry analysis revealed the presence of Cry 1Aa, 1 Ba, 1Ca, 1D, and 9E toxins in the crystals of MPU B9 strain. The bioassays demonstrated activities of the crystals as well as Vip3Aa58 and Vip59 against *S. exigua*, *C. pomonella* and *D. pini.* No significant differences in the activity against *S. exigua* and *C. pomonella* between Vip3Aa58 and Vip3Aa59 were found and these proteins were more active than the crystals of MPU B9 isolate. In contrast, Cry toxin was more active against *D. pini* than Vip3Aa59 (Table 1).

Insecticidal activity of toxin combinations

Insecticidal activity of mixtures containing Bt crystals and Vip proteins against *C. pomonella* is presented in Table 2. The results showed that synergism occurred in a few combinations. For all mixtures with Cry toxins in concentration 250 ng cm^{-2} (less than half of LC$_{50}$ value), synergism was observed upon addition of Vip toxins (10, 50 and 100 ng cm^{-2}). Even the addition of 10 ng cm^{-2} of Vip toxins to 250 ng cm^{-2} of MPU B9 Cry proteins caused the death of 60% or more insect larvae. Using higher dose of Cry toxins in the mixture, closer to the concentration that causes the death of 50% of the larvae, resulted in additive effect only.

Synergism was also observed between Cry and Vip toxins against *S. exigua* (Table 3). High insecticidal activity was observed at a dose as low as 10 ng cm^{-2} MPU B9 crystals and 2.5 ng cm^{-2} of Vip3Aa58 toxin. For a mixture containing 100 ng cm^{-2} of crystals and 2.5 ng cm^{-2} of Vip3Aa58 protein, strong synergism and high insecticidal activity were observed (mortality higher than 90%). Toxin combination containing the same amount of crystal toxin but with Vip3Aa59 protein also demonstrated synergism between these compounds, but the activity was lower. However, it still resulted in the death of more than 50% of the insect larvae.

Assessment of insecticidal activity of crystals and Vip toxin mixtures to *D. pini* was difficult because the insect cannot be continuously reared in laboratory and it is necessary to collect larvae in the field and quarantine them. This resulted in lower number of test insects available for research and slightly different approach regarding toxin concentration arrangement. Since our previous studies have shown higher activity of Vip3Aa59 than Vip3Aa58 to *D. pini* (Baranek et al. 2015), we applied only the former in activity tests. The values of LC$_{50}$ for *D. pini* were significantly higher than those for *S. exigua* and *C. pomonella* (Table 1). However, we also observed synergistic activity of some Cry and Vip combinations against *D. pini* (Table 4). Synergistic effect against *D. pini* was observed for mixtures containing crystals/Vip3Aa59 at the dose of 91/84 ng cm^{-2} (total amount of toxins = 175 ng cm^{-2}) and 182/167 ng cm^{-2} (total amount of toxins = 349 ng cm^{-2}). In toxicity assays for all three of the tested insects, for all toxin combinations except one, the observed mortality of larvae was higher than expected. However,

Table 1 Insecticidal activity of *Bacillus thuringiensis* toxins against pests

Insect	Toxin	LC$_{50}$ (ng cm^{-2})	95% fiducial limits	
			Lower	Upper
C. pomonella	MPU B9 crystals	590	460	750
	Vip3A58	210	110	390
	Vip3Aa59	240	130	450
S. exigua	MPU B9 crystals	570	460	720
	Vip3Aa58	15	2	60
	Vip3Aa59	17	5	50
D. pini	MPU B9 crystals	760	600	950
	Vip3Aa59	1450	1230	1700

Table 2 Efficacy of *Bacillus thuringiensis* MPU B9 crystals, Vip3Aa58, Vip3Aa59 and their combinations against *Cydia pomonella*

Toxin	Concentration (ng cm^{-2})	Mortality (%)		p (Fisher's exact test)
		Observed ($\pm$SE)	Expected	
MPU B9 crystals	250	30 ($\pm$4)	–	–
	500	50 ($\pm$13)	–	–
Vip3Aa58	10	10 ($\pm$2)	–	–
	50	30 ($\pm$0)	–	–
	100	40 ($\pm$19)	–	–
Vip3Aa59	10	5 ($\pm$2)	–	–
	50	20 ($\pm$4)	–	–
	100	25 ($\pm$8)	–	–
MPU B9 crystals + Vip3Aa58	250 + 10	65 ($\pm$2)	37	0.014
	250 + 50	75 ($\pm$4)	51	0.020
	250 +100	100 ($\pm$0)	58	<0.001
	500 + 10	65 ($\pm$19)	55	0.406
	500 + 50	80 ($\pm$17)	65	0.173
MPU B9 crystals + Vip3Aa59	250 + 10	60 ($\pm$6)	33	0.014
	250 + 50	67 ($\pm$0)	44	0.040
	250 +100	85 ($\pm$2)	48	<0.001
	500 + 10	58 ($\pm$13)	53	0.538
	500 + 50	75 ($\pm$8)	60	0.190

only some of the cases were statistically significant. There was no case of antagonistic interaction.

Discussion

Mass spectrometry studies of *B. thuringiensis* MPU B9 crystal composition showed the presence of Cry 1Aa, 1Ba, 1Ca, 1D, and 9E. Earlier gene identification by PCR in the genome of this strain showed also the presence of *cry1I*, *cry2Ab* and *cry9B* (Konecka et al. 2007). The absence of Cry1I toxins was not surprising because, unlike other Cry proteins, they are produced as soluble proteins during vegetative growth of bacteria and do not accumulate as crystals (Tailor et al. 1992). The lack of Cry2Ab in crystals indicates that there was no expression of the *cry2* gene in MPU B9 strain. This leads to a conclusion that estimation of the toxin composition in crystal solely on the basis of the PCR identification of genes in bacterial DNA is insufficient.

The composition of the proteins forming crystals of *B. thuringiensis* MPU B9 strain suggested that they should have high activity against Lepidoptera, especially since earlier studies have pointed at the synergism possibility between these toxins. Synergistic interactions have been proven between Cry1Aa and Cry1C toxins against *Helicoverpa armigera* and *S. exigua* (Xue et al. 2005; Li and Bouwer 2014), as well as Cry1C and Cry9Aa against *H. armigera* (Li and Bouwer 2014). In addition, toxicity of Cry1A against *Agrotis ipsilon* and *S. exigua* and Cry1C to *S. exigua* can be increased by *Manduca sexta* cadherin fragments which have synergistic properties (Abdullah et al. 2009). Gao et al. (2010) have revealed synergy between Cry1Aa toxin and Cry1Ab, Cry1Aa as well as Cry1Ca, Cry1Ac and Cry1Ca, Cry1Ac and Cry1Cb, Cry1Ab, and Cry1Ca, Cry1Ab and Cry1Ba, Cry1Ac and Cry1Ba against *Chilo suppressalis*. On the other hand, the mixture of Cry1Aa and Cry1Ab was antagonistic in toxicity to *Sesamia inferens*. There is no data about the interaction of Cry1A and Cry1D as well as Cry1B and Cry9. Konecka et al. (2015) have observed synergistic activities of mixtures consisting of crystals isolated from two isolates of *B. thuringiensis* against *C. pomonella*. These mixtures displayed

Table 3 Efficacy of *Bacillus thuringiensis* MPU B9 crystals, Vip3Aa58, Vip3Aa59 and their combinations against *Spodoptera exigua*

Toxin	Concentration (ng cm^{-2})	Mortality (%)		p (Fisher's exact test)
		Observed ($\pm$SE)	Expected	
MPU B9 crystals	10	3 ($\pm$2)	–	–
	100	8 ($\pm$4)	–	–
Vip3Aa58	0.1	0 ($\pm$0)	–	–
	0.5	8 ($\pm$0)	–	–
	2.5	38 ($\pm$4)	–	–
Vip3Aa59	0.1	0 ($\pm$0)	–	–
	0.5	6 ($\pm$2)	–	–
	2.5	15 ($\pm$2)	–	–
MPU B9 crystals + Vip3Aa58	10 + 0.5	15 ($\pm$6)	11	0.623
	10 + 2.5	61 ($\pm$2)	40	0.019
	100 + 0.1	10 ($\pm$2)	8	1.000
	100 + 0.5	24 ($\pm$8)	15	0.292
	100 + 2.5	94 ($\pm$6)	43	<0.001
MPU B9 crystals + Vip3Aa59	10 + 0.5	11 ($\pm$2)	9	0.735
	10 + 2.5	33 ($\pm$0)	18	0.036
	100 + 0.1	8 ($\pm$4)	8	1.000
	100 + 0.5	19 ($\pm$2)	14	0.380
	100 + 2.5	57 ($\pm$ 10)	22	<0.001

higher toxicity against insect than commercial biopesticide Foray based on *B. thuringiensis*, recommended for reduction of the number of pests in plant protection.

Vegetative insecticidal proteins Vip3A are also active against a broad range of lepidopteran pests. However, the range of insects susceptible to this toxin is not the same as Cry1. Since the discovery of Vip toxins, possible synergism between them and delta-endotoxin has been suggested (Donovan et al. 2001). Our study directly showed synergism between crystals containing Cry toxins and Vip3Aa proteins. Combinations of these toxins had high activity against economically important pests of woodlands (*D. pini*), orchards (*C. pomonella*) and crop fields (*S. exigua*). To date, only a few studies have identified interactions between certain Cry and Vip toxins against *S. exigua*. Zhu et al. (2006) have shown that *B. thuringiensis* YBT 1520 strain producing Cry1Aa, 1Ab, 1Ac, and Cry2 toxins after transformation with a plasmid containing the gene coding for synthesis of Vip3Aa7 had 10-fold more toxicity against *S. exigua*, than non-

transformed parental strain. However, the transformed strain had the same level of toxicity against *H. armigera* as that parental YBT 1520 strain.

The synergy and the high activity of the composition containing Cry and Vip3Aa toxins against *C. pomonella* was observed at much lower doses than for toxins administered separately. To our knowledge, there has been no study about the insecticidal activity of Cry and Vip proteins compositions towards this organism. Fighting *C. pomonella* is difficult because the larvae bore tunnels inside apples, pears, plums, and apricots. The only possible moment for the insecticide action is a short period when larvae dwell on the surface. Pests hidden within the fruit are unavailable for insecticides, including the most conventional chemical preparations and pesticides based on Baculovirus CpGV. Recently, sensitivity decrease of the insect to chemical preparations has been noted (Grigg-McGuffin et al. 2015), so demonstration the synergistic interaction between crystals and Vip proteins and high activity of Cry toxins and Vip3A mixtures is promising to fight *C. pomonella*. In our study,

Table 4 Efficacy of *Bacillus thuringiensis* MPU B9 crystals, Vip3Aa59 and their combinations against *Dendrolimus pini*

Toxin	Concentration (ng cm^{-2})	Mortality (%)		p (Fisher's exact test)
		Observed ($\pm$SE)	Expected	
Vip3Aa59	84	0 ($\pm$0)		
	167	2 ($\pm$1)		
	334	5 ($\pm$0)		
	668	21 ($\pm$4)		
	1336	48 ($\pm$6)		
	2672	68 ($\pm$15)		
	5348	93 ($\pm$8)		
	16,043	100 ($\pm$0)		
MPU B9 crystals	91	0 ($\pm$0)		
	182	13 ($\pm$4)		
	364	37 ($\pm$13)		
	728	48 ($\pm$6)		
	1458	68 ($\pm$15)		
	2917	78 ($\pm$10)		
	5834	92 ($\pm$0)		
MPU B9 crystals + Vip3Aa59	91 + 84	10 ($\pm$6)	0	0.027
	182 + 167	42 ($\pm$4)	14	0.001
	364 +334	57 ($\pm$15)	40	0.100
	728 + 668	73 ($\pm$13)	59	0.123
	1458 + 1336	90 ($\pm$10)	83	0.421
	5834 + 5348	100 ($\pm$0)	99	1.000

insecticidal activity of *B. thuringiensis* MPU B9 crystals and vegetative insecticidal protein (used separately and in combination) was also tested against *D. pini*. LC$_{50}$ of Vip3Aa59 toxin and was nearly two-fold higher than the Cry toxins. Moreover, a strong synergistic interaction was found between these two factors towards *D. pini*, indicating that formulations containing both proteins can be effective in combating this dangerous pest of conifers.

Several authors have also observed synergisms and high insecticidal activity of Vip and crystal toxin mixtures against different lepidopteran pests: *Anticarsia gemmatalis, Chrysodeixis includes* (Crialesi-Legori et al. 2014), *S. albula, S. cosmoides, S. frugiperda* (Bergamasco et al. 2013), *S. exigua, C. suppressalis* (Yu et al. 2012) and *Diatraea saccharalis* (Lemes et al. 2014). However, the authors have also noticed antagonistic Cry/Vip interactions in other insects. In silico analysis has revealed that the Vip3Aa-Cry1Ac fusion protein has strong affinity against receptors of

A. ipsilon, Pectinophora gossypiella, S. exigua and *S. litura*. Therefore, it should have high activity against lepidopteran cell receptors in general, and hence has a potential to be efficient broad-range insecticidal protein (Ahmad et al. 2015). However, fusion gene encoding Cry1Ac-Vip3Aa14 led to acquisition of chimeric protein, which retained toxicity of Cry1A, but partially lost that of Vip3Aa14 when tested against *H. armigera, S. litura* and *Plutella xylostella* (Saraswathy et al. 2008). Other chimeric protein obtained after fusing Vip3Aa7 with N terminus of Cry9Ca improved toxicity against *P. xylostella* larvae (Dong et al. 2012).

In our study we did not observe any antagonism, though the synergism occurred only in some combinations. In the case of high amount (closing to LC$_{50}$ or exceeding it) of one or both toxins, synergic phenomenon was not noticed. Similar results were obtained by Wei et al. (2015) for *Helicoverpa armigera*. Probably there was a significant damage

done to the intestinal epithelial cells caused by one component and the second additive had little significance. The fact that the same combination of proteins act synergistically or antagonistically may be an indication that there are different types of interactions within the host, depending on the insect species. Several authors suggest that antagonism occurs when two or more toxins attach to the same receptor, whereas synergism occurs when toxins bind to different receptors. For example Cry1Aa (or Cry1Ac) and Vip3Aa toxins do not compete with each other because they bind to different cell receptors (Sena et al. 2009; Qian et al. 2015).

Development of insect-resistant transgenic plants expressing the *B. thuringiensis* toxin-encoding genes provided effective control against important pests and cost reduction associated with spraying (Koch et al. 2015). However, constant pressure of one-toxin plants on insects causes resistance occurrence. Recently, Tabashnik et al. (2013a) have revealed reduced efficacy of Bt crops for some populations of pest species examined, compared with data from 2005. To delay the onset of resistance in insects, multi-toxin crops can be used. Simultaneously expression of Vip3A and different Cry proteins in plants is a beneficial feature because crops are more effectively control against pests and additionally risk of resistance development is reduced (Carrière et al. 2015; Chen et al. 2010).

In our opinion similar effects can be obtained when composition of toxin crystals and vegetative insecticidal proteins will be used as a spray. *B. thuringiensis* insecticide formulations usually contain a high proportion of living spores which could cause an imbalance in the natural bacterial population. Bacterial spores can survive in forest ecosystem for several months (Konecka et al. 2014). The use of insecticides that do not contain endospores is safer for the environment (Sanchis et al. 1999). Expression of *B. thuringiensis* toxin genes in heterologous bacterial hosts, for example in *Pseudomonas fluorescens*, and killing the bacteria after the formation of the crystalline inclusions allowed to get insecticides without spores. Such preparations are already commercially available. The Mycogen company produce insecticide MVP® used in controlling insects of the order Lepidoptera and formulation M-Trak effective against insects of the order Coleoptera (Sanchis 2012). Recently, delta-endotoxin production by a sporeless

B. thuringiensis strain was developed and obtaining inexpensive insecticide containing no spores can be a lot easier (Khedher et al. 2014). We believe that the addition Vip3Aa toxin to insecticides containing only *B. thuringiensis* crystals contribute to the better pests control and reduce the risk of resistance development.

In conclusion, this study revealed that the application of an insecticide containing the crystals of Cry toxins and Vip3A proteins seems to be a promising alternative to chemical preparations and bioinsecticides containing only one type of *B. thuringiensis* toxin. Occurrence of the synergism between two components depends on the proportion of their concentration in mixtures. The mixtures used as a spray are particularly justified in the protection of forests, crops grown on small fields and areas where the use of transgenic plants which produce insecticidal toxins is not feasible. Furthermore, due to the different mechanism of Cry and Vip toxins action, the risk of insect resistance to the preparation is low.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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