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Originalarbeit

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Investigations on residues of XenTari® (*Bacillus thuringiensis* subspec. *aizawai*) on greenhouse tomatoes

Untersuchungen zu Rückständen von *Bacillus thuringiensis* an Gewächshaustomaten

Abstract

XenTari® (*Bacillus thuringiensis* (*B.t.*) subspecies *aizawai*) is an important biological plant protection agent for the control of Noctuidae larva on tomato fruits in greenhouses and belongs to the group of presumptive *Bacillus cereus* species. In general, food control agencies do not routinely differentiate between *B.t.* and *B. cereus* and a threshold of 10^5 colony forming units (cfu)/g fresh weight is applied for presumptive *B. cereus* in official food control. As no data exists on the expected residues of *B.t.* spores after application, residual experiments were conducted on tomatoes in greenhouses. In the greenhouse experiment, five applications of XenTari® were applied at weekly intervals. The concentration of *B.t.* spores on the tomato fruits ranged in all experiments between 4.9×10^4 and 8.5×10^4 cfu/g fresh weight. For single application of *B.t.*, a maximum spore concentration of 4.7×10^4 cfu/g fresh weight was measured. None of the experiments reached the threshold for *B. cereus* of 1×10^5 cfu/g, although treatments were applied in a very narrow window. The findings were confirmed by additional laboratory experiments and by experiments conducted on a commercial tomato farm. To prove the degradation of *B.t.* spores under protected greenhouse conditions over time, a series of samples was taken after the last application over one week. Over all, the experiments demonstrated that the concentration of *B.t.* spores was reduced within one week to between 46% and 77% of the initial spore concentration. Therefore, in comparison to open field condition the degradation of *B.t.* spores under greenhouse condition was limited. When only the upper parts of the tomato plant were treated with XenTari® a distinct reduction of *B.t.* spores of up to 90% of *B.t.* spores with a concentration of 1.85×10^3 cfu/g fresh weight on the marketable tomatoes was achieved.

Key words: *Bacillus thuringiensis*, residue, tomato

Zusammenfassung

XenTari® (*Bacillus thuringiensis* subspecies *aizawai*) ist ein bedeutendes biologisches Pflanzenschutzmittel zur Bekämpfung von Noctuidenraupen im Tomatenanbau unter Glas. Da *B. thuringiensis* (*B.t.*) zur Gruppe der präsumptiven *Bacillus cereus*-Arten gezählt wird, in der Lebensmittelüberwachung im Allgemeinen aber kein Unterschied zwischen *B.t.* und *B. cereus* gemacht wird und für präsumptive *B. cereus* ein Grenzwert von 10^5 Kolonie-bildende Einheiten (KbE)/g Frischgewicht (FG) gilt, wurde experimentell überprüft, welche maximalen KbE-Konzentrationen an Gewächshaustomaten bei Anwendung von *B.t.*-Präparaten erreicht werden können. In Gewächshausversuchen mit fünf XenTari® Anwendungen im wöchentlichen Abstand wurden Rückstände von $4{,}9 \times 10^4$ bis $8{,}5 \times 10^4$ KbE/g FG ermittelt. Somit wurden in keinem der Versuche die Richtwerte für präsumptive *B. cereus*-

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Konzentrationen von 10^5 KbE/g FG erreicht, obwohl eine praxisunübliche und sehr enge Spritzfolge appliziert wurde. Ergänzende Labor- und Praxisversuche bekräftigten diese Ergebnisse. Wurde die Persistenz der Sporen auf dem Erntegut untersucht, so nahm die Sporenkonzentration innerhalb der ersten Woche nach Applikation auf 46% bis 77% der anfänglichen Konzentration ab. Durch Spritzdüseneinstellungen nur auf das obere beblätterte Pflanzensegment – unter Aussparung der unten hängenden unbeblätterten erntereifen Früchte – konnte die Keimbelastung des Ernteguts nach einmaliger Anwendung von XenTari® von $2{,}05 \times 10^4$ KbE/g FG auf $1{,}85 \times 10^3$ KbE/g FG reduziert werden. Daher könnten anwendungstechnische Maßnahmen, wie die Nichtbehandlung erntbarer Früchte – die entsprechende Applikationstechnik ist in der modernen Tomatenproduktion mittlerweile Standard – als ergänzende Maßnahmen dienen, die Belastung des Ernteguts mit *B.t.* weiter zu reduzieren.

Stichwörter: *Bacillus thuringiensis*, Rückstände, Tomate

Introduction

Isolates of *Bacillus thuringiensis* (*B.t.*) are one of the most important biocontrol agents worldwide and have been used for decades to control agricultural and forest insect pests, including mosquitoes. *B.t.* products are authorized to be used in organic agriculture and play an important role in integrated pest management (IPM).

Because of their safety to non-target insects, *B.t.* products are particularly efficient at protecting crops. For example, when cultivating tomatoes in greenhouses, a combination of beneficial insects, such as pollinating bumble bees and the beneficial insects *Macrolophus caliginosus* or *M. pygmaeus*, are used in combination with *B. thuringiensis* subspecies *aizawai* (XenTari®), which enable the reduction of chemical insecticide treatments to a minimum concentration. In 2012, nearly 100% of commercial tomato production in North Rhine-Westphalia greenhouses was treated using this biological control agent (Scholz-Döbelin, 2014).

B.t. products for lepidopterean control contain insecticidal parasporal crystalline endotoxins and alive spores, which may germinate under favorable conditions. Due to their genetic relationship, *B. thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus cereus* (sensu strictu, s.str.) are considered to belong to the presumptive *B. cereus* group *or B. cereus* (sensu lato, s.l.) (Helgason et al., 2000). Some *B. cereus* strains can cause food poisoning, such as vomiting and/or diarrhea (see review Ceuppens et al., 2013), others are considered as beneficial bacteria and are being used as probiotics (Hoa et al., 2000). For decades, the safety of commercial *B.t.* preparations on mammalians and other non-targets has been extensively tested and reviewed (Fisher and Rosner, 1959; McClintock et al., 1995). The European Food Safety Agency (EFSA, 2005) considers concentrations of *B. cereus* of > 10^5 cfu/g fresh weight a potential risk to human health. For *Bacillus* species others than *B. cereus* a critical threshold of 10^6 cfu/g fresh weight is recommended.

Because standard methods for detection and enumeration of *B. cereus* applied in foodstuff control do not distinguish *B. cereus* from other Bacillaceae, such as *B.t.* and *B. weihenstephanensis*, a general threshold of 10^5 cfu/g fresh weight is applied in Germany and some other European countries by food control agencies, irrespective of the food contains pathogenic or non pathogenic *B. cereus* s.l. strains. Because data regarding *B.t.* spore residues on tomatoes cultivated in greenhouses were not available, there may be a risk that the tomatoes are not marketable due to high *B.t.* spore (= *B. cereus* s. l.) concentrations. Therefore, this study was conducted to evaluate *B.t.* spore residues on fresh tomatoes after applying a maximum application rate under laboratory, experimental field station and professional grower conditions.

Materials and Methods

B.t. *product and experimental site*

The *B.t.* product XenTari® (*B. thuringiensis* subsp. *aizawai*, strain ABTS 1857) was kindly provided by BIOFA AG, Germany, for experimental testing. According to the registration of XenTari®, a worst-case scenario (maximum application rate and harvesting immediately after the last application) was chosen. The laboratory experiments and sample evaluation were performed at JKI Darmstadt, whereas the greenhouse experiments were conducted at the Chamber of Agriculture North Rhine-Westphalia, Experimental Station Horticulture in Straelen. Additionally, one experiment was conducted on a commercial tomato farm in Tönisvorst, North Rhine-Westphalia.

Laboratory experiment

Laboratory experiments were set up to obtain initial data on the expected range of cfu numbers per gram fresh weight of tomatoes. Because the greenhouse experiments were designed for the tomatoes to be harvested and stored at −20 °C until the enumeration of colony forming units, laboratory experiments were conducted to prove the influence of freezing on the cfu concentration.

Single tomato fruits were treated with a concentration of XenTari® and application adjustments were made in accordance with the application rate used for the greenhouse experiments: 600 l/ha, 3 bar, speed of 3.5 km/h, Teejet 8003 EVS nozzle and an application rate of 1000 g XenTari® per ha. For this experiment, 1.6 g XenTari® was suspended in 1000 ml tap water and 200 ml of suspension was transferred to the applicator (Spraylab 240/120 SPS, Schachtner, Germany). Marketable tomatoes on the vine with four to six tomatoes (80–100 g/fruit) were fixed on a tripod and were treated with one nozzle on each side, ensuring that all sides of the tomato were treated. When the application layer was touch dry, *B.t.* spores of half of the tomatoes were enumerated imme-

diately the other half were frozen at −20 °C and were enumerated on the following day. Only tap water was used as a control treatment. The experiment was independently repeated three times with five tomatoes per repetition.

To measure the application rate, an iron plate with defined weight and size was fixed in the middle of the applicator. Before and after application, the plate was weighted and based on the weight difference the application rate was calculated.

Greenhouse experiment

Greenhouse experiments were conducted at the Experimental Station Horticulture in Straelen. Two cabinets were cultivated with tomatoes on the vine, and two cabinets with cherry tomatoes. For plant sizes higher than 125 cm, 2.0 kg XenTari®/ha suspended in 1200 l water per ha was applied. The whole plant was treated. For application, a parcel spraying device with Airmix 11002 nozzles was used. The application was repeated five times at weekly intervals. First treatment of tomatoes was at a growth stage of BBCH 720 and fruit harvesting was at BBCH 723 or BBCH 724. Samples of about one kg of tomatoes on the vine (70–100 g/fruit), or cherry-tomatoes (30–50 g/fruit), were collected before the first application, before and after the last application, and 1, 2, 3, and 7 days after the last application. Similarly, samples were taken from an untreated control.

The experiments were conducted between July 18th and 24th, 2013 and August 20th and 27th, 2013. From the initial application to harvest, the temperature in and outside of the greenhouse and the sum light in Klux/h were measured. All fruits were transferred to a freezer (−18 °C) immediately after sampling. After all samples were taken, the number of cfu per gram fresh weight was enumerated. Five tomatoes were analyzed per sampling.

On-farm experiment

An un-repeated experiment was conducted in the greenhouse of a commercial tomato grower in Tönisvorst, Germany. The application was conducted using a half automatic application robot (Meto, Berghortimotive), at an application rate of 860 l/ha and 2 kg XenTari®/ha on September 17th, 2013 (Fig. 1, left). Within one application, either the whole plant (tomatoes on the vine) or only the upper part of the plant (leaves and young tomato fruits), were treated (Fig. 1, right) by closing the relevant lower nozzles. The weather on the day of application was cloudy and rainy. Samples of 1 kg were taken immediately after application and after 1, 2, 3, and 7 days, and were treated and analyzed as described above.

Enumeration of B.t. *spores*

For enumerating the *B.t.* residues, the sprayed tomatoes were weighed and transferred into a sterile bottle with

Fig. 1. Application of XenTari® with a half automatic application robot (Meto, Berghortimotive) (left), and with reduced numbers of opened nozzles (right).

250 ml autoclaved isotonic solution (9 g NaCl in 1 l sterile deionised water), and were shaken for 10 min on a reciprocal shaker (Köttermann, Germany). Afterwards, 15 ml of the supernatant was transferred into Falcon tubes, and was heat-treated for 20 min in a water bath at + 80°C. This and one additional decimal dilution were plated on three tryptic soy agar (TSA) Petri dishes (3% tryptic soy broth, 1.5% g agar (w/v) in deionised water) per dilution, using a spiral plater. Following the instructions for analyzing the cfu concentration with a spiral plater, segments with around 40 colonies were counted after 20 h incubation at 25°C and the number of cfu per gram fresh weight was calculated. From each sample five tomatoes were analyzed. The number of cfu of each tomato was enumerated on three TSA Petri dishes.

Statistical analysis

Data was statistically analyzed with the software SAS System for Windows v9.3. The laboratory experiment was analyzed using the generalized linear model. For separation of the means, original data was compared with the Student-Newman Keuls (SNK) test. The homogeneity of variance was tested with the Levene test ($p < 0.1$). For the greenhouse experiments, only the treated samples of all four experiments were statistically analyzed together. Because no homogeneity of variance was achieved, a non-parametric test was conducted. With the NPAR1WAY procedure, the sample taken after the last application was compared pair wise with the other samples. The on-farm experiment was not statistically analyzed.

Results

Laboratory experiment

Application with a laboratory applicator resulted in a mean volume and standard deviation of 613 (± 16.1) l/ha, and an application rate of 993 ± 47.8 g XenTari® per ha. Concentrations of about 5×10^4 cfu/g fresh weight were found on the fruits after a single treatment with XenTari®. When the spore concentration was compared before and after freezing of the tomatoes, no significant difference was measured (Fig. 2). This result indicated that freezing of fruits did not influence the spore enumeration.

Greenhouse experiment

During the experiment, the temperature inside the greenhouse ranged from 16.6 to 35.2°C with a daily mean temperature of between 20 to 29°C. The daily mean sum of light was 334 (± 112) Klux/h.

In untreated control, a mean concentration of 63.9 cfu/g fresh weight, with a maximal concentration of 8.7×10^2 cfu/g fresh weight, was observed throughout the entire experiment (Fig. 3). When XenTari® was applied five-times at weekly intervals, the mean concentration of colony forming units on tomato fruits ranged between 4.9×10^4 and 8.5×10^4 cfu/g fresh weights across all of the experiments (Tab. 1). Within one of four experiments (Fig. 3d), one of five tomato samples reached a concentration of 1.03×10^5 cfu/g fresh weight. For all other samples immediately taken after the last application, the concentration was below 1×10^5 cfu/g fresh weight. When the residues of tomatoes on the vine (80–100 g/fruit) and cherry tomatoes (30–60 g/fruit) were compared, cherry tomatoes had on average 36% higher residues than the larger tomatoes on the vine.

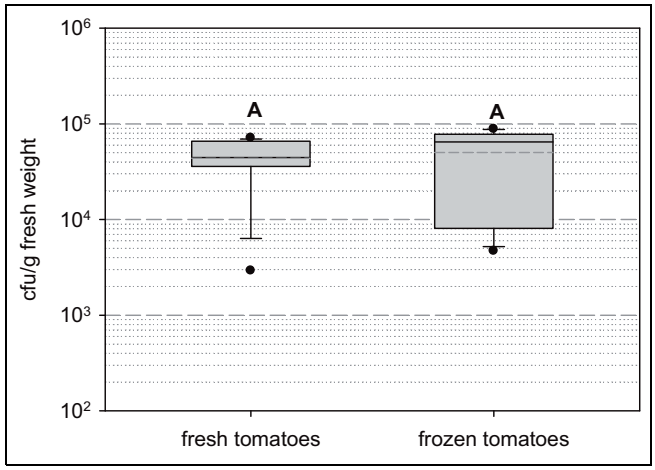

Fig. 2. Comparison of the number of colony forming units (cfu) in laboratory on tomatoes after application of XenTari®, before (fresh tomatoes), and after freezing (frozen tomatoes). The box plots show the first and third quartiles, the median (solid line) and the arithmetic mean (dash line). Whiskers (SD bars) above and below the box indicate the 90th and 10th percentiles. Dots are showing each outlier. Each treatment was repeated three times with five tomatoes. Means of the data with same letter are not significantly different according to the Student Newman Keuls test ($p < 0.05$).

To prove whether *B.t.* spores degrade over time under protected greenhouse conditions, samples were taken 1, 2, 3 and 7 days after the last application. In all experiments, the concentration of *B.t.* spores decreased to between 46% and 77% of the initial spore concentration immediately after the last spray (Tab. 1). A significant reduction of residues was measured two days after the last *B.t.* application (Wilcoxon, $p < 0.05$).

The concentration achieved by a single application can be calculated by the difference between the cfu concentration before and after the last application. In all greenhouse experiments, a mean cfu concentration after one application of 2.87×10^4 (minimum 2.1×10^4 cfu/g fresh weight, maximum 4.7×10^4 cfu/g fresh weight) was recorded (Tab. 1). This concentration corresponds with the results obtained during the laboratory experiments (5×10^4 cfu/g fresh weight), and the experiments carried out at the commercial farm (2.1×10^4 cfu/g fresh weight), where comparable application rates were used. One week after the last application the concentration declined stepwise to 58%. This result corresponds with the decline of 65% in the on-farm experiment.

On-farm experiment

In the untreated control a maximum concentration of 1.9×10^2 cfu/g fresh weight was achieved, whereas a single application of XenTari® on the whole plant resulted

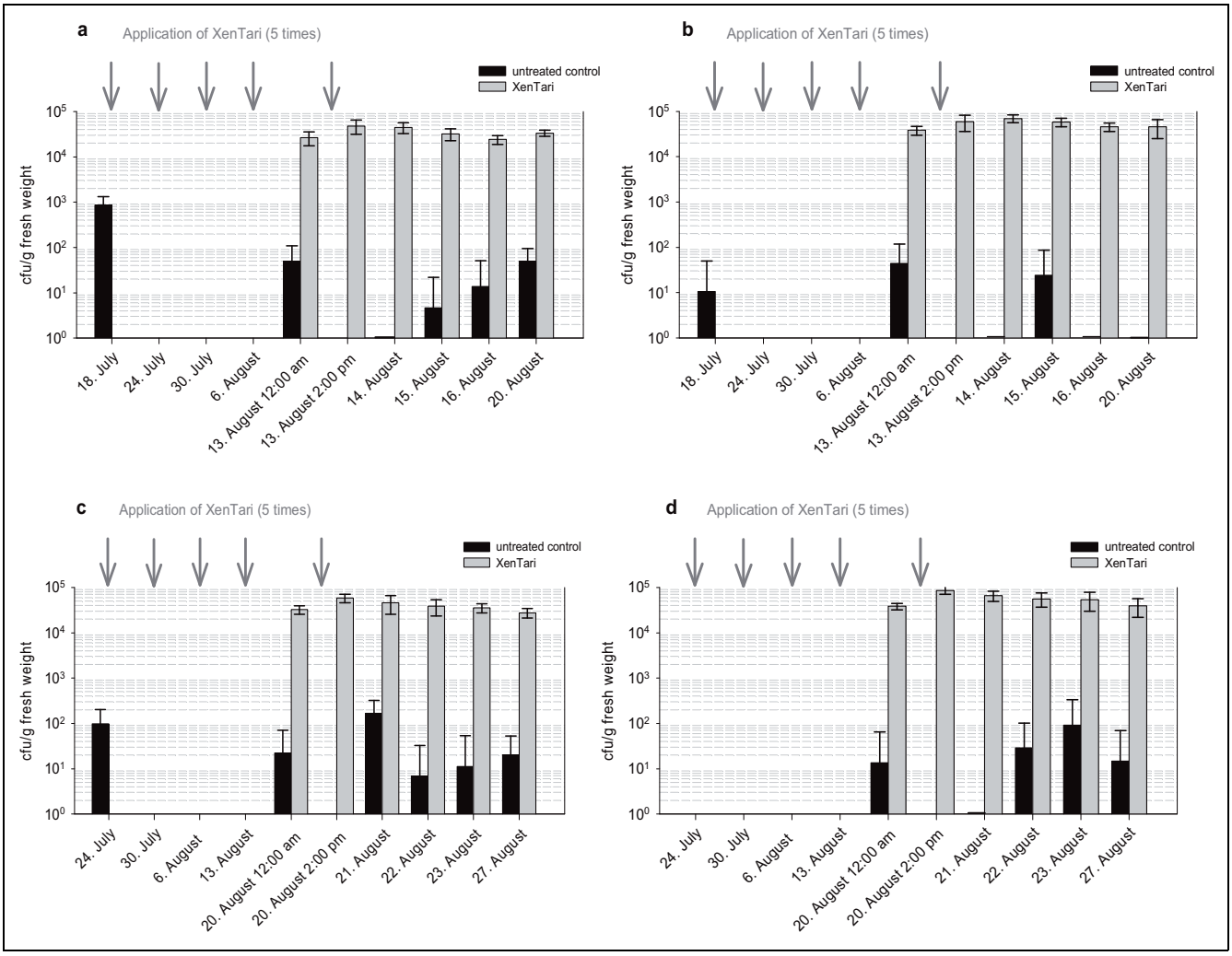

Fig. 3. Mean counts (SD bar) of colony forming units (cfu) per gram fresh weight of marketable tomatoes after five applications of XenTari® in greenhouse experiments; tomatoes on the vine (80–100 g/fruit) (panel a and c) and cherry tomatoes (30–50 g/fruit) (panel b and d). Five tomatoes were analyzed per sampling.

Tab. 1. Summary of the greenhouse experiments on residues of colony forming units (cfu) on tomatoes after five applications of XenTari® (arithmetic mean, SD and median of four independent experiments on tomatoes on the vine and cherry tomatoes)

	Number of cfu (x 10^4) per gram fresh weight and residues based on the cfu after last application (%)					
	Before last appl.	Days after last application				
		0	1	2	3	7
Arithmetic mean	3.39	6.25	5.63	4.61	3.93	3.63
± SD	± 0.91	± 2.20	± 2.20	± 1.82	± 1.78	± 1.15
Median	3.16*	6.14	6.01	4.56*	3.79*	3.16*
Calculated residues (%)		100	90.10	73.80	63.60	58.10

* The median of the sample is significantly different to the median of the sample taken after the last application (Day 0) following the Wilcoxon signed rank test ($p < 0.5$).

in a concentration of 2.1×10^4 cfu/g fresh weight. Within one week the concentration declined to 1.3×10^4 cfu/g fresh weight (Tab. 2).

When only the upper part of the cultivar (leaves and green tomatoes) was treated with XenTari®, only 1.85×10^3 cfu/g fresh weights were detected, equaling only 9%

Tab. 2. Concentration of colony forming units (cfu) on tomatoes after one application of XenTari® in two different application strategies. The on-farm application was performed on September 17th, 2013

	Number of cfu per gram fresh weight (mean ± SD) and calculated residues (CR) based on the cfu after immediately application (%)					
	Before	Days after application				
	appl.	0	1	2	3	7
Untreated	32.9 ± 61.1	165 ± 203	87.3 ± 90.7	40.2 ± 56.6	190 ± 153	106 ± 129
treatment of whole plant	129 ± 118	2.05 ± 0.66 x 10^4	1.79 ± 0.39 x 10^4	1.37 ± 0.35 x 10^4	1.02 ± 0.32 x 10^4	1.33 ± 0.48 x 10^4
CR (%)*		100	87.3	66.5	49.8	64.5
treatment of leaf area	58.7 ± 121	0.18 ± 0.12 x 10^4	0.33 ± 0.18 x 10^4	0.31 ± 0.11 x 10^4	0.11 ± 0.46 x 10^4	0.12 ± 0.49 x 10^4
CR (%)		100	181	165	60	65

of the concentration found on fruits from whole plant application. Within one week, 65% of the starting value was achieved.

Discussion

In Germany, the use of the *B. thuringiensis* subspecies *aizawai* based product XenTari® is authorized for the control of free feeding Lepidoptera and Noctuids larvae in fruit and vegetables in the field and in greenhouses. In greenhouses, a maximum of five applications can be made in 5–7 day intervals, and an application rate dependent on the height of the cultivar (1 kg XenTari®/ha in 600 l/ha (up to 50 cm), 1.5 kg/ha in 900 l/ha (50 to 125 cm height) or 2 kg/ha in 1200 l/ha (over 125 cm height) is authorized; BVL, 2013).

Based on the EFSA's (2005) opinion that food contaminated with *B. cereus*, at a concentration of > 10^5 cfu/g fresh weight, is considered not safe for human consumption, food commodities with concentrations of presumptive *B. cerus* (including *B.t.*) higher than > 10^5 cfu/g fresh weight are considered un-marketable. The EFSA report however, did not recognize that in contrast to *B. cereus* s.str., commercial *B.t.* strains were negatively tested for their capacity to express enterotoxins during the fermentation process at relevant levels, though they contain and may express the enterotoxin genes at a low rate. Although most *B.t.* strains are able to produce enterotoxins, it was shown by DAMGAARD (1995), that the commercial *B. thuringiensis* subspecies *aizawai* strain of XenTari® produced 70-fold less enterotoxins compared to a pathogenic *B. cereus* strain when cultured in Brain Heart Infusion Broth. Also, acute and short-term toxicity studies using rats did not exhibit any adverse effect with any of the commercial *B.t.* subspecies at concentrations of > 10^8 cfu per animal (MCCLINTOCK et al., 1995). So far, there is no evidence that commercial *B.t.* strains are able to produce enterotoxins at a biological relevant level after consumption by animals or humans. There are incidental associations with cases of food-borne illness, but it was not shown whether commercial *B.t.* strains were responsible (JACKSON et al., 1995; MCINTYRE et al., 2008).

In the field, *B.t.* sprays are rapidly degraded. Reported half-life times of *B.t.* spores on foliage range from a few hours to 2 days (PEDERSEN et al., 1995; IGNOFFO and GARCIA, 1978; PINNOCK et al., 1971; KRIEG et al., 1980). Thus, no or minimum residues of *B.t.* sprays are expected. In greenhouse applications, however, where fruit vegetables, such as tomatoes, are being continuously harvested for several months, any UV inactivation is unlikely. To gain initial information about the maximum residue levels of *B.t.* to be expected on greenhouse tomatoes at harvest, different experiments including a laboratory spray on tomatoes, a worst-case greenhouse experiment, in which the maximum authorized application rate was applied within minimum intervals of 7 days, and a single application in a commercial tomato farm were conducted. Cfu's exceeding the critical concentration of > 10^5 per gram fresh weight were not observed in any of the experiments.

Very little data analyzing *B.t.* residues on agricultural crops is available. BAE et al. (2004) investigated eight commercial vineyards sprayed with the *B.t.* product Delfin. *B.t.* was isolated in all vineyards throughout the period of grape cultivation concentrations of 10^2–10^6 cfu/g. At harvest time, residues varied between 10^2 and 10^4 cfu/g. FREDERIKSEN et al. (2006) tested 991 fruit and vegetable samples for the presence of *B. cereus* and *B.t.*. They identified 129 presumptive *B. cereus* strains (13%), 50 *B.t.* strains (5%) of which 14 strains were not distinguishable from commercial *B.t. kurstaki* and 9 strains (0.9%) were indistinguishable from commercial *B.t. aizawai*. The latter were predominantly found from Dutch greenhouse peppers (5), tomatoes (3), and cucumbers (1), with concentrations between 10 to 4.6 × 10^3 cfu/g fresh weight. On the other hand, spore forming Bacillaceae

including *B.t.* are ubiquitous, naturally occurring *B.t.* strains have been reported from soil and plant surfaces all over the world (BERNHARD et al., 1997). Natural populations of *B.t.* on the leaves are about 10^2 cfu/cm of leaf (SMITH and COUCHE, 1991; MADUELL et al., 2002; COLLIER et al., 2005). Even in the untreated controls of our greenhouse experiment background levels of spore forming Bacillaceae of up to 10^3 cfu/g fresh weight were observed (Fig. 2).

In our experiments we evaluated the stability of *B.t.* sprays under protected greenhouse conditions over one week, after a repetitive application of XenTari®. Throughout all of the experiments, the concentration of *B.t.* spores decreased to between 46% to 77% of the initial spore concentration, within one week. Hence, degradation of *B.t.* spores exists but does not play an important role under greenhouse conditions relative to field conditions. Nevertheless, our results indicate that a limited degradation of *B.t.* spores prevents an accumulation of *B.t.* spores over time.

Since Noctuid larvae are predominantly found in the younger parts of the plants, the application of XenTari® was modified for the on-farm experiment, so that only the leaf area and young fruits on the top; but not the ripening fruits at the bottom of the plants, were sprayed. Through this adjustment, it was possible to reduce the *B.t.* concentration on the marketable tomatoes by a factor of 10 compared to whole plant application. This finding may prove useful for designing methods of reducing the application rates, and residues of plant protection agents. However, further experiments need to be conducted to confirm these results, and to guarantee the control efficacy. Further actions, such as the implementation of pre harvest intervals are not practicable, because in commercial tomato production, carried out in greenhouses, the fruits are continuously harvested in short intervals and the degradation of spores is very slow.

In conclusion, the experiment indicated that even at maximum application rates the average concentrations of residues on tomatoes was 6.25×10^4 cfu per gram fresh weight. However, in practice, even with a high pest insect incidence, usually two applications within ten days are applied. Therefore, under commercial growing conditions, lower concentrations of *B.t.* residues can be expected.

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