J. Appl. Ent. 127, 146–152 (2003)

ISSN 0931-2048

Ingested particles reduce susceptibility of insect larvae to *Bacillus thuringiensis*

E. Ben-Dov[1,2], D. Saxena[1,3], Q. Wang[1], R. Manasherob[1,2], S. Boussiba[2,4] and A. Zaritsky[1,2,3]
[1]Department of Life Sciences, Ben-Gurion University of the Negev, Be'er-Sheva, Israel; [2]BioSan Ltd., Ariel, Israel; [3]The Research Institute, The College of Judea and Samaria, Ariel, Israel; [4]Department of Dryland Biotechnologies, Microalgal Biotechnology Laboratory, The Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede Boqer, Israel

Ms. received: October 28, 2001; accepted: May 7, 2002

Abstract: Susceptibility to *Bacillus thuringiensis* of mosquito and lepidopteran larvae is affected by feeding behaviour and nutritional value of the available food. Reduced mortality is attributed to feeding inhibition and dilution of the pathogen in the presence of nutritional and inert particles, which limit the amount of ingested toxin. These reasons are, however, not sufficient to explain the data presented here. Values of LC_{50} (the concentration that kills 50% of exposed population) of *B. thuringiensis* subsp. *israelensis* (Berliner) against *Aedes aegypti* (L.) larvae and of *B. thuringiensis* subsp. *kenyae* (Berliner) against *Spodoptera littoralis* (Boisduval) larvae were about 20–217 and 2.3–44-fold higher, respectively, in the presence of nutritional or biologically inert (non-nutritional) particles than without. The number of *B. thuringiensis* spores in carcasses of *B. thuringiensis* -killed *A. aegypti* and *S. littoralis* larvae were between 1.9 and 5.6-fold and between 8.5 and 12-fold higher, respectively, in the presence of particles than without. In all cases, non-nutritional particles better protected the exposed larvae than nutritious particles. We propose that another basic mechanism exists, that ingested particles protect midgut epithelial cells by covering their surface and thus preventing availability of the toxin to the gut receptors. Understanding the defence mechanisms of insects against *B. thuringiensis* toxicity may lead to improved pest management methods.

1 Introduction

Various subspecies of *Bacillus thuringiensis* (Berliner), the most effective microbial insecticides, are classified according to their target organisms (Hofte and Whiteley, 1989; Feitelson et al., 1992), serotype (de Barjac and Frachon, 1990), and homology of δ-endotoxin polypeptides (Crickmore et al., 1998; http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html). Their efficacy is influenced by environmental factors such as sunlight and temperature, amounts and composition of food, water quality, larval density and co-habitation with other filter feeding organisms competing for food (Ramoska and Pacey, 1979; Khawaled et al., 1988; Mulla, 1990; Mulla et al., 1990; Becker et al., 1992; Kondo et al., 1995). Toxicity is modified by variations in food consumption through feeding behaviour, food availability or nutritional value (Ramoska and Pacey, 1979; Gaugler and Molloy, 1980; Aly, 1983; Merritt et al., 1992; Kondo et al., 1995). Dilution of the pathogen and feeding inhibition in the presence of particles are considered the major causes for the reduced mortality response observed (McGaughey, 1976; Ramoska and Pacey, 1979; Gaugler and Molloy, 1980; Aly, 1983; Salama and Abdel-Razek, 1992). Rate of ingestion is faster in the presence of food than with non-nutritive particles, indicating a role for phagostimulation by chemical compounds (Dadd, 1968; Rashed and Mulla, 1989), but is inhibited in proportion to the concentration of both types of particles (Gaugler and Molloy, 1980). This implies similar differences in the length of time during which the filtered material stays in the larval gut: the rate of clearance is proportional to ingestion rate. Non-nutritious particles indeed remain in the gut for longer periods of time (Dadd, 1970).

Many ecological studies have been performed with *B. thuringiensis* subsp. *israelensis* (Berliner) and with *B. sphaericus*, which are very effective against Diptera (Porter et al., 1993). When mosquito larvae are presented with a mixed suspension of bacteria and latex beads (as markers), they filter at a constant rate for a period that depends on the species and stop after ingesting a certain amount of toxin (Aly, 1988). Feeding inhibition by toxin has also been observed in Lepidoptera and Coleoptera with other species of *B. thuringiensis* (Herbert and Harper, 1987; Hoy and Hall, 1993; Regev et al., 1996).

Kondo et al. (1995) have recently found that larvae of the euryphagous chironomid *Pentapedilum tigrinum* (Kieffer) (Dip., Chironomidae), which are sensitive to *B. thuringiensis* subsp. *israelensis*, exhibited altered susceptibility when reared on different food materials prior to treatment. The authors explained this observation by the quality of food presented causing changes of the proteolytic conditions in the larval midgut. Exposing larvae of the black fly *Simulium*

 0931–2048/2003/2703–0146 $ 15.00/0

vittatum (Zetterstedt) (Dip., Simuliidae) to a rich fish food prior to treatment by *B. thuringiensis* subsp. *israelensis*, which caused feeding inhibition, significantly reduced toxicity level (Gaugler and Molloy, 1980). Post-treatment feeding inhibition by food particles raised toxicity, apparently due to longer retention time of the δ-endotoxin in larval gut.

Lower mortalities of target organisms by sublethal concentrations of *B. thuringiensis* can be explained by physiological defence mechanisms. For example, damaged midgut epithelial cells of the rice moth *Corcyra cephalonica* (Stainton) (Lep., Pyralidae) larvae are replaced, and the newly made cells are protected from toxicity of *B. thuringiensis* subsp. *kurstaki* HD-1 by a quickly produced mucous layer covering their surface (Chiang et al., 1986). Chitin, a major component of the cuticle, can serve as a defence agent against pathogens as well (Sampson and Gooday, 1998); it is part of the peritrophic membrane (that contains also proteins, glycoproteins and carbohydrates), a protective sleeve lining the gut of many insects (Beaty and Marquardt, 1996). Pathogens such as *B. thuringiensis*, which infect through the gut, must penetrate this chitin-rich barrier. Addition of chitinases enhances the insecticidal activity of *B. thuringiensis* by allowing easy access of the bacterial toxins to the gut epithelium for their easier binding to gut receptors (Sneh et al., 1983; Regev et al., 1996; Wiwat et al., 1996; Sampson and Gooday, 1998).

Larvae of *Aedes aegypti* (L.) (Dip., Culicidae) and *Spodoptera littoralis* (Boisduval) (Lep., Noctuidae), ingesting high doses of δ-endotoxin, exhibited reduced toxicities in the presence of particles, which cannot be explained by feeding inhibition or by dilution of the pathogen. It is suggested that ingested particles protect midgut epithelial cells of insect larvae by covering their surface and thus preventing availability of the toxin.

2 Materials and methods

2.1 *Bacillus thuringiensis* strains

Two subspecies of *B. thuringiensis* were used, *israelensis* (Berliner) (Bactimos Primary Powder 1990, fun 89C06D, Duphar B.V., Weesp, Holland; 12 000 International Toxic Units/mg) and *kenyae* (Berliner) HDB-23 (kindly supplied by Dr D. R. Zeigler, Bacillus Genetic Stock Center, Columbus, OH).

2.2 Rearing mosquito larvae

Dry strips of paper bearing eggs of *A. aegypti* were submerged as described by Khawaled et al. (1988) in 1 l of sterile tap water supplemented with 1.5 g of Pharmamedia, a cottonseed-derived protein nutrient (Traders Protein, Memphis, TN). Third instar larvae were selected for bioassays after 3–4 days of incubation at 28 ± 2°C.

2.3 Rearing *Spodoptera* larvae

Pupae of *S. littoralis* were surface-sterilized using 10% formalin and placed in a container with soil or vermiculite inside a cage. Upon eclosion, the adult moths were fed 10% sucrose solution. The cage was lined with white cotton cloth serving as an egg-laying surface. Eggs were collected daily and incubated in ventilated containers (10-cm diameter, 7-cm height) at 26 ± 2°C with 60 ± 5% relative humidity. After 2–4 days, when neonate larvae emerged, some were preserved for colony maintenance and the rest were used for bioassays.

Artificial larval diet (for 1 l) included cracked wheat (50 g), wheat germ (37.5 g), semolina (31.25 g), casein (25 g), ascorbic acid (3 g), sorbic acid (1.5 g), *para* -aminobenzoic acid (62.5 mg), formaldehyde (8.75 ml of 10%) and linseed oil (6.25 ml). The ingredients were blended, heated to 70°C, and added to (800 ml) boiled sterile distilled water with agar (25 g). The mixture was blended (2 min) and poured into bioassay trays (1.5–2 ml/well) or vials (5 ml) for larval rearing.

2.4 Non-nutritional particles

Carmine-vital dye (Natural Red 4) was purchased from Sigma Chemical Co. (St Louis, MO) and activated charcoal powder from BDH Chemicals Ltd. (Poole, England).

2.5 Bioassays for *Aedes aegypti* larvae

The *B. thuringiensis* subsp. *israelensis* powder was suspended in sterile distilled water [1 mg/ml, *ca.* 10^9 colony-forming units (CFUs)] and heat shocked (10 min, 70°C). Twenty third-instar *A. aegypti* larvae were incubated (28°C) in 100-ml sterile tap water (in 170-ml disposable plastic cups) with appropriate *B. thuringiensis* subsp. *israelensis* dilutions, alone or with particles (Pharmamedia, charcoal, carmine). Larval mortality was determined after 24 h. Each bioassay was independently performed at least three times in duplicate. Values of LC_{50} (concentration of spores with toxin that kills 50% of exposed population in a standard bioassay) were determined with six concentrations. LC_{50} and 95% fiducial limits (FL) were obtained using probit analysis developed by Daum (1970). Mortality of control larvae (without *B. thuringiensis* subsp. *israelensis*) never exceeded 5%.

To determine the lethal dose (LD) of *B. thuringiensis* subsp. *israelensis* against *A. aegypti*, larvae were pre-incubated with Pharmamedia or charcoal for 2 h. *B. thuringiensis* subsp. *israelensis* was added in approximately the respective LC_{50} concentrations. Single larvae were withdrawn upon death, washed in sterile distilled water four times and frozen in 0.2 ml of water. Each larva (in 30 replicates from three experiments) was homogenized in 1 ml of water, sonicated (MSE Sonifier, three times of 30 s duration and at 30-s intervals, 4°C), and samples were plated on Luria-Bertani broth (LB). The number of CFUs is correlated to the amount of toxin, thus representing the LD values (in spore count).

2.6 Bioassays for *Spodoptera littoralis* larvae

Bacillus thuringiensis subsp. *kenyae* was grown in LB broth at 28°C with shaking (200 rpm) for 4–5 days (1.4×10^9 CFU/ml). Harvested spores and crystals were washed with sterile saline (0.85% NaCl) and dried to a powder at 50°C. The powder was suspended at the same concentration (1.4×10^9 CFU/ml) in saline with 0.01% Tween 80, sonicated for two cycles of 45 s at 4°C and serially diluted. One hundred microlitres of each dilution was evenly spread onto solidified freshly prepared insect diet, which had been set for 2 h in a bioassay tray, and air-dried for about 30–60 min.

Sixteen freshly hatched larvae of *S. littoralis* were released, one per well, for each treatment and control. The trays were incubated at 60–70% relative humidity, 26°C, and in a 16 h light/8 h dark photoperiod. Larval mortality was recorded

after 5–7 days and LC_{50} and 95% FL were obtained using probit analysis (Daum, 1970). The larvae were considered live even if they had not grown significantly as long as they responded to touch by moving. Bioassays with control mortality exceeding 10% were discarded. In addition to the usual bioassay, LC_{50} values were determined when a dried suspension of *B. thuringiensis* subsp. *kenyae* (in 100-μl dilution buffer per well) was presented, and when it was evenly mixed with agar (2.5%) alone. Charcoal (0.5 mg/ml) was added whenever stated.

Fresh cotton leaves were cut into circular discs, dipped in various dilutions of *B. thuringiensis* subsp. *kenyae*, and kept in petri dishes into each of which eight neonate larvae were released; four such plates were used for each dilution. Powder of cotton leaves (0.5 mg/ml) was prepared by drying distilled water-washed leaves at 37°C and crushing them by mortar and pestle. The fine powder was mixed with *B. thuringiensis* subsp. *kenyae* and dried.

Total viable spores were counted for LD calculation. For each diet, 96 neonate *S. littoralis* larvae were singly exposed to the respective LC_{50} of *B. thuringiensis* subsp. *kenyae*. At predetermined times, three live and three dead larvae were washed separately with sterile distilled water, homogenized in 500 μl of 0.1% Tween 80 and sonicated for 45 s at 4°C at maximum energy for two cycles. The sonicated homogenates were plated (before and after heat shock, 10 min at 70°C) on LB agar, incubated at 30°C and the number of colonies was counted after 24 h.

3 Results

3.1 Ingested particles by *Aedes aegypti* larvae

Exposure of third instar larvae to about LC_{90} (35 ng/ml) of *B. thuringiensis* subsp. *israelensis* for different times (during 5 h) before adding (1 mg/ml) of either charcoal, carmine or Pharmamedia yielded similar mortalities, reaching a plateau at 1 h (fig. 1). During the first 20 min, toxicity was lower with the non-nutritional particles than with Pharmamedia, but none affected toxicity later on, implying an immediate action of *B. thuringiensis* subsp. *israelensis* upon ingestion. The mortality at 50 ng/ml ($>LC_{100}$ in 24 h) dropped substantially when *B. thuringiensis* subsp. *israelensis* was applied simultaneously with particles (1 mg/ml), to 5% with charcoal, 30% with carmine, and 45% with Pharmamedia (fig. 2). An even larger difference was observed when the larvae were incubated with 0.5 mg/ml particles for various times before adding 350 ng *B. thuringiensis* subsp. i*sraelensis*/ml (fig. 3). Here too, both non-nutritious particles protected the larvae better than the nutritious particles, most likely by pathogen dilution and feeding inhibition, according to the conventional opinion.

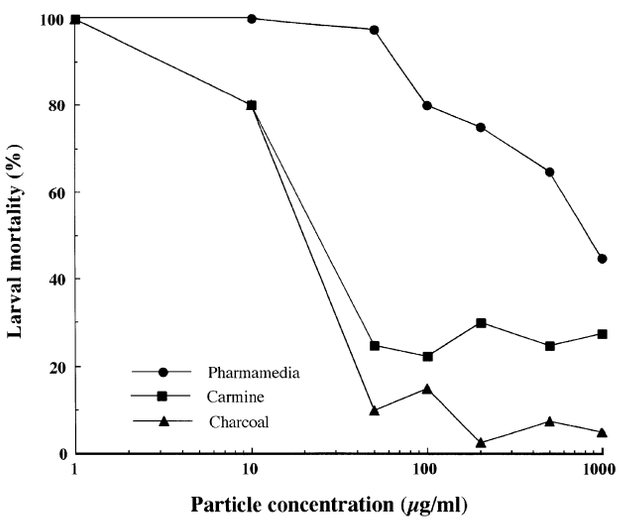

Fig. 2. *Mortality of* Aedes aegypti *larvae (third instar) exposed to* Bacillus thuringiensis *subsp.* israelensis *(50 ng/ml) simultaneously with different concentrations of the indicated particles (1–1000 μg/ml)*

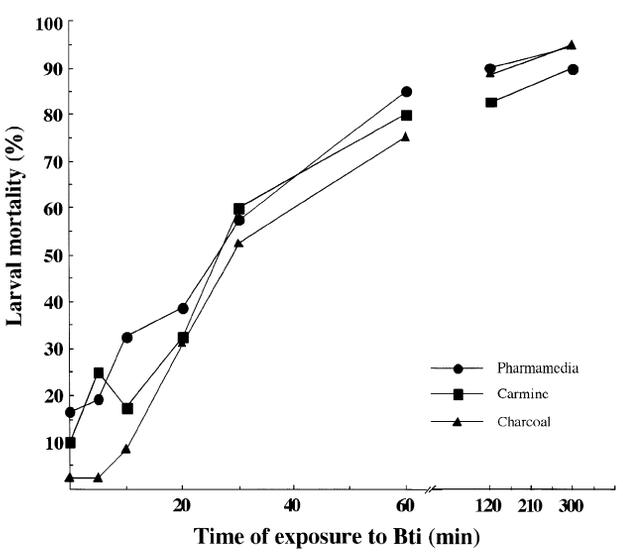

Fig. 1. *Mortality of* Aedes aegypti *larvae (third instar) exposed to* Bacillus thuringiensis *subsp.* israelensis *(35 ng/ml) for different times before adding the indicated particles (1 mg/ml)*

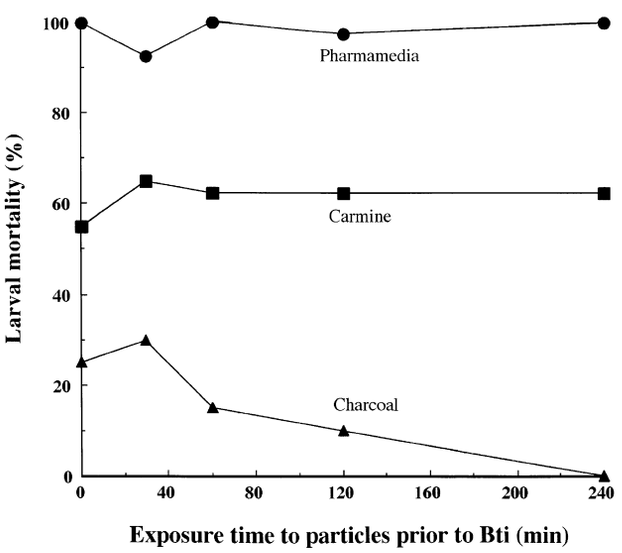

Fig. 3. *Mortality of* Aedes aegypti *larvae (third instar) exposed to the indicated particles (0.5 mg/ml) for different times before adding* Bacillus thuringiensis *subsp.* israelensis *(350 ng/ml)*

Table 1. *Colony-forming units of* Bacillus thuringiensis *subsp.* israelensis *in carcasses of* B. thuringiensis *subsp.* israelensis-*killed* Aedes aegypti *larvae*

Treatment	Without particles (14.8 ng/ml); mortality, 62 ± 4%*	With Pharmamedia (290 ng/ml); mortality, 65 ± 2%*	With charcoal (3217 ng/ml); mortality, 47 ± 5%*
CFU†	509 ± 49	956 ± 119	2837 ± 266
Ratio‡	1	1.9	5.6

* Percentage mortality represents average of three independent bioassays with their standard errors (each with 200 larvae) as described in Materials and Methods at indicated concentrations (LC_{50}) of *B. thuringiensis* subsp. *israelensis*.
† Colony forming units are average values of 30 individual larvae from three independent bioassays, with their standard errors.
‡ Relative to the value obtained without particles.

The LC_{50} of *B. thuringiensis* subsp. *israelensis* in standard bioassays, 14.8 (FL between 12.8 and 18.2) ng/ml, increased by about 20-fold to 290.1 (200.1–472.7) ng/ml in the presence of nutritious Pharmamedia, and by 220-fold to 3217.2 (2470.5–4776.3) ng/ml in non-nutritional charcoal.

The number of *B. thuringiensis* subsp. *israelensis* spores (509 per larva) recovered from killed larvae at the LC_{50} value right after death (table 1) increased by 1.9- and 5.6-fold in the presence of Pharmamedia (956 spores per larva) and charcoal (2837 spores per larva), respectively.

3.2 Ingested particles by *Spodoptera littoralis* larvae

The LC_{50} of *B. thuringiensis* subsp. *kenyae* to exposed neonate larvae on agar was 2.3-fold higher with artificial diet (293 ng/well) than without (127 ng/well) and 44-fold higher without both (6.7 ng, suspended in water and dried in the well) (table 2). Addition of charcoal to agar, with or without artificial diet, did not affect mortality, but in dried suspension of *B. thuringiensis* subsp. *kenyae* charcoal increased LC_{50} by 17-fold (116 ng/well) (table 2). Leaves of cotton (whether fresh or dry) that serve as natural food to larvae of *S. littoralis* reduced toxicity of *B. thuringiensis* subsp. *kenyae* to a lesser degree (LC_{50} of 76 and 52 ng/well, respectively).

The number of *B. thuringiensis* subsp. *kenyae* (*ca.* 500 spores per larva) recovered from killed or live larvae at the LC_{50} value when applied alone was 12- and 8.5-fold lower than in agar, whether with artificial diet (*ca.* 6000 spores per larva) or without (*ca.* 4400 spores per larva) (table 3).

Table 2. *LC_{50} of* Bacillus thuringiensis *subsp.* kenyae *to neonate larvae of* Spodoptera littoralis *with different diet compositions*

Diet/particles	LC_{50}, ng/well*
Agar + artificial diet	293.2 (164.7–557.3)
Agar + artificial diet + charcoal	286.8 (171.8–499.8)
Agar	127.5 (75.5–201.1)
Agar + charcoal	132.7 (76.6–213.7)
Water	6.7 (4.3–11.6)
Water + charcoal	115.7 (69.5–187.1)
Fresh leaves	76.1 (51.9–107.4)
Dry leaves	52.4 (28.6–84.7)

* LC_{50} expressed in nanograms of *B. thuringiensis* subsp. *kenyae* biomass per well andthe 95% fiducial limits (in parentheses).

4 Discussion

The results with *A. aegypti* larvae (figs 1 and 2) are consistent with previous conclusions that mortality caused by δ-endotoxins is reduced in the presence of particles (nutritional or non-nutritional) due to diluting the pathogen and feeding inhibition (e.g. McGaughey, 1976; Ramoska and Pacey, 1979; Gaugler and Molloy, 1980; Aly, 1983; Salama and Abdel-Razek, 1992), but cannot be fully explained by these explanations. The results are consistent with the hypothesis that ingested particles protect midgut epithelial cells from the larvicidal effect of δ-endotoxins by covering their surface and thus preventing toxin approach to the gut receptors. This phenomenon would be analogous to the natural protection by chitin and mucous layers (Chiang et al., 1986; Sampson and Gooday, 1998).

The higher *B. thuringiensis* subsp. *israelensis* toxicity in the presence of nutritious particles (Pharmamedia) than in charcoal or carmine (figs 2 and 3) confirms previous reports that ingestion rate is faster in the presence of food than with non-nutritive material (Dadd, 1968; Rashed and Mulla, 1989). Toxicity was inversely proportional to the concentrations of both types of particles, consistent with a positive correlation between particle concentration and feeding inhibition (Gaugler and Molloy, 1980). In addition, the rate of clearance is also proportional to ingestion rate: inert particles remain in the gut of mosquito larvae for longer periods of time (Dadd, 1970); hence, they might be considered better protectors than nutritional particles. Another possible explanation for the lower values of LC_{50} and number of *B. thuringiensis* subsp. *israelensis* spores recovered from killed larvae in the presence of nutritious particles is that they raise the gut content of trypsin-like proteases that activate *B. thuringiensis* subsp. *israelensis* protoxins. One cannot exclude, on the other hand, that gut proteases degrade the toxins and reduce toxicity (MacIntosh et al., 1990; Kondo et al., 1995).

It has been claimed (Dadd, 1968; Gaugler and Molloy, 1980) that non-nutritional particles such as carmine and charcoal, which have been extensively used for pathogen dilution and feeding inhibition, affect the physiological state of larvae (e.g. pH) to decrease *B. thuringiensis* toxicity. Particles such as latex beads that adsorb solubilized polypeptides have even been used to raise the toxicity of *B. thuringiensis* subsp. *israelensis* crystal proteins (Visser et al., 1986). The size and number per weight of particles affect larval mortality as well

Table 3. *Colony forming units (CFUs) of* Bacillus thuringiensis *subsp.* kenyae *in live and in carcasses of* B. thuringiensis *subsp.* kenyae-*killed* Spodoptera littoralis *larvae**

		Artificial diet in agar (293.2 ng/well)†		Agar (alone) (127.5 ng/well)†		Dried *B. thuringiensis kenyae* (6.7 ng/well)†	
Larvae	Time (h)	CFUs without HST	CFUs with HST	CFUs without HST	CFUs with HST	CFUs without HST	CFUs with HST
Live	0.5	610	600	530	500	110	100
	1	870	820	980	940	220	200
	2	2250	2100	2230	2200	590	560
	4	4410	4200	4080	3900	640	610
	6	5280	4800	4370	4200	690	620
	8	5880	5100	5380	5000	630	570
Dead	12	6150	5400	5680	5100	590	540
	16	6380	5500	4950	5000	510	440
	20	6180	5050	4760	4800	460	360
	24	5510	3600	4560	4700	490	280
	36	6130	2100	4360	3100	570	40
	48	6450	1500	5100	1700	600	13
	72	5950	26	2800	11	510	6

* Colony forming units are the average of three individual larvae.
† *B. thuringiensis* subsp. *kenyae* biomass concentration of LC_{50} added per well.
HST, heat shock treatment.

(Skovmand et al., 1997), and may explain the different reactions to charcoal and carmine (fig. 3).

The simplest explanation for increased number of *B. thuringiensis* subsp. *israelensis* spores recovered from killed larvae in the presence of particles (table 1) is that they physically protect the epithelial cells from toxin binding to their specific receptors. Non-nutritional particles are indeed known to cover the peritrophic membrane by an additional layer (see Dadd, 1968). Dissolution, dispersion and digestion of the nutritional particles can explain their lower protection.

The peritrophic membrane completely separates the food bolus from the intestinal epithelium and is usually permeable to molecules around 30–40 kDa. To reach their target, digestive enzymes must cross it and digestion products (including *B. thuringiensis* protoxins that are activated by trypsin-like proteases) must traverse this membrane back to reach the absorptive epithelium (Beaty and Marquardt, 1996). The peritrophic membrane has been reported to serve as a solid support to digestive enzymes: as food moves along the digestive tract, it is digested by these immobilized enzymes (Beaty and Marquardt, 1996). Rees, Jarret and Ellar (pers. comm.) demonstrated that *B. thuringiensis* toxins bind to a wide size range of proteins in the peritrophic membrane of several lepidopteran larvae, some of which may be external receptors for toxins to pass the peritrophic membrane. An additional layer of particles (whether nutritional or not) physically covering the peritrophic membrane may delay proteolytic processing of *B. thuringiensis* protoxins and traversing of the activated toxins to reach the absorptive epithelium as necessary for binding to their specific receptors.

Dilution and feeding inhibition by particles slow down ingestion rate of spores and toxins (Ramoska and Pacey, 1979; Gaugler and Molloy, 1980; Aly, 1983) to reach sublethal concentrations for extended periods, allowing the larvae enough time to repair and replace damaged midgut epithelial cells (Chiang et al., 1986). The concept that food particles increase the dose necessary to kill larvae by faster regeneration of cells damaged by δ-endotoxin (Khawaled et al., 1988) should still be tested.

LC_{50} of *B. thuringiensis* subsp. *israelensis* against third instar *A. aegypti* larvae increased by about 20-fold in the presence of nutritious Pharmamedia and by 220-fold in non-nutritional charcoal particles (see Results). On the other hand, LD (the number of *B. thuringiensis* subsp. *israelensis* spores recovered from killed larvae at the LC_{50} value right after death) increased by 1.9- and 5.6-fold only in the presence of Pharmamedia and charcoal, respectively (table 1). Dilution and feeding inhibition can explain the decreased toxicity (higher LC_{50}) in the presence of particles (figs 2 and 3; Ramoska and Pacey, 1979; Gaugler and Molloy, 1980; Aly, 1983), but the moderate increase of LD needs an additional explanation. We reckon that this increase in LD is caused by an additional layer of particles (irrespective of their nutritional value) covering the peritrophic membrane, which prevents or delays traversing of the activated toxins to midgut epithelial cells.

The lethal dose in the presence of activated charcoal (known as an excellent adsorbent) is about threefold higher than in the presence of Pharmamedia (table 1), most likely because charcoal adsorbs the toxin. This is supported by immediate reduction of mortality upon addition of charcoal, even when compared to addition of carmine (figs 2 and 3). This difference may be further affected by a difference in size and number per weight (Skovmand et al., 1997) between charcoal and carmine particles. Whatever the reason(s) is (are), the end result is that higher amounts of ingested toxin are required to kill mosquito larvae when particles are present.

These conclusions were not restricted to *B. thuringiensis* subsp. *israelensis* and mosquito larvae. They were

also derived from a series of similar experiments that were performed with larvae of the terrestrial (lepidopteran) species *S. littoralis* (tables 2 and 3). All four sorts of particles, artificial diet and cotton leaves (nutritious) as well as agar and charcoal (non-nutritious), serve as protectors, though at different levels (table 2).

The number of ingested *B. thuringiensis* subsp. *kenyae* spores and toxic crystals that killed a larva on agar, with artificial diet or without, was about 10-fold higher than on dried toxin preparation alone and proportional to the concentration of *B. thuringiensis* subsp. *kenyae* applied (table 3). Therefore, neither dilution of *B. thuringiensis* subsp. *kenyae* nor feeding inhibition by food or non-nutritious particles is sufficient to explain reduction of its toxicity (McGaughey, 1976; Salama and Abdel-Razek, 1992). Hence, we believe that ingested particles protect midgut epithelial cells from the larvicidal effect of *B. thuringiensis* δ-endotoxins by covering their surface and preventing toxin binding to their specific receptors. This finding opens up a new set of possible explanations for modified toxicity by particulate matter, which is based on physical properties of the ingested material.

Acknowledgements

This investigation was partially supported by a grant (No. 801-8) from the Israel Ministry of Environment and a grant (No. 97-00081) from the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel (A.Z. and S.B.), and a post-doctoral fellowship (E.B.-D.) from the Israel Ministry of Science. Part of this work was planned by Q.W., in 1996, during the visit of A.Z. to the Huazhong Agricultural University, Wuhan, P.R. China. The warm hospitality of Prof. Yu Ziniu and the financial support of UNESCO (under the MIRCEN Biotechnology Programme) are gratefully acknowledged. Yoel Margalith is gratefully acknowledged for free supply of *Aedes aegypti* eggs, and Amos Navon for *Spodoptera littoralis* larvae. Michael Friedlander was helpful in shaping the version of the manuscript.

References

Aly, C., 1983: Feeding behavior of *Aedes vexans* larvae (Diptera: Culicidae) and its influence on the effectiveness of *Bacillus thuringiensis* var. *israelensis*. Bull. Soc. Vector Ecol. **8,** 94–100.

Aly, C., 1988: Filter feeding of mosquito larvae (Diptera: Culicidae) in the presence of the bacterial pathogen *B. thuringiensis* var. *israelensis*. J. Appl. Entomol. **105,** 160–166.

de Barjac, H.; Frachon, E., 1990: Classification of *Bacillus thuringiensis* strains. Entomophaga **35,** 233–240.

Beaty, B. J.; Marquardt, W. C., 1996: The Biology of Disease Vectors. Niwot: University Press of Colorado.

Becker, N.; Zgomba, M.; Ludwig, M.; Petric, D.; Rettich, F., 1992: Factors influencing the activity of *Bacillus thuringiensis* var. *israelensis* treatments. J. Am. Mosq. Contr. Assoc. **8,** 285–289.

Chiang, A. S.; Yen, D. F.; Peng, W. K., 1986: Defense reaction of midgut epithelial cells in the rice moth larva (*Corcyra cephalonica*) infected with *Bacillus thuringiensis*. J. Invertebr. Pathol. **47,** 333–339.

Crickmore, N.; Zeigler, D. R.; Feitelson, J.; Schnepf, E.; Van Rie, J.; Lereclus, D.; Baum, J.; Dean, D. H., 1998: Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62,** 807–813.

Dadd, R. H., 1968: A method for comparing feeding rates in mosquito larvae. Mosq. News **28,** 226–230.

Dadd, R. H., 1970: Comparison of rates of ingestion of particulate solids by *Culex pipiens* larvae: phagostimulant effect of water-soluble yeast extract. Entomol. Exp. Appl. **13,** 407–419.

Daum, R. J., 1970: Revision of two computer programs for probit analysis. Bull. Entomol. Soc. Am. **16,** 10–15.

Feitelson, J. S.; Payne, J.; Kim, L., 1992: *Bacillus thuringiensis*: insects and beyond. Biotechnology **10,** 271–275.

Gaugler, R.; Molloy, D., 1980: Feeding inhibition in black fly larvae (Diptera: Simuliidae) and its effect on the pathogenicity of *Bacillus thuringiensis* var. *israelensis*. Environ. Entomol. **9,** 704–708.

Herbert, D. A.; Harper, J. D., 1987: Food consumption by *Heliothis zea* (Lepidoptera: Noctuidae) larvae intoxicated with ß-exotoxin of *Bacillus thuringiensis*. J. Econ. Entomol. **80,** 593–596.

Hofte, H.; Whiteley, H. R., 1989: Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53,** 242–255.

Hoy, C. W.; Hall, F. R., 1993: Feeding behavior of *Plutella xylostella* and *Leptinotarsa decemlineata* on leaves treated with *Bacillus thuringiensis* and esfenvalerate. Pest. Sci. **38,** 335–340.

Khawaled, K.; Barak, Z.; Zaritsky, A., 1988: Feeding behavior of *Aedes aegypti* larvae and toxicity of dispersed and of naturally encapsulated *Bacillus thuringiensis* var. *israelensis*. J. Invertebr. Pathol. **52,** 419–426.

Kondo, S.; Ohba, M.; Ishii, T., 1995: Comparative susceptibility of chironomid larvae (Dipt., Chironomidae) to *Bacillus thuringiensis* serovar *israelensis* with special reference to altered susceptibility due to food difference. J. Appl. Entomol. **119,** 123–125.

MacIntosh, S. S.; Kishore, G. M.; Perlak, F. J.; Marrone, P. G.; Stone, T. B.; Sims, S. R.; Fuchs, R. L., 1990: Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. J. Agric. Food Chem. **38,** 1145–1152.

McGaughey, W. H., 1976: *Bacillus thuringiensis* for controlling three species of moth in stored grain. Can. Entomol. **108,** 105–112.

Merritt, R. W.; Dadd, R. H.; Walker, E. D., 1992: Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. Annu. Rev. Entomol. **37,** 349–376.

Mulla, M. S., 1990: Activity, field efficacy and use of *Bacillus thuringiensis israelensis* against mosquitoes. Bacterial Control of Mosquitoes and Black Flies. Ed. by de Barjac, H. Sutherland, D. J. New Brunswick, NJ: Rutgers University Press, 134–160.

Mulla, M. S.; Darwazeh, A. H.; Zgomba, M., 1990: Effect of some environmental factors on the efficacy of *Bacillus sphaericus* 2362 and *Bacillus thuringiensis* (H-14) against mosquitoes. Bull. Soc. Vector Ecol. **15,** 166–176.

Porter, A. G.; Davidson, E. W.; Liu, J.-W., 1993: Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol. Rev. **57,** 838–861.

Ramoska, W. A.; Pacey, C., 1979: Food availability and period of exposure as factors of *Bacillus sphaericus* efficacy on mosquito larvae. J. Econ. Entomol. **72,** 523–525.

Rashed, S. S.; Mulla, M. S., 1989: Factors influencing ingestion of particulate materials by mosquito larvae (Diptera: Culicidae). J. Med. Entomol. **26,** 210–216.

Regev, A.; Keller, M.; Strizhov, N.; Sneh, B.; Prudovsky, E.; Chet, I.; Ginzberg, I.; Koncz-Kalman, Z.; Koncz, C.; Schell, J.; Zilberstein, A., 1996: Synergistic activity of a *Bacillus thuringiensis* δ-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl. Environ. Microbiol. **62,** 3581–3586.

Salama, H. S.; Abdel-Razek, A., 1992: Effect of different kinds of food on susceptibility of some stored products insects to *Bacillus thuringiensis*. J. Appl. Entomol. **113,** 107–110.

Sampson, M. N.; Gooday, G. W., 1998: Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. Microbiology **144,** 2189–2194.

Skovmand, O.; Hoegh, D.; Pedersen, H. S.; Rasmussen, T., 1997: Parameters influencing potency of *Bacillus thuringiensis* var. *israelensis* products. J. Econ. Entomol. **90,** 361–369.

Sneh, B.; Schuster, S.; Gross, S., 1983: Improvement of the insecticidal activity of *Bacillus thuringiensis* var. *entomocidus* on *Spodoptera littoralis* (Lepidoptera, Noctuidae) by addition of chitinolytic bacteria, a phagostimulant and a UV-protectant. Z. Angew. Entomol. **96,** 77–83.

Visser, B.; van Workum, M.; Dullemans, A.; Waalwijk, C., 1986: The mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis* is associated with Mr 230 000 and 130 000 crystal proteins. FEMS Microbiol. Lett., **30,** 211–214.

Wiwat, C.; Lertcanawanichakul, M.; Siwayapram, P.; Pantuwatana, S.; Bhumiratana, A., 1996: Expression of chitinase-encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* in *Bacillus thuringiensis* subsp. *israelensis*. Gene **179,** 119–126.

Authors' addresses: Eitan Ben-Dov (corresponding author), R. Manasherob, A. Zaritsky and Q. Wang, Department of Life Sciences, Ben-Gurion University of the Negev, PO Box 653, Be'er-Sheva 84105, Israel; S. Boussiba, Department of Dryland Biotechnologies, Microalgal Biotechnology Laboratory, The Blaustein Institute for Desert Research, Ben-Gurion University of the Negev at Sede Boqer 84990, Israel; D. Saxena, The Research Institute, The College of Judea and Samaria, Ariel 44837, Israel. E-mail: ariehz@bgumail.bgu.ac.il