Journal of Industrial Microbiology & Biotechnology (1997) 18, 370–378

Quantitative bioreduction assays for calibrating spore content and viability of commercial *Bacillus thuringiensis* insecticides

VL Seligy[1,2], RW Beggs[1,2], JM Rancourt[1] and AF Tayabali[1,2]

[1]*Environmental and Occupational Toxicology Division, Environmental Health Centre, Department of Health Canada;*
[2]*Environmental Chemistry and Toxicology Program, Biology Department, Carleton University, Ottawa, Canada*

The redox dyes MTT (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide, thiazolyl blue) and XTT (sodium 3′- {1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis{4-methoxy-6-nitro} benzene sulphonic acid hydrate) were used as dosimetry reporters in liquid (multi-well) and solid support (membrane) assays to estimate spore viability and content of commercial BT products derived from fermentation of *Bacillus thuringiensis* subsp *kurstaki (Btk)* and subsp *israelensis (Bti)*. QC tests on five BT products were done using spore, protein and gene contents, and morphology (scanning electron microscopy) as indicators. Spore levels (6–40 $\times$ 10^9 colony forming units (CFU) ml^{-1}) were approximately equivalent when based on International Units (IU) of potency. Spore viability was highly stable over a broad range of temperatures and pHs but germination and growth were restricted (optima: pH $\approx$ 7.5 and 37°C). Quantitative bioreduction activity (QBA) of MTT and XTT correlated with vegetative cell production. Depending on manipulation of pre-assay conditions, both dyes could discriminate doses from ~2 to 10^9 spores (or 10^{-3} to 10^6 IU). Non-toxic effects of XTT and its formazan product enabled automated collection of data on growth and dose. Solid support assays also reliably estimated product dosage by *in situ* detection of CFU. With appropriate reference dilutions of microbe-containing products the QBA assays can provide high throughput QC monitoring of product comparisons and field release in aerial spray or water injection applications.

Keywords: biopesticides; gentamicin; MTT; PCR; SDS-PAGE; tetrazolium; XTT

Introduction

Most commercial BT insecticides in global use are essentially fermentation concentrates of the Gram-positive, spore-forming *Bacillus thuringiensis* subspecies *kurstaki (Btk)* and subspecies *israelensis (Bti)* [1,4,7,25,27]. As such, these crude, water-based formulations contain proprietary additives, traces of growth medium, cellular debris and metabolites, and two major sporulation products, the spore and parasporal inclusion body (PIB). The PIB, whether intact as an aggregated crystalloid or partly assembled (or degraded), is the primary source of various pro-δ-endotoxins and their derivatives [3,12]. From the point of view of BT product efficacy in the field, the spores are important in the toxification of insects [2,4,8,9,14,18,19,23,28–31]. However, clarification of their pathogenesis has been largely ignored in favour of exploiting the δ-endotoxins, particularly their engineering and use in transgenic plants [3,6,12,16]. Information on spores in BT products is germane to furthering the understanding of several issues [1,27]: (a) the long-term, environmental impact due to large-scale, localized release of 'commercialized' microbe species; (b) the spore's role in target (and non-target) animal cell toxification; and (c) development of effective, target species-specific, biological control products.

We are primarily interested in BT products as surrogates for the development of large-scale environmental assessment methodologies and the collection of data relating to release, exposure and persistence of microbe-containing biotechnology products (MBPs). Development of standardized, inexpensive and rapid quantification methods for monitoring QC and large-scale release of commercial MBPs like BT depends heavily on details about individual products which are largely unavailable because of proprietary controls. At present, several strategies have been used to assess spray deposits based on one or more of the bioconstituents (spores, crystalloid structures of intact PIBs, δ-endotoxins polypeptides) or formulation additives and tracer dyes [1,11,17,25]. As an assessment priority, release of high levels of spores has the greatest potential for environmental impact because of their hardiness, growth properties (this study) and genetic relatedness to known animal pathogens [1,5,26,27]. In the present study we report on the spore properties of several BT products including germination-growth-responsive changes in electron transport (bioreduction) activity. Based on these analyses, two types of relatively simple assays are described which offer considerable potential for use in semi-automated QC analysis and field monitoring of MBP deposition and exposure.

Correspondence: Dr VL Seligy, Mutagenesis Section, Environmental Health Centre, Health Canada, Tunney's Pasture, Ottawa, Canada K1A 0L2
Received 18 December 1996; accepted 11 March 1997

Materials and methods

Sampling methods and characterization of BT products

Products tested are listed in Table 1. Great care was given to making accurate product dilutions by extensive mixing to ensure homogeneity of aliquots. Diluents included filter-sterilized deionized distilled water, standard bacterial growth medium (LB at pH 7.4, Gibco/BRL, Burlington,

Table 1 Characteristics of commercial BT products used in this analysis

Commercial product (supplier)	Formula type IU ml^{-1}	Subspecies and strain of *B. thuringiensis*	Solids % product (v/v)[b]	Spores ml^{-1} $\times 10^9$ (spores IU^{-1})	Major polypeptides, kilo Daltons	Gene detection by PCR[c] Cry/Ribosome
[a]Dipel (DIPel*WP, Abbott, Quebec)	Powder 4.2×10^6	*kurstaki*, HD1	22	5.9 ± 1.3 (~1400)	133–137, 63–67	1Ab + / 16S + 1Ac +
Foray 48B (Novo /Abbott)	Liquid 12.7×10^6	*kurstaki*, HD1	24	31.5 ± 7.0 (~2480)	133–137, 63–67	1Ab + / 16S + 1Ac +
Foray 76B (Novo /Abbott)	Liquid 20×10^6	*kurstaki*, HD1	26	39.8 ± 8.7 (~2460)	133–137, 63–67	1Ab + / 16S + 1Ac +
Thuricide (CIL, Laval, Quebec)	Liquid 4.2×10^6	*kurstaki*, HD1	20	10.0 ± 2.1 (~2380)	133–137, 63–67	1Ab + / 16S + 1Ac +
Vectobac 12AS (Abbott)	Liquid	*israelensis*, HD14	24	29.4 ± 7.6 (–)	134, 78, 28	ND / 16S + ND

[a]Liquid equivalent made up as described in [28].
[b]Pellet volume measured after 25 000 × *g*, 60 min.
[c] + = Presence of diagnostic PCR fragments (see Figure 1C). ND = not detected. All other assays are described in Methods.

Ont, Canada) and SE (0.01% SDS–0.1 mM ETDA, pH 8.0). Growth medium was made with/without 50 μg ml^{-1} gentamicin (Gibco/BRL). Other media included: Grace's insect medium [28] and human cell culture medium (Dulbeco's MEM, Gibco/BRL).

Putative contents (spores, vegetative cells, proteins and DNA) of BT products were examined after various periods of incubation in diluents by a variety of techniques: light and scanning electron microscopy (SEM), differential centrifugation, protein and nucleic acid biochemistry and plate counts. SEM was conducted using a JSM6400 (Joel, Boston, MA, USA) and gold-shadowed fresh mounts on aluminum foil. Spores and PIB structures were partitioned from the liquid phase of the commercial formulations by centrifugation (horizontal microfuge at 12 500 × *g*, 15 min or preparative centrifuge, Beckman SW50.1 rotor at 25 000 × *g*, 30–60 min). Pellets were washed three times with 10 volumes of sterile water or TE (10 mM Tris-HCl and 1 mM EDTA, pH 9.5) and reconstituted to the original product volume by mixing them extensively with water or buffer.

Extraction of PIB proteins from the spore-rich, TE-washed pellets was done by mixing the pellets in 0.2% SDS and 0.5% proteinase K (Sigma, St Louis, MO, USA), followed by an incubation (12 h, 37°C with mixing) and repeated washing of the recovered pellets in TE and water. Detection of toxin (*cryl*Ab and *cryl*Ac) and 16S ribosomal genes [28; V Seligy, manuscript in preparation] was performed by polymerase chain reaction (PCR) using product dilutions, single colony forming units (CFU) and extracted cellular DNA. Thermocycler (Model 9600) and reagents were obtained from Perkin-Elmer (Mississauga, Ont, Canada). Quantity and quality of δ-endotoxin proteins, the 132- and 67-kDa polypeptides associated with BT products, were assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [28]).

Total bacteria (spores) and spores IU^{-1} of product (*Btk* products) or equivalent based on CFU as in the case of *Bti* product, were deduced by scoring CFU after spread-plating of linear or Log_{10} serial dilutions (50- to 100-μl aliquots) on agar plates containing LB medium (Gibco/BRL). Alter-

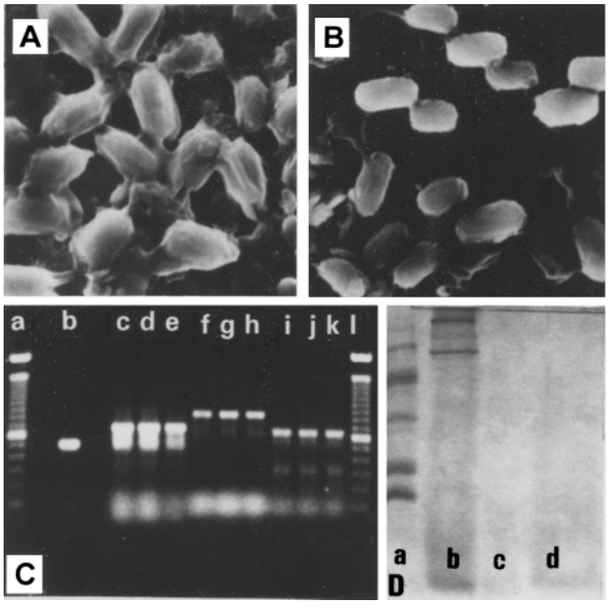

Figure 1 Quality analysis of commercial BT products. Example is Foray 48B®. (A,B) SEM of 10^{-4} product dilution before (A) and after (B) protease treatment showing spores as the major component. Spores are approximately 1.2 μm long. (C) Ethidium bromide-stained agarose gel (1.5%) electrophoretic patterns of DNA markers (100-bp ladder, lanes, a, l) and PCR products from λ DNA control (0.56 kbp, lane b) and BT product: total untreated (lanes c, f, i) and washed (12 500 × *g* pellets) before (lanes d, g, j) and after protease digestion (lanes e, h, k) using primers to identify δ-endotoxin genes (*cry*1Ab, 0.65 kbp, lanes c–e; *cry*1Ac, 0.83 kbp, lanes f–h) and 16S rDNA (0.6 kbp, lanes i–k). (D) Coomassie blue-stained 12% polyacrylamide gel of electrophoretically separated high-low molecular weight markers (left, lane a) and total BT product (3000 IU) before (lane b) and after (lane d) protease treatment (see B) and 12 500 × *g* 15 min supernatant of total product (lane c). Marker proteins in kDa are: myosin (214), phosphorylase B (111), bovine serum albumin (68), ovalbumin (45), α-chymotrypsinogen (24), β-lactoglobin (18) and lysozyme. Further details are given in Methods.

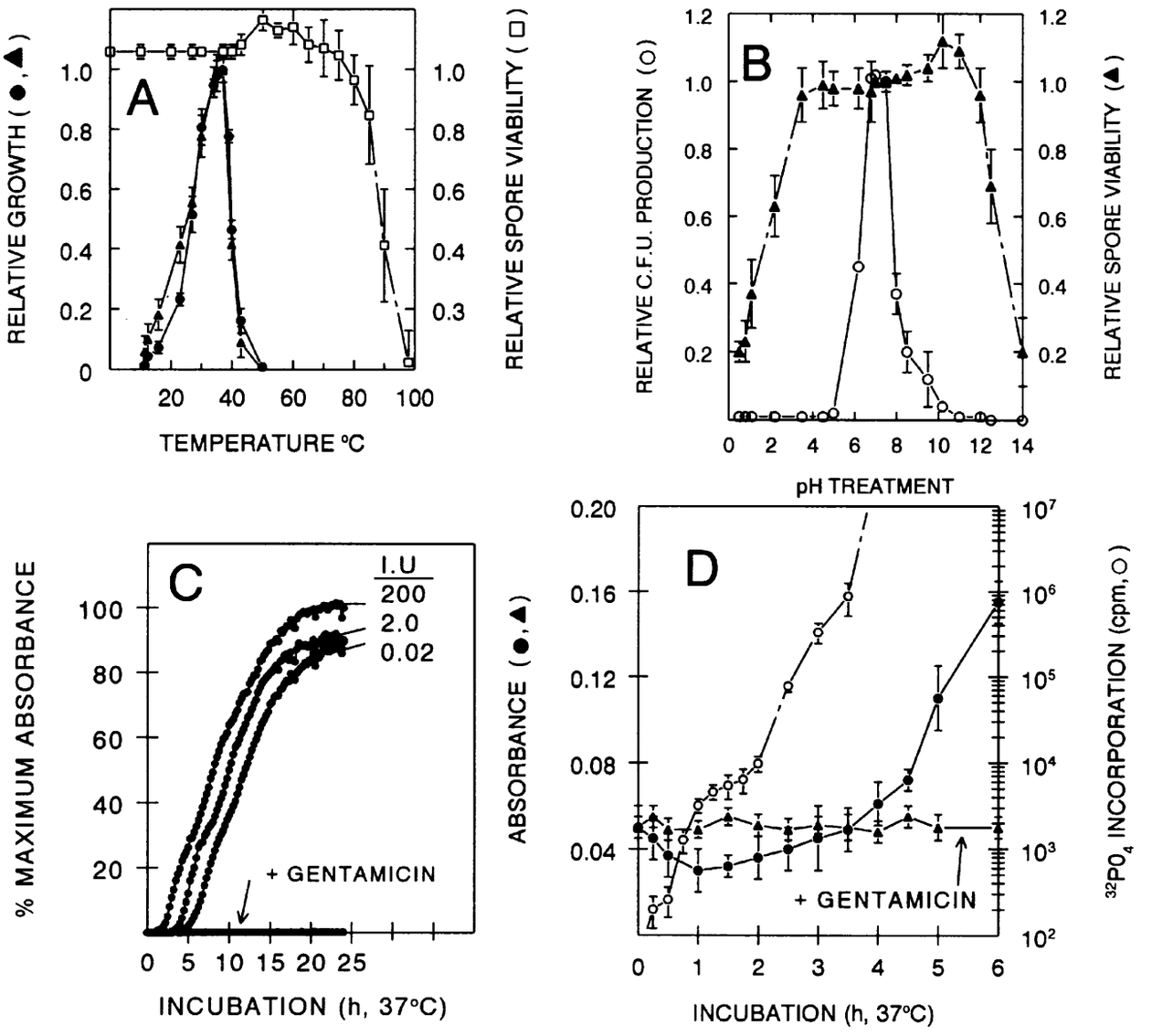

Figure 2 Properties of spore component of BT products. (A) Effect of temperature on *Btk* growth and spore viability. Growth was determined over a 36-h period by measuring increase in colony size (▲) or optical density at 450 nm (●), at 37°C, using BT product at 0.2 IU and 200 IU, respectively. Spore viability was determined by pre-treatment of a suspension containing 0.2 IU for 1 h at different temperatures before plating, and incubating the plates at 37°C. (B) Effect of pH on spore viability and growth rate, measured by plate assay, relative to pH 7.0, using 0.2 IU at 37°C. (C) Viable spore content of BT product illustrated by use of an automated microtitreplate spore germination-growth assay monitored at 450 nm. (D) Spore activation, germination and growth as determined by monitoring ^{32}-PO_4 incorporation and optical density at 450 nm. See Methods for details on radiolabelling of cells.

natively, spore contents were determined by trapping (10- to 100-μl samples per 10 ml of SE) on sterile membranes (0.45-μm pore size, 4.5 cm diameter or 10 cm × 13.2 cm, Zeta probe, Biorad, Mississauga, Ont, Canada) and incubation on LB agar plates or in sterile multiblot trays (8.5 cm × 12.5 cm, Robbins Scientific) containing either a layer of LB agar or an insert of LB-soaked gel blot paper (GB002, Schleicher & Schuell, Keene, NH, USA). Tests for spore viability were conducted by obtaining CFU number (spread plate) or computer monitoring of growth profiles (at 480 nm or 600 nm) over time (0–24 h) after exposure to a range of temperatures (–80, –20 and 4–100°C), pHs (0.5–12) and freeze-thaw cycles (40, 30-min cycles of –40 to +50°C). Temporal activity (germination and growth) was examined at different temperatures (4–100°C), nutrient medium concentrations (0, 0.01, 0.1, 1 and 2×) and pHs (3–12), using programmable incubators (Kloolatron chamber, VWR Scientific hybridization oven, London, Ont, Canada; PCR thermocycler and microwell plate reader, Spectramax 250, Molecular Dynamics, Sunnyvale, CA, USA). The pH was adjusted using stocks of HCl, H_3PO_4, H_2NaPO_4, HNa_2PO_4 and NaOH. Microassays of growth profiles for dose-time experiments were computer-monitored. Product dilutions or spores (1–10^9) were dispensed by multichannel pipette as 10- or 20-μl aliquots into 90 or 190 μl of medium, respectively. Controls consisted of diluent and medium with/without BT product and antibiotic to block growth [26,28]. In some cases ^{32}P-PO_4 (1 μ Ci ml^{-1}, Amersham, Oakville, Ont, Canada) was used to determine uptake into 5% TCA-insoluble cellular products. Aliquots (10 μl) were

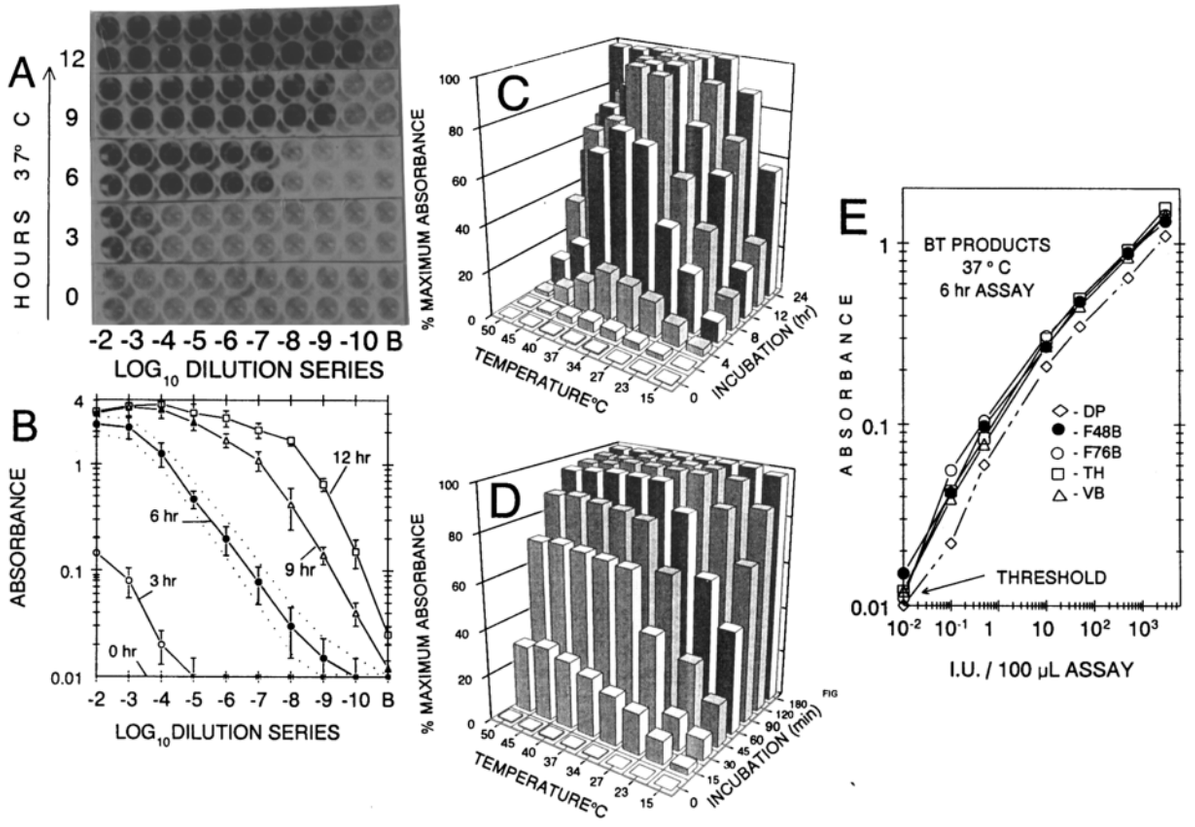

Figure 3 Optimization of MTT-based bioreduction assay of BT products. (A) Formazan produced after 2.4 mM MTT was added (2 h, 37°C) to product dilutions pre-incubated in 100 μl LB for 0–12 h at 37°C. (B) Absorbance readings (490 nm) are averages of those shown in (A) and four other experiments. Shaded area highlights semi-linear response at 6 h. (C) Effect of pre-incubation (12.7 IU product) time and temperature on MTT-formazan produced in a 2-h assay at 37°C. Maximum absorbance occurred with a 12-h pre-incubation. (D) Optimization of formazan produced from MTT relative to the 120-min point at 37°C. (E) Bioreduction based on product IU specified by manufacturer (DP, Dipel; F, Foray and TH, Thuricide). The VB (Vectobac) equivalent was based on 2400 CFU ≈ IU. MTT was added after a 6-h pre-incubation. Data points are the means of three separate experiments.

trapped on nylon membranes and counted using a scintillation spectrophotometer (Beckman Inst, LS3800, Fullerton, CA, USA).

Tetrazolium-based bioassays

Stocks of ≤24 mM MTT (MW 414.3), ≤1.62 mM PMS (MW 306.3) and ≤7.42 mM XTT (MW 673.5) were made with reagents (Sigma) dissolved in sterile PBS, growth medium or water, depending on usage. Scanning spectrophotometry (Beckman DU70 and Spectramax 250) was used to evaluate wavelength optima for monitoring formazan products. Preliminary tests indicated that the absorption spectra of formazan made by *Btk* and *Bti* cells were very similar. Formazan production was optimized by addition of different concentrations of MTT or XTT (± PMS) and incubation at 23–45°C. BT dose-dependent assays were done using dilutions of BT product added at various times (up to 24 h) prior to start of redox assays (time = 0). At *t* = 0, 10 or 20 μl of various stock dilutions of MTT or XTT were added to each well, followed by a 0- to 5-h incubation to assess analytic sensitivity. Effects of solubilization of MTT-formazan associated with *Btk* and *Bti* cells was tested by mixing one sample volume of dimethyl sulfoxide (DMSO) or 20% SDS and 50% dimethylformamide pH 4.7 [10], directly to samples or to pellets recovered by centrifugation (12 500 × *g*, 10 min). After addition of solubilizers, microtitre plates were agitated intermittently over a 5-min interval prior to measuring absorbance. Absorbance measurements were taken at <3.2, the upper limit of reliability of the detector. Samples with readings >3.2 were assessed by making appropriate dilutions. Background readings were taken before addition of redox dye. Assays were repeated at least three times per experiment and processed using Softmax Pro (Molecular Dynamics) and Excel 5.0 (Microsoft) software.

Results and discussion

Characterization of BT commercial products

Information on exact contents of commercial BT products is generally not available. This applies particularly to spore content. Some practical QC analysis of individual products is given in Table 1. Comparison of data for all *Btk* formulations showed that they had similar CFU contents based on number of IU specified by manufacturers. Also the *Bti*-based product (Vectobac) was similar in CFU content. Heat treatments (viable counts after 20 min at 37°C *vs* 80°C) indicated that the CFU content originated from spores (>99.9%) and not vegetative cells. This was confirmed by examination of 100× to 10 000× SEM images of air-dried

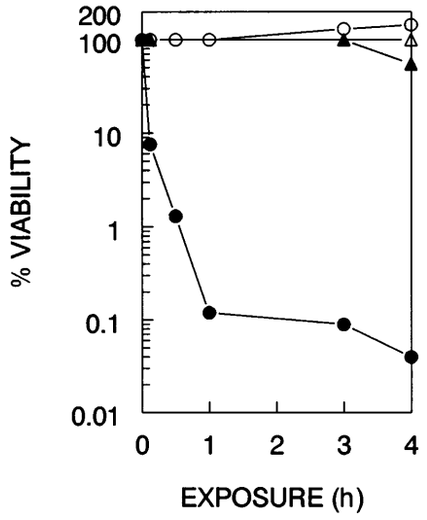

Figure 4 MTT toxification of spores (△,▲) and vegetative cells (○,●). Aliquots of 0.2 IU BT product were incubated with the addition of 2.4 mM MTT (closed symbols) or without MTT (open symbols) before diluting and plating the suspension on LB agar plates. Vegetative growing cells were derived by pre-incubating 0.2 IU of product for 4.5 h at 37°C. Per cent viability (CFU after 24 h) was based on control values at zero time, just prior to MTT addition. Data are the means of three experiments and are not adjusted for vegetative cell increases observed in the 4-h control incubations with no MTT.

products (0.5–5 μl) at dilutions from 0 to 10^{-6}. Product spreads at dilutions of $\leq 10^{-4}$ were found to be generally free of aggregates that consisted mainly of compact, rod-shaped spores (~0.75 μm wide × 1.2 μm long) (Figure 1A). The spores were the only structures remaining after proteinase K digestion and washes (Figure 1B). Authenticity of *Btk*-containing products was confirmed by predicted size of PCR-generated products [28], using boiled product and pellets recovered after washes with/without protease treatment (Figure 1C). As expected, only the 16s rRNA PCR probe was positive for *Bti*-based Vectobac product. Some product-specific differences in pellet sublayers were apparent before and after protease treatment, depending on conditions used for centrifugation. At 25 000 × *g* (60 min), supernatants from all products were free of spores, granules of any size and protein (Figure 1D, lanes a and b). Protease treatment reduced the pellet volume by ~50% which could be accounted for in part by spore loss (<20%) and practically all other components such as various sized amorphous granules, the characteristic bipyrimidal-shaped PIB crystalloid structures (~0.6 μm × 1.4 μm) and polypeptides ranging from ~60 kDa to 137 kDa (Figure 1D, lanes a and c). Treatments with alkaline buffers and proteases are used to solubilize and process pro-δ-endotoxins [16,31].

Growth properties of spores from BT products

All BT products examined here were highly concentrated. Polypeptide and viable spore content did not change over a 4-year period of intermittent testing (data not shown). Results shown in Figure 2A and B indicate that the spores were viable following exposure to a broad range of temperatures (–80 to +90°C) and pH (0.5–12). However, conditions for germination and growth were restricted. Growth was optimal at 37 ± 2°C and pH = 7.5 ± 1.0. The 5–10%

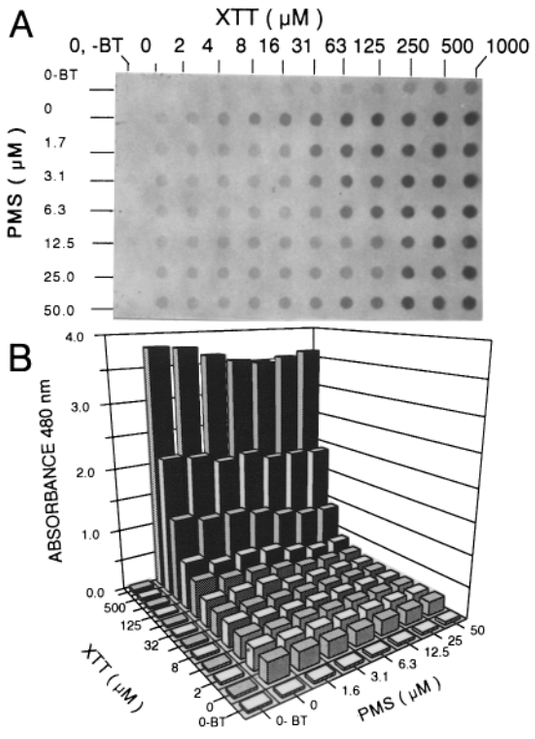

Figure 5 Effects of XTT and PMS electron donor concentrations on BT-bioreduction. The assay demonstrated here was one of three conducted using a 96-well plate with and without (0-BT) product at 10^{-4} dilution in LB at 37°C. After 4 h, XTT was added and incubated for 1 h before membrane entrapment of cells. (A) Photograph of membrane filter with entrapped cells revealed by associated formazan. (B) Quantification of XTT-formazan by sampling at 480 nm.

increase in CFU count at high temperature (50–80°C) and pH (8–12) may be related to increased efficiency of spore activation [31]. Biomass production in insect cell culture medium was higher than in LB broth by ~30% (data not shown). However, productivity was zero once these media were diluted to ≤0.1×. Continuous monitoring of growth with a computerized, thermally regulated, multi-well plate reader demonstrated that spores of a given product dose germinated and grew more-or-less in phase. Regularity of the dose-dependent, time-lag differences (growth curve displacements), shown in Figure 2C, indicate that initial spore content (CFU) or IU of a given dose can be approximated. As a baseline we used gentamicin (50 μg ml^{-1}) to inhibit growth [26,28]. Although not shown, the relative displacement of the growth curves of *Btk* and *Bti* products at comparable dilutions were consistent with the difference in CFU ml^{-1} estimated by plating (Table 1). For all products, little or no growth activity was recorded if CFU content per microwell assay was ≥5 × 10^9. The earliest changes in absorbance readings (over the range from 450 to 600 nm) occurred at about the same time as uptake of $^{32}PO_4$ into TCA insoluble product (Figure 2D). The initial drop in absorbance which began between 0.3 to 0.8 h is considered an indicator of spore activation [31].

Bioreduction activity measurements using MTT and XTT liquid assays

Both MTT and XTT have been tested against a variety of microbial species [10,13,24] but not using methods

reported here or with either laboratory strains of *Bacillus thuringiensis* or spores of actual commercial BT products. In an earlier study involving toxification of insect cells by BT products, MTT and XTT were reduced by the vegetative cells arising from spore germination but the spores themselves had little or no reducing capability [28]. Further investigations were directed at the possible use of these dyes in assays to provide information on spore activation and to facilitate a rapid calibration of BT products in terms of spore viability and content.

A battery of tests established that 1.0–2.4 mM MTT was sufficient to detect bioreducing activity by vegetative cells generated from any BT product dose after an appropriate preincubation from 2 to 16 h using either animal cell or bacterial (LB) growth medium. The media alone did not generate any appreciable background reduction of MTT. As shown in Figure 3 (A–C), MTT-formazan production essentially mirrored the vegetative cell activity data (Figure 2C and D). Near-linear dose responses could be obtained over several orders of magnitude of product dilution by manipulating duration (Figure 3B) and temperature (Figure 3C) at which the pre-assay incubation was conducted. However, similar variation of assay conditions did not affect the end-point of MTT reduction (Figure 3D). At temperatures >34°C the bioreduction was essentially complete by ~60 min. Data in Figure 3E demonstrate the marked similarity in bioreduction capability of various BT products when compared at equivalent IU by an assay which combined a 6-h pre-incubation to enable initiation of vegetative cell production and a 1-h MTT exposure. The reproducibility of this assay depended on making accurate product dilutions and also controlling cell duplication while MTT conversion to formazan took place. The results of experiments summarized in Figure 4 established that spores did not germinate in the presence of MTT and that their toxification was relatively low in comparison to vegetative cells. With a tLD_{50} of ~10 min, vegetative cell proliferation was terminated rapidly and therefore would not enhance the level of formazan produced within the 60- to 120-min assay period as in the case of XTT (*vide infra*). MTT-formazan crystal deposits within cells and membranes probably cause lethality. For animal cells, these deposits are refractive and require solubilization prior to quantification of the formazan deposits [10,15,20,28]. However, in the case of *Btk* or *Bti*, semi-quantification of MTT-formazan by the plate reader could be done with a moderate loss in efficiency (<30%), if we used 530 nm as the detection wavelength (data not shown).

In comparison to MTT, XTT and its water-soluble formazan product are non-toxic to a variety of organisms [20,21,24]. However efficient metabolism of XTT may sometimes require an electron coupler such as phenazine methosulphate (PMS). In our studies, no loss in viability was observed in plating and liquid growth assays when either *Btk* or *Bti* spores (or vegetative cells) were incubated in the presence of XTT and XTT-formazan. The optimization of XTT and PMS concentration requirements revealed that only XTT was important. Sensitivity increased with XTT concentration but background reduction was substrate-dependent and could be a serious problem depending on the duration of the assay and medium used [20]. For this reason brain heart infusion broth was not useful unless XTT was ⩽500 μM. Such background effects can be circumvented by monitoring the bound formazan of vegetative cells harvested from the liquid assays by a multiwell membrane filtration apparatus. This is shown for a XTT and PMS optimization study in Figure 5A and B. The lack of cell inhibition by XTT limits its use in obtaining accurate

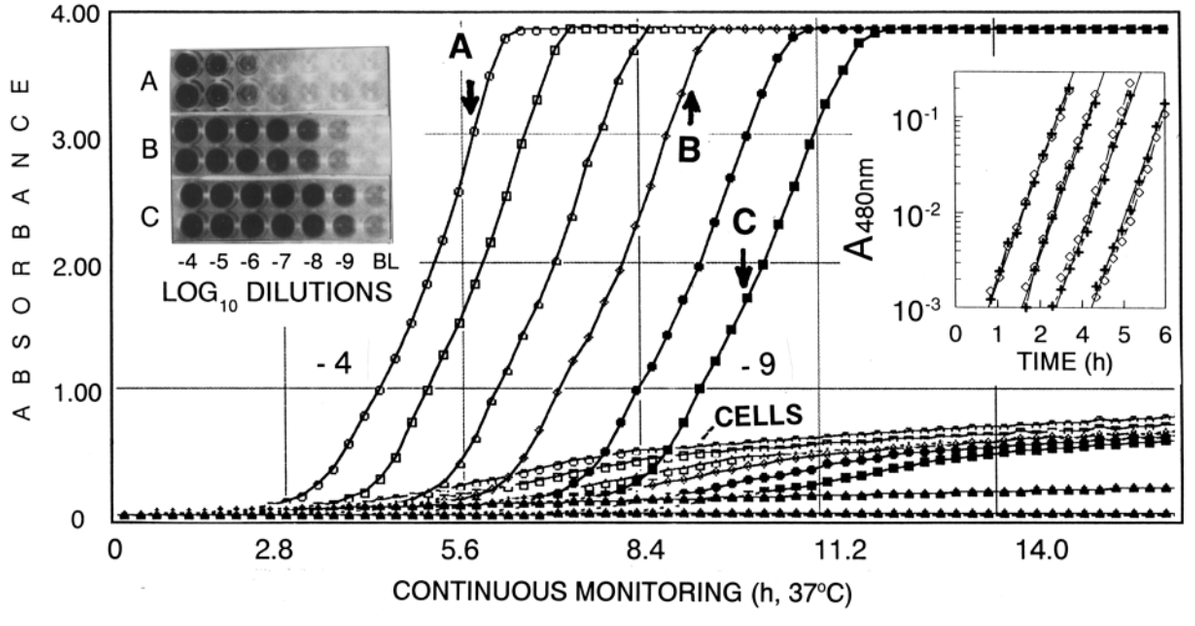

Figure 6 Computer monitoring of XTT-formazan and vegetative cell production from BT-product. The collection of readings resulting from spore activation and vegetative cell production at 37°C (log_{10} product dilution series). Letters A, B and C with arrows show intervals when the 96-well plate assay was photographed (left inset). The assay was conducted in triplicate but not all replicas are shown. The right inset shows part of the high-resolution scale results of formazan production after backgrounds (cells and blanks) were subtracted.

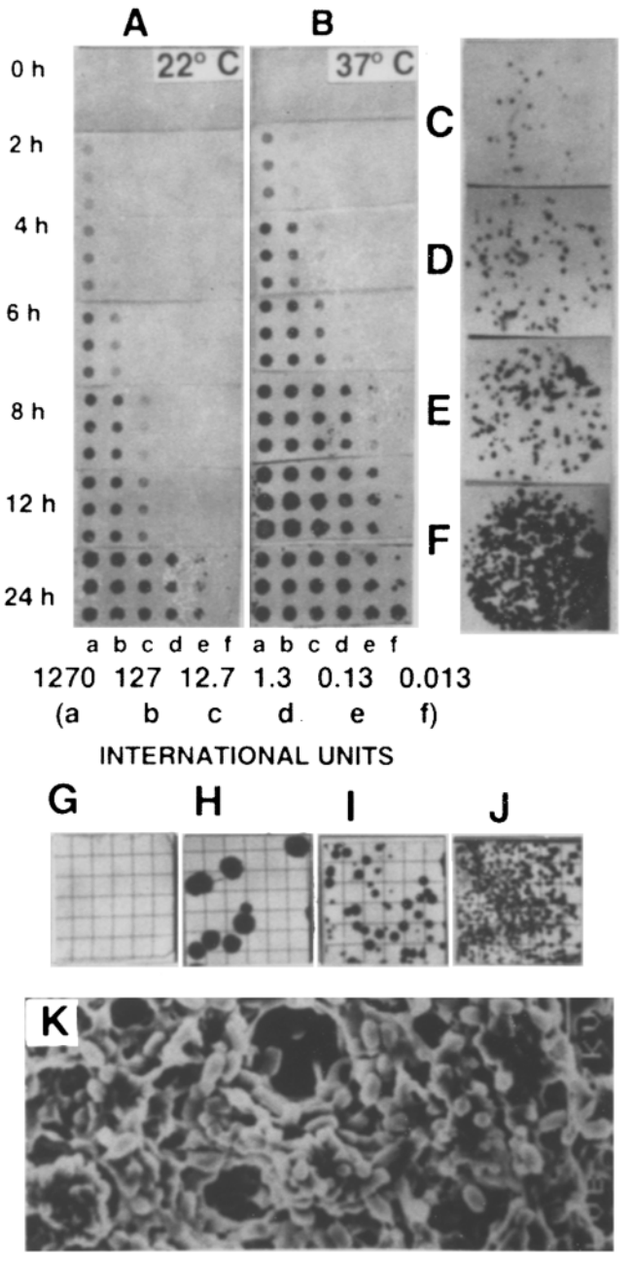

Figure 7 Solid support *in situ* MTT-bioreduction. (A, B) MTT-formazan production from membrane-trapped dilutions of 0.013–1270 IU. Triplicate dilutions (100 μl LB medium well^{-1}) were filtered through sterile membranes and subsequently incubated on LB-soaked absorbent paper at 22°C or 37°C for various times before placing them onto MTT-soaked absorbent paper for 30 min at 37°C. (C–F) Formazan deposits of putative colonies from 0.13, 1.3, 12.7 and 127 IU dilutions, respectively, after 6 h pre-incubation at 37°C. (G) Aliquot of 1270 IU filtrate incubated for 24 h before MTT assay showing no leakage of spores occurred. (H–J) Membranes with grids indicating colony sizes detected by formazan after pre-incubating for 24 h (0.01 IU), 12 h (0.20 IU) and 8 h (10 IU). (K) SEM image of a membrane containing 1270 IU (~3×10^6 spores) of trapped product. Spores are approximately 1.2 μm long.

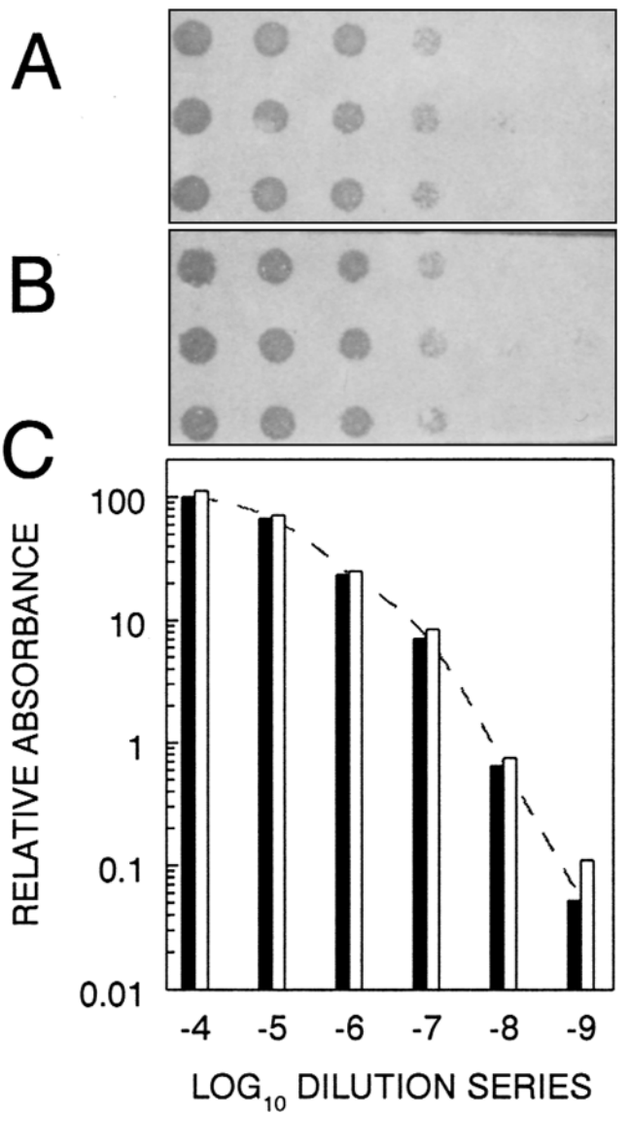

Figure 8 Formazan production from immobilized BT product spores. Membrane assays were conducted at 37°C in triplicate and incubated for 6 h on an absorbent support containing LB broth without (A) or with 500 μM XTT (B). After 6 h, membrane A was placed on a similar XTT-soaked paper and incubated for 2 h. Both membranes were then dried, photographed and scanned to quantify production of formazan (C) relative to that of the 10^{-4} dilution in experiment A.

intermediate growth measurements such as in Figure 3 for MTT. However, with continuous monitoring results such as those shown in Figure 6 can be obtained. In these dose-dependent assays, XTT is present at all stages of the assay and formazan build-up as well as cell growth can be measured more-or-less simultaneously. The contribution to turbidity from cells alone is relatively minor and can be corrected too, if need be, by appropriate controls (cultures without XTT). The XTT-formazan data in Figure 6 reveal sharp, growth-dependent sigmoidal curves for each spore concentration (dose) which are not easily apparent from visual observation at any single time-point (left inset). The bioreduction profiles resolved at high resolution (right inset) are each offset by ~45 min for successive $\log_{10}$ dilutions which makes it possible to estimate the initial dose. The XTT-formazan sigmoidal curves resemble those for cell accumulation (Figures 6 and 2C) but are at least five times more sensitive. This XTT assay format has not

been reported previously for use with other microorganisms, possibly because appropriate equipment was not available. Preliminary comparison of inhibitory concentrations (MIC) of antibiotic using bioreduction and growth profiles as indicators shows that the bioreduction profiles generated from *Btk* and *Bti* spore inoculation are very distinct in comparison to other bacterial species such as *E. coli* [22].

Formazan production from solid support assays

The use of MTT and XTT as possible *in situ* stains for early detection and quantification of *Btk* and *Bti* colony growth was examined. As summarized by experiments using multiwell trapping procedures (Figure 7 A–F), appearance of formazan deposits correlated favourably with CFU trapped. In this trapping procedure, cell retention was 100% even at the highest practical dose (~10^4 spores mm^{-2}), since filtrates, when recultured or spotted directly on membranes, did not produce colonies or formazan (Figure 7G). The size of individual formazan deposits was dependent on temperature and time, factors which correlated with cell production (growth rate and colony size). The membrane support assay was about as sensitive as the liquid plate assay but it required less than 10 min for maximum formazan production. Once developed, the dried filters can be conveniently stored like photographs. Minor problems with this assay are: the additional time required in setting up sterile membranes and incubation chambers, the necessity to determine appropriate dye exposure times to regulate colony size differences (Figure 7 H–J) and the multilayering or overlapping of the seed spores due to lack of mixing and channelling through the membrane's irregular pore structures (Figure 7K). Calculations based on the size of vegetative cells suggest that the largest and most easily detected deposits (>0.5 mm diameter) seen within the first 6 h are probably produced by 10 or more clustered microcolonies. Using the same assay method with XTT we could demonstrate similar but less defined microcolonies with little or no effect observed on cell viability or formazan production (Figure 8A and B). However, with XTT, quantification of formazan by surface scan was not always possible because of masking by background production of formazan. This background rose significantly in a linear fashion after about 6 h and was probably related to a diffusible enzymatic activity (Beggs and Seligy, unpublished observations).

Applications of QC and QBA assays for monitoring BT products and their field release

To our knowledge, this study in conjunction with another [28], provides the first multiparameter characterization of contemporary commercial BT products. The difference between the *Btk*-based products at equivalent IU is insignificant from the point of view of spore and polypeptide content, indicated by SEM, CFU count (most probable number), QBA assays and SDS-PAGE analysis. With equivalent spore contents, we have found that dose-responses of three of these BT products were virtually the same when tested against two different insect cell lines [28]. The results from spore viability assays and assays to determine optimal vegetative cell growth are more comprehensive than earlier observations gleaned from reports on different laboratory strains [25]. The overlap in growth parameters (temperature and pH) with mammalian cells has not been clearly documented before. Use of this information with the introduction of semi-automated MTT and XTT bioreduction activity assays provides a means for practical, low cost QC testing of microbe-containing samples and products such as BT products and probiotics. The assays, whether liquid (Figures 3, 6) or matrix support type (Figures 7, 8), can discriminate spore content over a relatively broad range of product doses using an antibiotic to control growth if needed for background reference or species selection. Resolution of such assays depends mainly on the care in which accurate logarithmic or linear dilution standards are made up.

The areas in which we are currently attempting to validate QBA assays include the assessment of product efficacy [28], antibiotic sensitivity [22], virulence [5] and environmental exposure using commercial BT products released by aerosolization (Foray48B®) and injection into water (Vectobac®) to control infestations of spruce budworm and blackflies, respectively. Specific details of these studies will be reported at a later date. So far, MTT has proven useful for both liquid and solid support membrane-type assays in which high microsample throughputs are required. These single end-point determinations are relatively cost-efficient and sensitive due to intense staining characteristics and growth inhibition by the insoluble formazan product. Without the computer monitoring capability of our present equipment, the XTT was less suitable because of medium-dependent dye reduction (background), its cost and potential overestimation of QBA by failure to curtail cell proliferation during XTT-formazan production. However, unlike other bacteria [24], *Btk* and *Bti* cells do not require an electron coupler which would increase the assay costs and manipulation time. The XTT assay shows particular promise for obtaining growth profiles in microassays and rapid determination of MICs of various antibiotics and target organisms including microbe-based biotechnology products and testing of electron transport inhibitors [22].

Acknowledgements

We thank members of the Department of Health, Drs A Godfrey (Biotechnology Section) and A Laszlo (WHO Collaborating Centre for Tuberculosis Bacteriology Research, LCDC) for technical review and advice. This research was supported by Health Canada, Industry Science and Technology (National Biotechnology Strategy) and NSERC (to VLS).

References

1 Addison JA. 1993. Persistence and nontarget effects of *Bacillus thuringiensis* in soil: a review. Can J For Res 23: 2329–2342.
2 Aly C. 1985. Germination of *Bacillus thuringiensis* var *israelensis* spores in the gut of *Aedes* larvae (Diptera: Culicidae). J Invert Path 45: 1–8.
3 Aronson AI. 1993. The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. Mol Microbiol 7: 489–496.
4 Beegle CC and T Yamamoto. 1992. Invitation paper (CP Alexander Fund): History of *Bacillus thuringiensis* Berliner research and development. Can Entomol 124: 587–612.

5 Beggs RW, AF Tayabali, JM Rancourt and VL Seligy. 1996. Virulence enhancing potential of extracellular metabolites produced by commercially-formulated strains of *Bacillus thuringiensis*. Cell & Mol Biol 42: S63–S64.
6 Brand RJ, DE Pinnock, KL Jackson and JE Milstead. 1976. Viable spore count as an index of effective dose of *Bacillus thuringiensis*. J Invert Path 27: 141–148.
7 Bryant JE. 1994. Commercial production and formulation of *Bacillus thuringiensis*. Agric Ecosyst Environ 49: 31–35.
8 Burges HD, EM Thompson and RA Latchford. 1976. Importance of spores and delta endotoxin protein crystals of *Bacillus thuringiensis* in *Galleria mellonella*. J Invert Path 27: 87–94.
9 Chiang AS, DF Yen and WP Peng. 1986. Germination and proliferation of *Bacillus thuringiensis* in the gut of rice moth larva, *Corcyra cephalonica*. J Invert Path 48: 96–99.
10 Comley JCW and CH Turner. 1990. Potential of a soluble tetrazolium/formazan assay for the evaluation of filarial viability. Int J Parasitol 20: 251–255.
11 Dubois NR, K Mierzejewski, RC Reardon, W McLane and JJ Witcosky. 1994. *Bacillus thuringiensis* field applications: effect of nozzle type, drop size, and application timing on efficacy against gypsy moth. J Environ Sci Health B29: 679–695.
12 Gill SS, EA Cowles and PV Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu Rev Entomol 37: 615–636.
13 Gomez-Flores R, S Gupta, R Tamez-Guerra and RT Mehta. 1995. Determination of MICs for *Mycobacterium avium–M. intracellulare* complex in liquid medium by a colorimetric method. J Clin Microbiol 33: 1842–1846.
14 Ignoffo CM. 1962. The effect of temperature and humidity on mortality of larvae of *Pecinophora gossypiella* (Saunders) injected with *Bacillus thuringiensis* Berliner. J Insect Pathol 4: 63–71.
15 Marshall NJ, CJ Goodwin and SJ Holt. 1995. A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. Growth Regul 5: 69–84.
16 Masson L, G Prefontaine, L Peloquin, PCK Lau and R Brousseau. 1989. Comparative analysis of the individual prototoxin components in P1 crystals of *Bacillus thuringiensis* subsp *kurstaki* isolates NRD-12 and HD-1 . Biochem J 269: 507–512.
17 McGuire MR, BS Shasha, LC Lewis, RJ Bartelt and K Kinney. 1990. Field evaluation of granular starch formulations of *Bacillus thuringiensis* against *Ostrinia nubilalis* (Lepidoptera:Pyralidae). J Econ Entomol 83: 2207–2210.
18 Moar WJ, L Weste, A Osbrink and JT Trumble. 1986. Potentiation of *Bacillus thuringiensis* var *kurstaki* with thuringiensin on beet armyworm (Lepidoptera:Noctuidae). J Econ Entomol 79: 1443–1446.
19 Miyasono M, S Inagaki, M Yamamoto, K Ohba, T Ishugoro, R Takeda and Y Hayashi. 1994. Enhancement of delta-endotoxin activity by toxin-free spore of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella*. J Invert Path 63: 111–112.
20 Nargi FE and TJ Yang. 1993. Optimization of the L-M cell bioassay for quantitating tumor necrosis factor alpha in serum and plasma. J Immunol Meth 159: 81–91.
21 Paull KD, RH Shoemaker, MR Boyd, JL Parsons, PA Risbood, WA Barbera, MN Sharma, DC Baker, E Hand, DA Scuderio, A Monks, MC Alley and M Grotte. 1988. The synthesis of XTT: a new tetrazolium reagent that is bioreducible to a water-soluble formazan. J Heterocyclic Chem 25: 911–914.
22 Rancourt JM, RW Beggs, G Coleman, AF Tayabali and VL Seligy. 1996. Quantitative redox assay applications for assessing antibiotics against the microbe component of commercial *Bacillus thuringiensis* biopesticides. Cell & Mol Biol 42: S64–S65.
23 Raun ES, GR Sutter and MA Revelo. 1966. Ecological factors affecting the pathogenicity of *Bacillus thuriengiensis* var *thuringiensis* to the European corn borer and fall army worm. J Invert Path 8: 365–375.
24 Roslev P and GM King. 1993. Application of a tetrazolium salt with a water-soluble formazan as an indicator of viability in respiring bacteria. Appl Environ Microbiol 59: 2891–2896.
25 Rowe GE and A Margaritis. 1987. Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis*. CRC Crit Rev Biotechnol 6: 87–127.
26 Samples JR and H Buettner. 1983. Corneal ulcer caused by a biologic insecticide (*Bacillus thuringiensis*). Am J Opthalmol 95: 258–260.
27 Swadener C. 1994. Insecticide Fact Sheet: *Bacillus thuringiensis* (B.T.). J Pesticide Reform 14: 13–20.
28 Tayabali AF and VL Seligy. 1995. Semiautomated quantification of cytotoxic damage induced in cultured insect cells exposed to commercial *Bacillus thuringiensis* biopesticides. J Appl Toxicol 15: 365–373.
29 van Frankenhuuzen K. 1994. Effect of temperature on the pathogenesis of *Bacillus thuringiensis* Berliner in larvae of the spruce budworm, *Choristoneura fumiferana* Cem (Lepidoptera:Tortricidae). Can Entomol 126: 1061–1065.
30 Walther CJ, GA Couche, MA Phannensteil, SE Egan, LA Bivin and KW Nickerson. 1986. Analysis of mosquito larvacidal potential exhibited by vegetative cells of *Bacillus thuringiensis* subsp *israelensis*. Appl Environ Microbiol 52: 650–653.
31 Wilson GR and TG Benoit. 1993. Alkaline pH activates *Bacillus thuringiensis* spores. J Invert Path 62: 87–89.