A STUDY OF THE ENTOMOTOXIC ACTIVITY OF SELECTED COMMERCIAL FORMULATIONS OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS (BTI)* UTILIZING A NOVEL TITRATION PROCEDURE WITH AN AQUATIC MICROBIAL FLORA

L. J. GOLDBERG AND I. FORD

Naval Biosciences Laboratory, University of California, Berkeley, CA 94720

ABSTRACT. A test challenge procedure has been outlined, using 3rd instar *Culex pipiens* larvae held in a defined aquatic microbial test flora AS 2(7), which provides for a standardized bioassay of the entomotoxic activity per cell of *Bacillus thuringiensis* var. *israelensis (Bti)*. AS 2(7), a selected soil microbial isolate, following inoculation and incubation with a sterilized Tetramin® suspension in dilute alfalfa infusion, provides an excellent mosquito larval nutritional food source as evidenced by an 89% adult emergence rate.

Since the rate of mosquito larval filter feeding is nominally 10 ml/hr, a larval test challenge using 2 larvae held in 4 ml of fluid results in near complete larval filter feeding of a test challenge within less than one hour. As a consequence, a larval test challenge can be directly expressed in terms of ingested dose. Using the outlined standardized larval test challenge procedure a comparative evaluation of several commercial formulations of *Bacillus thuringiensis* var. *israelensis (Bti)*, both liquids and wettable powders, has been reported.

INTRODUCTION

IPS-78, a wettable dry powder formulation of *Bacillus thuringiensis* var. *israelensis (Bti)*, has been proposed as an International Standard by the World Health Organization. In July 1980, a summary of a one-year study on the larvicidal stability of IPS-78 stored at room temperature (± 27°C) and utilizing 3rd instar *Culex pipiens* (Linn.) as test larvae was reported (Goldberg 1980, unpublished data). A loss of 6–7 fold in the entomotoxic activity of IPS-78 was observed between 5/2/79 and 6/25/80 for both our original samples as well as a fresh duplicate sample obtained from the Pasteur Institute, Paris, France. Additionally, the activity of Bactimos®, lot No. 676 (wettable powder) produced 6/2/80 by Biochem was tentatively labeled as 6000 i.u./mg. If, however, one corrects for the estimated loss in larvicidal activity of IPS-78 during the period of 5/2/79 until 6/28/80, the labeled activity of Bactimos lot No. 676 would be defined as ca. 1000 i.u./mg.

This unfortunate observation of long-term sensitivity to storage at even moderate room temperatures for all commercially available formulations of *Bti*, has led to the publication of experimental data with this time-temperature storage effect compounded within published results (de Barjac and Coz 1979, Frommer et al. 1981, Garcia and Desrochers 1979, Goldberg and Margalet 1977, Goldberg and Ford 1980, Guillet and de Barjac 1979, Hembree et al. 1980; Ignoffo et al. 1981, Lacey and Lacey 1981, Undeen and Nagel 1978, Undeen and Berl 1979, Undeen and Colbo 1980, Undeen et al. 1981, Van Essen and Hembree 1980). A further complication occurs if a commercial formulation is stored refrigerated or a field test requires the formulation to be subjected to elevated field temperatures.

These problems demonstrated the requirement for the development of a standardized test procedure to clearly define comparative activities of *Bti* formulations following various required conditions of storage prior to larvicidal testing. This paper outlines a *Bti* standardization as well as comparative entomotoxic test procedure. Based on the proposed standardizations of *Bti*, data on the relative larvicidal activity of stored test aliquots of several commercial formulations of *Bti*

are reported; i.e.: 1) Sandoz (402) WDC-I and WDC-A, both liquid formulations of *Bti* (these are commercially designated as Teknar®); 2) Biochem–Bactimos (lot No. 676) a wettable dry powder formulation of *Bti;* 3) Abbott (ABG-6108D lot No. 6478–194) a wettable dry powder formulation of *Bti* and 4) IPS-78 a wettable dry powder formulation of *Bti* and the current proposed International Standard for *Bti:* defined as 1,000 i.u./mg.

OUTLINE OF A PROCEDURE FOR THE STANDARDIZATION OF BTI

(1) Preparation of BTI-NA/A, a liquid formulation of *Bti* for use as a reference standard for test challenge.

(a) *Alfalfa infusion.* The equivalent of 16 gm of fresh alfalfa leaves is added to 1 liter of distilled water and is autoclaved at 15 lbs/in.^{2} for 30–45 min. This fluid is filtered using glass wool (or equivalent). If stored for future use it should be resterilized as outlined above, and then can be stored at room temperature for several weeks.

(b) *NA/A:* Nutrient agar (Difco) is prepared with alfalfa infusion substituted for the required amount of water. Twenty ml of this agar is used in a 100 mm petri dish to provide an adequate agar layer for microbial surface growth.

(c) A surface inoculum of 0.25 ml/plate containing ca. 10^8 spores of *Bti* is uniformly distributed over the agar surface. These plates are incubated in an inverted position at 30°C for 24 hrs. This should result in a confluent surface growth of ca. 1.5×10^{10} cells (spores) per plate. The surface growth of 2 such BTI-NA/A plates is suspended in 10 ml of sterile alfalfa infusion. This provides a convenient laboratory suspension of *Bti* for subsequent test challenge (ca. 3×10^9/ml). We utilized room-temperature-stored aliquots of Sandoz 402 (WDC-I) as a source of inoculum. In retrospect, we would suggest that one obtain a fresh production sample and divide it into a number of small aliquots and keep them frozen until required for use.

(2) Preparation of larval test challenge fluid.

(a) Depending upon subsequent test challenge and daily larval feeding requirements, prepare several liters of alfalfa infusion (see 1a).

(b) *Tetramin®*[1] *suspension:* Using 4 gm of dry Tetramin/100 ml of distilled water, prepare as much Tetramin suspension as will be required during one week. This suspension is sterilized by autoclaving at 15 lbs/in.^{2} for 30–45 min.

(c) 900 ml of sterile alfalfa infusion plus 100 ml of sterile Tetramin suspension is asceptically transferred into a 2 liter Ehrlenmeyer flask and inoculated with AS2(7)[2], a gram-negative rod which has been isolated from a local fresh water stream. After 24 hr incubation at 27°C, this suspension provides an excellent food source for *Culex pipiens* larval growth, as evidenced by an adult emergence rate of ca. 89%.

This preincubated fluid is dispensed using 4 ml/well into clear plastic trays, each containing 25 wells in a 5×5 array. [The separated individual small plastic wells are used as 0.5 oz. jelly trays.] An empty weighted tray is used, inverted, as a lid to minimize surface evaporation and to contain adult mosquitoes.

3. Preparation of *Culex pipiens* egg rafts. Some 15 eggs rafts are surface sterilized by submerging for 15 min in a 100 ppm solution of sodium hypochlorite which has been adjusted to pH 7.0 (±0.5) using KH_2PO_4. A 150 mm sterile petri dish with a smaller (47 mm) sterile petri dish placed in the center of the larger dish facilitates this process. First, form a fine mesh stainless steel screen so it will act as a cover for the small petri dish. Partially fill this small petri dish with 100 ppm sodium hypochlorite solution (pH 7.0) and add ±15 egg rafts. Immediately cover with the stainless steel screen and

[1] A commercially available fish food.

[2] Lyophilized cultures of AS 2(7) can be obtained by writing the authors.

flood over with 100 ppm sodium hypochlorite solution in such a manner as to prevent the formation of air bubbles on the egg rafts. After 15 min, remove the screen and utilize these egg rafts within the next 2–3 minutes. A freshly broken end of a wooden stick will facilitate the removal of floating egg rafts. This procedure prevents added microbial contamination of AS 2(7), the desired aquatic microbial test flora, since surface sterilization of the egg rafts removes potentially undesirable microbial contamination.

4. Standard 100 mm sterile petri dishes are prefilled with ±20 ml of fluid which has been preinoculated with AS 2(7) (see 2a,b,c). One sterilized egg raft (see 3a) is transferred into each such prepared petri dish and covered. These dishes are then incubated at 27°C for 72 hours at which time one should observe some 100 1st to 2nd instar larvae per dish. Discard dishes with low yield, mortality, etc.

5. Larvae, as prepared in (4) are carefully selected and pooled. Using a sterile glass pipette, larvae are placed, 2 per well, in the prepared trays. Each well should contain 2 larvae plus 4 ml of 24-hr-AS 2(7) alfalfa-Tetramin suspension. These trays are held at 27°C until adult emergence. Larval feeding is as follows: Tetramin suspension and sterile alfalfa infusion are combined in the proportions of 20 ml of Tetramin suspension and 80 ml of alfalfa infusion. On the second and on each subsequent day, until pupation, each well is fed with 0.5 ml of this larval feeding suspension. This provides 2 mg of Tetramin per larva per day. Four days of feeding is normally required.

NOTE. On the day of test challenge larval feeding is delayed until a minimum of 1 hr post-challenge in order to standardize aquatic conditions for test challenge.

6. Standard test challenge procedure. Larvae are normally challenged at the 3rd instar. Egg rafts obtained on Friday provide for 1–2nd instar larvae on Monday. On Wednesday, they are at 3rd instar. By Friday, pupation is observed and by the following Monday adult emergence is well-along. Standard experimental larval holding temperature is 27°C with a 12-hour day/night lighting cycle.

(a) *BTI-NA/A Reference Test Challenge.* The surface growth from two 24-hr, 30°C, BTI-NA/A plates is suspended in 10 ml of sterile alfalfa infusion. This fluid is then diluted (using alfalfa infusion as a diluent) 1 to 100. It is then further diluted in 14 additional steps of 1 to 1.5 (usually 10 ml transferred into 5 ml of sterile diluent). These last 15 test dilutions of 1:1.5 are utilized for test challenge. A freshly flamed, cooled, blunt-cut 18 gauge needle will provide ca. 30 drops per ml. Larval challenge is one drop per larva or 2 drops per well. For a given preparation, 20 test larvae are used for each test dilution (total of 300 larvae per test preparation). This provides an acceptable level of statistical accuracy. The viable count of this test challenge series is estimated using Difco blood agar base. (18 hr, 37°C).

Suspension V.C. 3.64×10^9/ml (1–7–81).

(b) *IPS-78 Test Challenge.* 0.5 gr of dry powder is added to 1 ml of Jojoba oil (can also be safflower or soy cold pressed oils) and is mixed into a paste. Ten ml of alfalfa suspension are added and this entire mixture is emulsified into a colloidal suspension by rapid transfer between two 10 ml syringes with 18 gauge needles, attached together with suitable small bore rubber tubing. This initial step has been found to optimize the observed viable count but has no effect on the larvicidal activity (a 2 X increase in v.c. can be observed with older preparations). This suspension is diluted as outlined in (a) for larval test challenge.

V.C.: 6.7×10^{10}/gm of original dry powder (1–7–81)

Note: In the case of IPS-78, 48 hr incubation at 37°C is required to optimize viable counts due to petite colony formation resulting from prolonged storage.

(c) Bactimos (Lot No. 676, produced 6–2–80)

0.5 gr of dry powder utilized as

outlined in (b)
(v.c. 5.1×10^{10}/gr, 7–7–80)
v.c. 3.5×10^{10}/gr, 1–7–81.

(d) Abbott (ABG-6108D Lot No. 6478–194)
0.5 gr of dry powder utilized as outlined in (b).
v.c. 4.5×10^{10}/gr. 1–7–81.

(e) Sandoz liquid formulations (Teknar)
(1) Frozen aliquots of Sandoz (402) WDCI (held ± 1.5 years)
v.c. 2.5×10^{10}/ml, 1–7–81.
(2) Aliquots of Sandoz 402 WDCI held for 1.5 years at room temperature (± 27 C)
v.c. 2.5×10^{9}/ml, 1–7–81.
Note: Original independent assay 2.5×10^{10}/ml.
(3) Fresh production of Sandoz 402 WDC-A
v.c. 2.7×10^{10}/ml, 1–7–81.

The starting test suspension is prepared by adding 1 ml of a concentrate to 9 ml of sterile alfalfa infusion. Subsequent dilution is as previously outlined (see [a]).

All of the above test materials were used in a single parallel larval test challenge. Larval mortality was recorded at 1, 2, and 5 days post-challenge. All graphical data are 5 days post-challenge unless otherwise noted.

RESULTS

(1) *BTI-NA/A:* Proposed standard reference. Larval mortality as a function of post-challenge time and test challenge dose per larva are summarized in graphical format in Fig. 1. [Note 0/20 is indicated as an arrow at 5% mortality; i.e. (1/20 × 100); 20/20 is indicated as an arrow at 95% mortality; i.e. (19/20 × 100)].

At 1 day post-challenge the estimated ED_{50} dose is 4.7×10^4 cells per larva. At 2–5 days post-challenge, the estimated ED_{50} dose is 3.0×10^4 cells per larva.

Note: Upon referring to Fig. 7, which is a composite of all 5-day post-challenge dose responses, it can be noted that there was an 11% nonspecific mortality rate at all dose levels. Reported ED_{50} values are corrected for an 11% non-specific mortality rate.

(2) Comparison of wettable powder formulations, (a) Abbott (ABG-6108-D Lot No. 6478–194), (b) Bactimos (Lot No. 676), (c) IPS-78.

Figure 2 provides a graphical summary of the test results using IPS-78.
Est. ED_{50}: 3.5 μg per larva
or 2.4×10^5 cells per larva.

Figure 3 summarizes the test results using Bactimos (Lot No. 676).
Est. ED_{50}: 0.85 μg per larva
or 3.0×10^4 cells per larva.

Figure 4 summarizes the test results using Abbott (ABG-6108-D Lot No. 6478–194)
Est. ED_{50}: 3.5 μg per larva
or 1.6×10^5 cells per larva.

Figure 5 provides an overall graphical

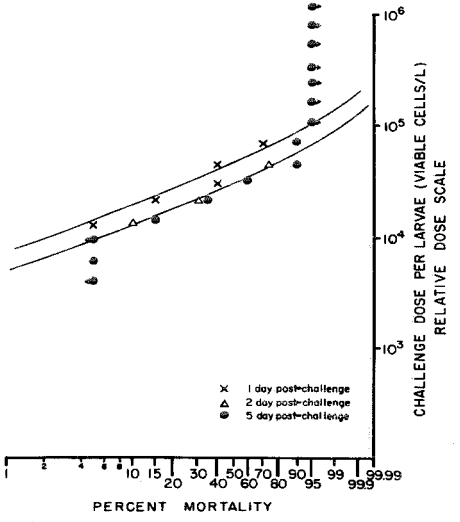

Fig. 1. Dose response to a liquid suspension of *Bacillus thuringiensis* var. *israelensis* obtained using a 24 hour, 30°C surface growth on NA/A, (Nutrient Agar, Difco) using 3rd instar *Cx. pipiens* larvae.

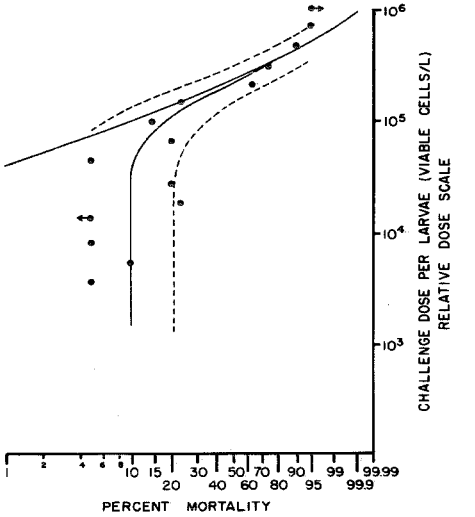

Fig. 2. Dose response to IPS-78, a dry powder formulation of *Bacillus thuringiensis* var. *israelensis*, using 3rd instar larvae of *Cx. pipiens*, evaluated in terms of viable count and dry weight of larval challenge dose.

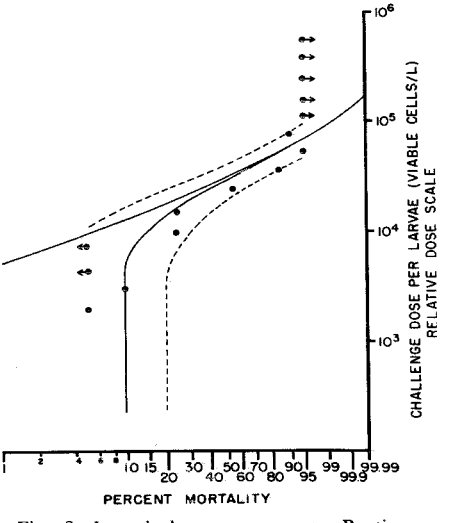

Fig. 3. Larval dose response to Bactimos (Lot No. 676), a dry powder formulation of *Bacillus thuringiensis* var. *israelensis*, using 3rd instar larvae of *Cx. pipiens* evaluated in terms of viable count and dry weight of larval challenge dose.

summary from the test results in Figs. 2, 3 and 4.

Using the experimentally observed ED_{50} dose per larva of BTI-NA/A as a reference standard, one standard entomotoxic unit (s.e.u.) = 3.0×10^4 cells of BTI/NA/A. Hence, for Bactimos (Lot No. 676) after 0.5 yr storage 1 gm = 1.2×10^6 s.e.u. On an equal weight basis, IPS-78 has ¼th the activity of Bactimos (1–7–81). If one notes that the original v.c. of IPS-78 was ± 3 X that of Bactimos, the loss per cell becomes 12-fold. Since Bactimos has lost activity in the ratio of 1 to 1.5 in the previous 6 mo., the overall loss in activity per cell for IPS-78 is estimated as some 18-fold since its original production in 1978.

This same estimated 18-fold loss in activity following initial production would also apply to Abbott (ABG-6108 D Lot No. 6478–194) wettable powder since the original v.c. is listed as 1.5×10^{11} cells/gm.

(3) Comparison of larvicidal activity following various storage conditions of Teknar, a liquid formulation of *Bti* produced by Sandoz. Figure 6 graphically summarizes the dose response using an aliquot of WDC-I which was kept frozen for ± 1.5 years prior to test vs. an aliquot stored at room temperature (±27°C) for ± 1.5 years vs. a fresh production lot (WDC-A) vs. the proposed reference standard BTI-NA/A.

For purposes of comparison the aliquot of WDC-I which was stored for 1.5 years at room temperature is graphically summarized in terms of its original assay; i.e., 2.5×10^{10} cells/ml. Using this graphical format, it is easy to note that the loss of entomotoxic activity per cell following 1.5 years of room temperature storage was 2.6 fold. Since the viable count decreased a factor of10 fold it is evident that entomotoxic activity of this stored liquid

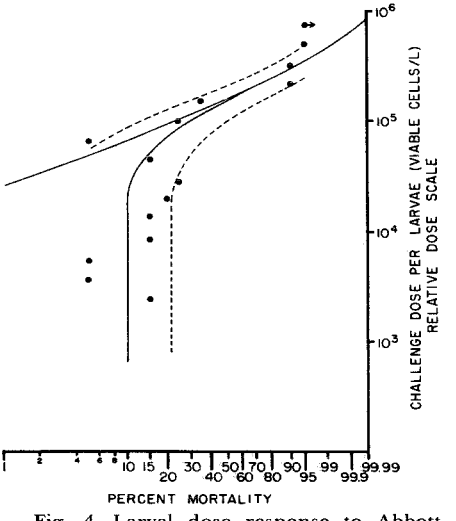

Fig. 4. Larval dose response to Abbott (ABG-6108D; Lot No. 6478–194), a dry powder formulation of *Bacillus thuringiensis* var. *israelensis*, using 3rd instar larvae of *Cx. pipiens* evaluated in terms of viable count and dry weight of larval challenge dose.

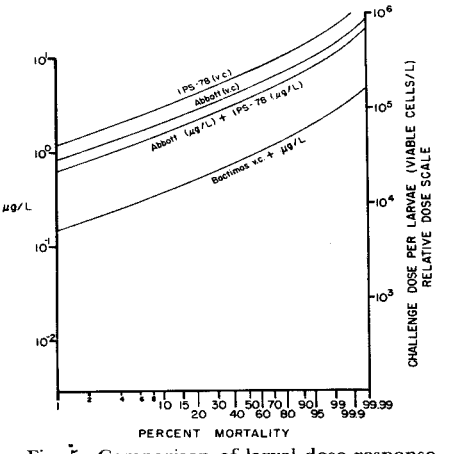

Fig. 5. Comparison of larval dose response to IPS-78, Abbott (BBG-6108-D, Lot No. 6478–194), and Bactimos (Lot No. 676), dry powder formulations of *Bacillus thuringiensis* var. *israelensis*, using 3rd instar larvae of *Cx. pipiens;* comparisons using both viable count and dry weight of larval challenge dose.

formulation does not parallel the observed decrease in viable count.

A comparison of the ED_{50} dose of 3.0×10^4 cells per larva using BTI-NA/A with the observed value of 2.8×10^4 cells per larva for both WDC-A and for WDC-I which was kept frozen for ± 1.5 years demonstrates 2 things:

(1) Stability of fermentation procedures over a period of ±1.5 year at Sandoz,

(2) Equivalence of larvicidal activity per cell of WDC-I (and of Bactimos) with the proposed reference BTI-NA/A.

DISCUSSION

BTI-NA/A, a 24-hr, 30 C surface growth of *Bacillus thuringiensis* var. *israelensis* on nutrient agar (Difco) prepared substituting an alfalfa infusion for water can provide a convenient laboratory reference standard for determining the relative larvicidal activity per cell of commercial formulations of *Bti*. One unit of activity (1 s.e.u.) has been defined in text as equivalent in toxin content to the ED_{50} dose, expressed in terms of ingested cells per larva, using 3rd instar *Culex pipiens* larvae and test challenged using BTI-NA/A within an aquatic test flora AS 2(7), as defined in the body of this paper. The estimated value of 1 s.e.u. is 3.0×10^4 cells of BTI-NA/A.

It has also been demonstrated that a 1.5 year-old, frozen aliquot of Sandoz 402 WDC-I as well as WDC-A, a fresh production lot, both demonstrate a larvicidal activity per cell equivalent to an $ED_{50} = 2.8 \times 10^4$ cells per larva. Bactimos (Lot No. 676) produced by Biochem demonstrates an $ED_{50} = 3.0 \times 10^4$ cells per larva.

Thus, these results provide strong cross-verification for the use of BTI-NA/A as a possible laboratory reference standard for *Bti*.

Using BTI-NA/A as a reference standard Bactimos would have been labeled with an activity of 1 gram = 1.2×10^6 s.e.u., and Teknar, a liquid formulation, would have been labeled as 0.96×10^6 s.e.u. per ml.

Using this procedure, the long-term stability of an entomotoxic standard of *Bti* is dependent upon a carefully preserved reference culture. An "absolute" system will depend upon the chemical definition of the unique toxin(s) produced by *Bti*. Using this proposed test procedure, relative changes in toxin yield per cell can be accurately estimated as a function of fermentation. It is important in this instance to note that it is the total toxin yield per fermentation batch rather than actual yield per cell, that is of practical importance; i.e., the product of total cell yield times activity per cell.

It is also important to keep in mind that this proposed standard test procedure purposely provides for near complete larval ingestion within less than one hour following test challenge. This results because larvae are held and challenged using 2 ml of water per larva and the larval (filter) feeding rate is ±10 ml per hr.

One can conceive of future formulation technology which provides for long-term shelf life and protects against aquatic biodegradation as well as minimizes the time required for larval toxin ingestion. Using a test challenge system which provides 100 ml of water per larva in combi-

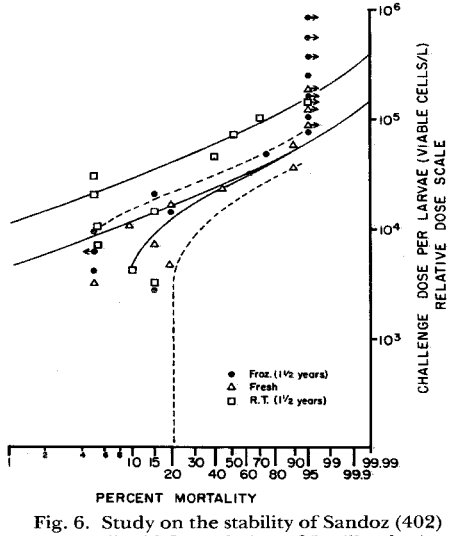

Fig. 6. Study on the stability of Sandoz (402) WDC-I, a liquid formulation of *Bacillus thuringiensis* var. *israelensis*, stored frozen and held at ± 27 C for ± 1.5 years; comparison with Sandoz (402) WDC-A, a recent production lot; using 3rd instar *Cx. pipiens* larvae.

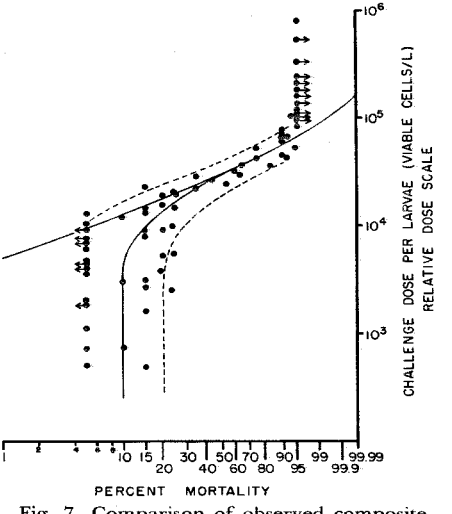

Fig. 7. Comparison of observed composite dose responses of larvae to *Bacillus thuringiensis* var. *israelensis* with a theoretical 3-hit dose response, modified by an 11% non-specific larval mortality rate.

——— 3 hit dose response (100% susceptible larval population.

——-—— 3 hit dose response modified 11% non-specific larval mortality.

– – – – defines 95% confidence area, i.e., graphical area in which one should expect to observe 95% of larval responses to test challenge when using a test group size of 20 larvae.

nation with a water depth of some 10 cm will provide a reasonable laboratory approximation to selected adverse aquatic field conditions. This also requires the selection of a suitable aquatic microbial test flora, which will probably vary with different test species of larvae. Routine laboratory testing using such large test volumes is costly in space, as well as in required hours of test observations, however, such an alternative added testing procedure would provide a more meaningful comparative labeling for a potential user.

Experimental comparative data on both liquid and wettable powder formulations of *Bti* clearly demonstrate the dependence of entomotoxic stability upon temperature during prolonged storage. For wettable powders, the possible absorption of water during prolonged storage adds another variable to be considered. Additionally, decreasing entomotoxic activity during prolonged storage does not necessarily parallel residual viable count.

It is important to note that a frozen aliquot of Sandoz WDC-1, a liquid formulation of *Bti*, provides a convenient current secondary reference standard, providing refrigeration can be reliably maintained.

One should expect a fresh commercial formulation of *Bti* with ca. 1.5×10^{11} spores per gm (or ml) to demonstrate an entomotoxic activity of ca. 5×10^6 s.e.u. per gm (or ml). With improved entomotoxic yields from advanced fermentation techniques, toxic activity may exceed 1×10^7 s.e.u. per gram.

ACKNOWLEDGMENT

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