BACILLUS THURINGIENSIS δ-ENDOTOXIN: ON THE RELATIVE ROLES OF SPORES AND CRYSTALS IN TOXICITY TO SPRUCE BUDWORM (LEPIDOPTERA: TORTRICIDAE)[1]

P. G. FAST

Insect Pathology Research Institute, Canadian Forestry Service, Sault Ste. Marie, Ontario P6A 5M7

Abstract

Can. Ent. 109: 1515–1518 (1977)

Newly moulted sixth instar larvae of spruce budworm, *Choristoneura fumiferana*, were force fed graded dosages of preparations containing (1) purified crystals, (2) purified spores, and (3) mg/mg mixtures of purified spores and purified crystals. The LD_{50} for purified crystals was 0.094 μg/larva and for the mixture 0.096 μg/larva but the latter preparation had an LD_{50} of 0.048 μg/larva based on crystals indicating that the presence of 2.4×10^5 spores enhanced the toxicity of crystals twofold. The LD_{50} spore:crystal corresponded to LD_{60} for crystals alone. The LD_{50} for spores treated with proteinase to remove residual crystals was 20 μg/larva, or 1.0×10^8 spores. The regression coefficients were not significantly different from each other in all three preparations indicating a common or similar mode of action. It is concluded that spores play little or no role in mortality of spruce budworm induced by *Bacillus thuringiensis* insecticides.

Résumé

Des larves du sixième stade to Tordeuse des bourgeons de l'Epinette (*Choristoneura fumiferana*) de mue récente ont été gavées au moyen de préparations à doses calibrées contenant (1) des cristaux purifiés, (2) des spores purifiées et (3) des mélanges mg/mg de spores purifiées et de cristaux purifiés. En cristaux purifiés, le LD_{50} "titrait" à 0.094 μg/larve, et en mélange, à 0.096 μg/larve, mais cette dernière préparation avait une puissance réelle en LD_{50} de 0.048 μg/larve, vu que la présence de 2.4×10^5 spores rehaussait 2 fois la toxicité des cristaux. Le LD_{50} spores:cristaux correspondait à LD_{60} pour les cristaux seuls. Pour les spores purifiées, le LD_{50} correspondait à 20 μg/larve ou 1.0×10^8 spores. Les coefficients de régression ne furent pas significativement différents l'un de l'autre dans les trois préparations, indiquant un mode d'action commun ou similaire. L'auteur conclut que les spores jouent un rôle à peu près nul dans la mortalité de la Tordeuse des bourgeons de l'Epinette produite par des insecticides contenant *Bacillus thuringiensis*.

Introduction

The use of insecticides based on *Bacillus thuringiensis* has long been confused by the presence of two potentially active ingredients: the bacterial spore and the δ-endotoxin crystal produced as a concomitant of sporulation. This confusion remains despite the evidence in early work that the δ-endotoxin played a significant, if not decisive, role in mortality of target insects. It was exacerbated by the fact that no good measure of endotoxin content was available whereas spore content was readily measured. Furthermore, it was widely held that spores are essential to observed mortality and that endotoxin content bore some more or less constant relationship to spore content and thus most field and laboratory studies were based on spore content.

Experiments using laboratory purified crystals or sterile dissolved and reprecipitated δ-endotoxin, have shown that endotoxin alone can kill both those insects that show general paralysis and 24 h mortality (type I) (Heimpel and Angus 1959) and those that show no general paralysis and little mortality at < 48 h (type II) (Rogers 1967). Rogers carefully studied the relative role of spores and crystals and concluded that spores play no role in mortality of *Pieris brassicae*. On the other hand, in *Galleria mellonella*, an absolute requirement for both spores and crystals to cause mortality has been demonstrated (Burges *et al.* 1976). Neither entity by itself has any effect.

Spruce budworm, *Choristoneura fumiferana*, has been a prime target for *Bacillus thuringiensis* insecticides since the early field trials of Angus *et al.* (1961) and of Smirnoff (1963). Smirnoff claimed that preparations containing 80% crystals did not kill spruce budworm. He interprets this as indicating crystals play little or no role in spruce budworm mortality (e.g. Smirnoff 1974). In 1970 Yamvrias and Angus published data showing that preparations of B.t. var. *sotto* with crystal:spore ratios of

[1]Contribution No. 330, Insect Pathology Research Institute.

https://doi.org/10.4039/Ent1091515-11 Published online by Cambridge University Press

7000:1 caused mortality and that sterile dissolved endotoxin also could kill spruce budworms at concentrations as low as .05% in the diet.

Since justification for the addition of chitinase to commercial B.t. insecticides used against spruce budworm at additional cost is based on the premise that spores profoundly influence mortality, and since much research is being carried out to devise formulations that will protect spores against environmental degradation in forest insect control, we have attempted to clarify the relative roles of spores and crystals in mortality of spruce budworm.

Materials and Methods

Pure cultures of *Bacillus thuringiensis* derived from the commercial HD-1 strain (Dipel, Abbott Laboratories) were grown as described by Fast *et al.* (in preparation) and purified on Renograffin (E. R. Squibb & Sons) gradients by the method of Milne *et al.* (1977). The crystal preparations contained 470 spores/μg and the spore preparations 5.05×10^6 spores/μg, and ~ 4% by weight alkali soluble protein presumed to be crystal contaminant and(or) toxic spore coated protein.

Newly moulted sixth instar larvae were force fed 2.0 ± 0.05 μl of test suspensions and then placed in coffee creamers on artificial diet (Grisdale 1970, 1973) and held under normal rearing conditions. Each assay consisted of at least five serial dilutions of each preparation with 20 or 25 insects treated at each dose, as well as controls that received water only. Mortality was assessed 96 h after dosing and the data submitted to computerized probit analysis. Test suspensions, diluted with water, consisted of the following: (1) purified crystals, (2) a mixture of purified spores and purified crystals (mg/mg), (3) purified spores, (4) purified spores treated with *Trichoplusia ni* gut proteinase (Murphy 1973) to remove residual crystal protein contamination.

Results

The LD_{50} for six assays of purified crystals against newly moulted sixth instar spruce budworm larvae varied over a 7× range. When the separate assays were combined and analyzed as recommended by Finney (1971, p. 175) the regression equation for purified crystals was

$$Y = 1.04\,(\pm.05) + .34\,(\pm 0.03) \log_e x,$$

where x is the dose in μg (numbers in parentheses are standard errors). Conversion of this equation to LD_{50} and LD_{95} with 95% fiducial limits are shown in Table I. The mortality data on which the equation is based was heterogeneous due to variation between assays but the regression was highly significant ($p < .001$). These crystal preparations contained 470 viable spores/μg. The LD_{50} dose therefore contained, on average, 44 spores.

The assays for purified spores were not replicated as extensively as those for crystals or spore:crystal mixtures. But when purified spores were compared with proteinase treated spores on the same batch of insects the LD_{50} for purified spores was

Table I. Toxicity and 95% fiducial limits of purified crystals, spore:crystal mixture, and proteinase treated spores

	LD_{50}(μg/larva)	Fid. limits	LD_{95}	Fid. limits
Crystals	0.094	0.136–0.068	11.3	37.8–4.7
Spore:crystal mixture	0.096	.128– .072	6.8	15.8–3.8
Spore:crystal mixture based on crystal content	.048	.064– .036	3.4	7.9–1.9
Proteinase treated spores	20.87	.336–5.6	1059	9.7×10^{11}–132
Proteinase treated spores as viable spore count	1.04×10^8	1.68×10^9–2.79×10^7	5.2×10^9	4.8×10^{18}–6.6×10^8

https://doi.org/10.4039/Ent1091515-11 Published online by Cambridge University Press

3 μg/larva and that for proteinase treated spores 20 μg/larva. The slope of 0.41 ± 0.12 of the line for proteinase treated spores did not differ significantly from that for purified crystals or spore:crystal mixtures below. Spores, therefore, were 200 times less toxic than crystals.

The individual assays (7) of spore:crystal mixtures (mg/mg) were as variable as those for crystals alone and it was not until purified crystals and spore:crystal mixtures were assayed on the same batch of larvae that we were able to recognize an effect of spores. When the assays were combined and analyzed as for crystals the regression equation was

$$Y = 1.17\,(\pm 0.05) + 0.39\,(\pm 0.03) \log_e x.$$

The data were again heterogeneous but the regression was significant ($p < .001$). LD_{50} and LD_{95} for spore:crystal mixtures are included in Table I. In Fig. 1 we have plotted the individual data and the derived regression lines. In this case the LD_{50} contained 240,000 spores. The LD_{50} based on crystal content (Table I) shows that the presence of spores resulted in a twofold increase in toxicity. The LD_{50} based on crystal content corresponds to an LD_{60} for purified crystals. Simple covariance analysis of the regression coefficient (the slope factor) showed that slope for spore:crystal mixture was not significantly different from that for crystals above ($p > 0.75$) and so the lines may well be parallel.

Discussion

Two factors that must be considered in evaluating our data are firstly, the slope of the regression line, that is the regression coefficient b, which defines the slope of the line, in the equation $Y = a + b \log_e x$; and secondly the LD doses calculated from the equation.

The regression coefficient b is a function of the mode of action of a toxin. If two regression lines for different toxins are parallel, i.e., if their regression coefficients are

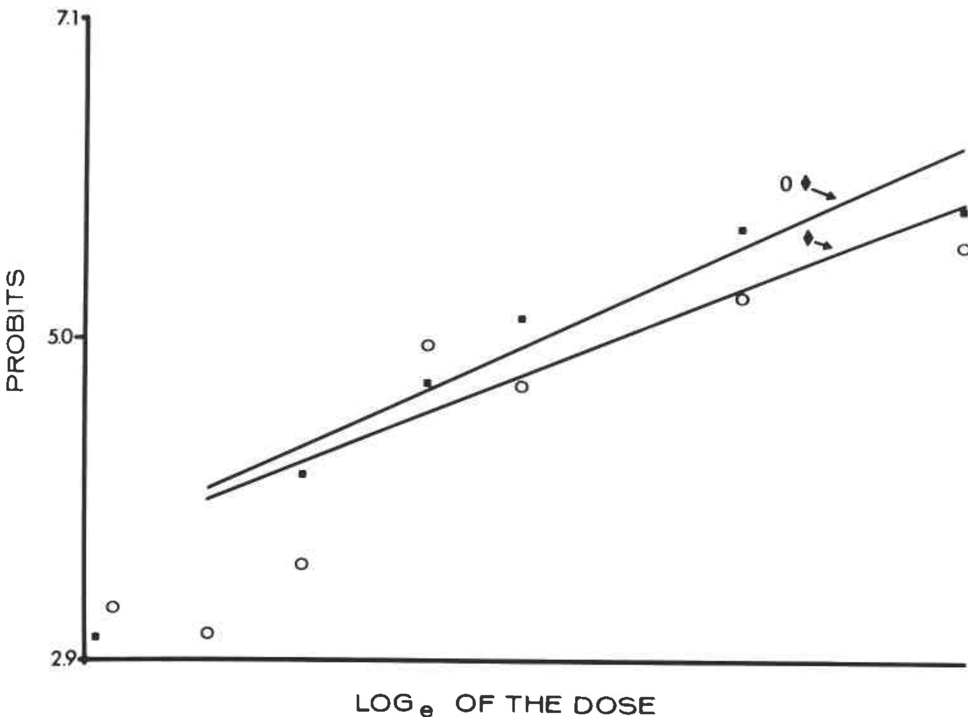

FIG. 1. Best fit probit regression lines for mortality due to crystals (■) and spore:crystal mixtures (○).

https://doi.org/10.4039/Ent1091515-11 Published online by Cambridge University Press

the same, it is likely that their mode of action is the same or very similar. If mode of action is not the same the relative potency of one vs. the other is quite large and the regression line of one component should curve as the other component is added (Finney 1971, Chap. 11).

In our studies the coefficients of regression are indistinguishable between spores, crystals, or mixtures of the two and the regression lines may, in fact, be parallel. Analysis of variance of the regressions indicates that the equations for the lines fit the data very well despite the deviation of the points at low concentration and the heterogeneity of the data due to batch to batch variations. Thus the mode of action is not significantly altered by the addition of spores to crystals.

Although the LD_{50} of purified crystals or mixtures of spores and crystals are indistinguishable it only takes half as many crystals to yield an LD_{50} when spores are present mg/mg. Thus spores do have an effect, small though it may be (the LD_{50} for the crystals corresponds to LD_{60} for the mixture). This corresponds with the qualitative observation of many workers including ourselves. We are thus led to conclude that spores act in the same fashion as crystals but the amount of toxic material in spores is so small that large quantities of spores are required to have any effect at all. The effect may be due to the spore toxin described by Somerville and Pockett (1975) which has been shown chemically to be very similar to crystal toxin.

Finally, the LD_{50} and LD_{95} found by us for mixtures correspond to 1.6 and 108 International Units of Dipel WP (16,000 IU/mg). Since each IU of Dipel contains 2000 spores and an equal number of crystals (G. Hansen, pers. comm.) the spores present in the $LD_{50} = 3200$ and in the $LD_{95} = 216{,}000$. The latter figure is less than the spores in the LD_{50} of our spore:crystal mixture so that spores will have less effect in commercial preparations than observed in these experiments.

Septicemia may occur in B.t. treated larvae in the laboratory and in the field. However, the effect of such septicemia is not detectable by altered regression coefficients within 4–6 days of receiving a dose and this does not appear to affect the mode of action.

References

Angus, T.A., A.M. Heimpel, and R.A. Fisher. 1961. Tests of microbial insecticide against forest defoliators. *Bi-mon. Prog. Rep. Can. Dep. For.* **17**(3): 1–4.

Burges, H.D., E.M. Thompson, and R.A. Latchford. 1976. Importance of spores and δ-endotoxin protein crystals of *Bacillus thuringiensis* in *Galleria mellonella*. *J. invert. Path.* **27**: 87–94.

Finney, D. J. 1971. Probit analysis. University Press, Cambridge. 3rd ed.

Grisdale, D. 1970. An improved laboratory method for rearing large numbers of spruce budworm *C. fumiferana* (Lepidoptera: Tortricidae). *Can. Ent.* **102**: 1111–1117.

——— 1973. Large volume preparation and processing of a synthetic diet for insect rearing. *Can. Ent.* **105**: 1553–1557.

Heimpel, A.M. and T.A. Angus. 1959. The site of action of crystalliferous bacteria in Lepidoptera larvae. *J. Insect Path.* **1**: 152–170.

Milne, R., D.W. Murphy, and P.G. Fast. 1976. *Bacillus thuringiensis* δ-endotoxin: An improved technique for separating crystals from spores. *J. invert. Path.* **29**: 230–231.

Murphy, D.W. 1973. The role of midgut proteases in determining the relative resistance of 3 susceptible species of lepidopterous larvae to the δ-endotoxin of *Bacillus thuringiensis* var. *thuringiensis*. Thesis, Univ. of California, Riverside.

Rogers, A.H. 1967. The bioassay of crystals of *Bacillus thuringiensis* using larvae of *Pieris brassicae*. PhD. Thesis, University of Leeds, U.K.

Smirnoff, W.A. 1963. Tests of *Bacillus thuringiensis* var. *thuringiensis* Berliner and *B. cereus* Frankland and Frankland on larvae of *Choristoneura fumiferana* (Clemens). *Can. Ent.* **95**: 127–133.

——— 1974. Three years of aerial field experiments with *Bacillus thuringiensis* plus chitinase formulation against the spruce budworm. *J. invert. Path.* **24**: 344–348.

Somerville, H.J. and H.V. Pockett. 1975. An insect toxin from spores of *Bacillus thuringiensis* and *Bacillus cereus*. *J. gen. Microbiol.* **87**: 359–369.

Yamvrias, C. and T.A. Angus. 1970. The comparative pathogenicity of some *Bacillus thuringiensis* varieties for larvae of the spruce budworm, *Choristoneura fumiferana*. *J. invert. Path.* **15**: 92–99.

(Received 25 January 1977)

https://doi.org/10.4039/Ent1091515-11 Published online by Cambridge University Press