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Effectiveness of Dead-Spore *Bacillus thuringiensis* Formulation Against Diamondback Moth

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Abstract

We developed a process of producing a dead-spore *Bacillus thuringiensis* Berliner var. *kurstaki* HD-1 formulation, Toarow CT, and studied several aspects to improve the productivity of crystal toxin and the insecticidal potency after sterilization to kill spores. The production of cell mass or crystal toxin was highly influenced by aeration and agitation of the culture. The culture broth, within 24 hours after the release of 90% of spores from sporangia, was suitable for formulation into commercial product. We tested a live-spore (Toarow) and a dead-spore (Toarow CT) formulations, both having the similar insecticidal potency, against silkworm. Both formulations were equally effective against diamondback moth, *Plutella xylostella* (L.) when tested by a leaf dip method. Also no differences in mortality and control ratio were observed in a field test either. The dead-spore formulation was thus satisfactory for the control of diamondback moth.

Introduction

Various strains of *Bacillus thuringiensis* Berliner (*Bt*) produce crystal toxins comprised of bipyramidal, cuboidal and irregular proteins. Some of the crystal toxins show selective toxicity to larvae of moths and butterflies and others show insecticidal activity against larvae of flies and mosquitos or show selective toxicity only to beetles represented by Colorado potato beetle (Himeno 1989). *Bt* strains have been classified according to flagellar antigens in addition to pathogenicity (De Barjac and Frachon 1990). *Bt* produces spores almost simultaneously with formation of crystal toxins. Therefore, *Bt* formulations contain both crystal toxins (active ingredient) and spores. The crystal toxins have a highly selective insecticidal effect against the target insect(s), while being harmless to humans, animals, birds and fish. On the other hand, spores are known to have no insecticidal effect (Angus 1954). We developed a technology to sterilize vegetative cells and spores of *Bt* with minimum loss of insecticidal activity of crystal toxins, and created a novel *Bt* insecticide, dead-spore *Bt* formulation (Japanese Pat. No. 831099). The formulation under the name of Toarow CT wettable powder was approved for use in insect control in 1981 (Reg. No. 14459) by the Ministry of Agriculture, Forestry and Fisheries of Japan. In this paper, we will present a number of findings in developing the process to manufacture the dead-spore *Bt* formulation and its effects against diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae).

Materials and Methods

Manufacturing process

The process of manufacturing a dead-spore *Bt* formulation (Toarow CT) is shown schematically in Fig. 1. The strain used is *B. thuringiensis* var. *kurstaki* HD-1, donated

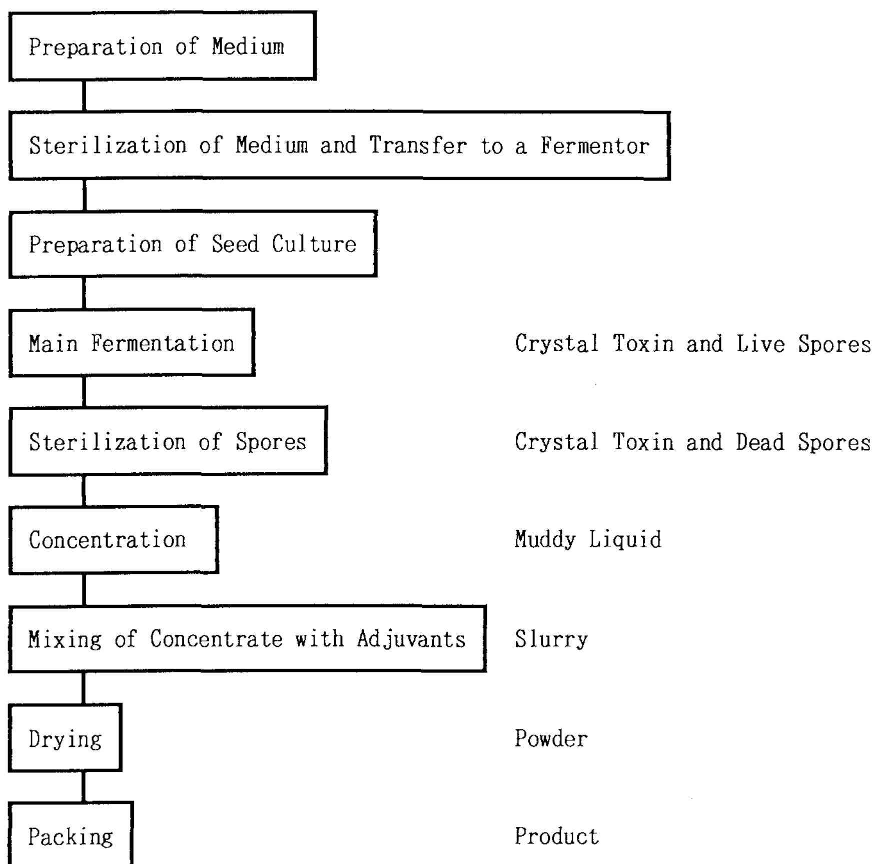

Fig. 1. Flow diagram of manufacturing process of Toarow CT wettable powder (a dead-spore *Bt* formulation).

by Dulmage (Dulmage 1970; De Barjac and Le Mille 1970). This strain has both bipyramidal insecticidal crystal protein (ICP) with high toxicity for lepidopteran larvae and cuboidal ICP with high toxicity for dipteran larvae (Iizuka 1990).

This microbe was cultivated in a conventional submerged fermentor (medium composition: glucose 3%, corn steap liquor 6% and tap water; pH = 7.0 ± 0.1; temp. = 30 ± 1°C). Prior to pilot-scale fermentation, we investigated some factor(s) influencing ICP production by using a 20 l jar-fermentor (Marubishi Co., Type MSJ-205). Agitation speed (rpm) of impeller, flow rate of air (vvm) and working volume were varied. Culture broth was drawn from a jar-fermentor and the precipitate obtained by centrifugation (4°C, 20000g, 10 min) was repeatedly dried in oven and cooled in desiccator until constant weight was obtained. With this value, we calculated a dry cell weight (g/l) of culture broth. The final culture broth was also employed in a bioassay with silkworm (*Bombyx mori* L.). We determined a proper harvesting time by microscopical examination of the culture broth at various stages. The ratios of free spores released from sporangia to total number of spores under five views were employed and the average values of these data were used as the index to judge an end-point of cultivation.

Sterilization of culture broth containing crystal toxins was carried out with a combination of physical treatment (heating, sonication, etc.) and chemical treatment (sterilizers, etc.) (Japanese Patent Publication (Kokoku) No. 51-5047). In industrial scale, sterilization was carried out continuously as follows: the mixture of culture broth and sterilizer was continuously introduced

into the inner part of a double-walled tube, while hot water flowed in reverse through the outer part of it. The number of viable spores and cells was estimated as follows: 1 ml of sample (the sterilized solution or the suspension of product in water, 0.5 g/10 ml) was placed in 10 ml of sterile saline. The number of viable spores (and cells) was determined by counting serial dilutions plated on nutrient agar medium (Difco).

Concentration, mixing of concentrate with adjuvants, drying and packing are carried out successively (Fig. 1) like a conventional insecticide formulation process.

Evaluation of insecticidal potency against silkworm

We adopted a bioassay with silkworm to evaluate ICP's productivity in culture and the two formulations; live spores and dead spores. The bioassay was carried out with third instar larvae of silkworm. A series of 5-6 dilutions of the sample or standard (U.S. standard HD-1-s-1971 of 18000 IU/mg) was added to 10 g of artificial diet (VitaSilk, Kyodo Shiryo), and the mixture was kneaded homogeneously and spread in a petri dish. Twenty silkworm larvae reared on artificial diet were placed on it. The number of dead larvae in each dilution was counted after 3 days of incubation at 28°C. LC_{50} was determined according to Probit Analysis (Finney 1974).

The insecticidal potency (IU/mg) of the sample was calculated as follows:

$$\text{Potency for sample (IU/mg)} = \underset{(= 18000\ \text{IU/mg})}{\text{Potency for standard}} \times \frac{LC_{50}\ \text{for standard (ppm)}}{LC_{50}\ \text{for sample (ppm)}}$$

The potency of the sample was determined as a mean value from three assays on separate days.

Evaluation of insecticidal potency against DBM

Prior to field trials of Toarow CT against DBM, we carried out several tests in the laboratory. The bioassay was made with first, second and third-instar larvae of DBM in accordance with specific purpose. We used the DBM OSS strain (Virapong et al. 1983) reared in laboratory (16L: 8D and 70-75% RH at 25 ± 1°C). The first, second and third instar larvae corresponded to 4-6, 6-7 and 7-8 days after egg hatch, respectively. A series of 5-7 dilutions of the sample with distilled water containing 0.03% of Triton X-100 was prepared. A cabbage leaf disk (5 × 5 cm) was dipped into 50 ml of each dilution and immediately pulled out and dried in the air at room temperature. Control leaves were dipped in Triton X-100 0.03% only. The dipped leaflet was inserted into a plastic container, at the bottom of which an absorbent paper (Whatman 40) was placed to absorb moisture, and on the top of which a perforated plastic cover was placed for ventilation. Twenty larvae of DBM reared on radish (*Raphanus sativus* var. Osaka 40 nich) seedlings were inserted into each container. The larval mortality was checked after 3 days (1 or 2 days in some cases) of incubation at 25°C. LC_{50} was determined in a manner similar to silkworm.

Field trials were carried out in Obu, Aichi, on cabbage, in which the efficacy of Toarow CT preparation (with dead spores) was compared with that of Toarow preparation (with live spores), under the identical insecticidal potency against silkworm, 10000 IU/mg. Treatments were arranged in a randomized complete block design with two replications using larval density before treatments as the control criterion. The plot was 5 × 15 m and consisted of 100 cabbage plants (at 7-8 weeks after transplanting) in four rows. The formulations were applied as aqueous suspensions at the concentrations of 0.05 and 0.1% with 0.033% of Tokuase (as spreader-sticker, Sankyo Co.). A single application of each preparation was made. One liter was applied for 20 plants per treatment with a manually operated, compressed-air sprayer. An untreated plot was prepared as a control plot. The number of DBM larvae per cabbage plant in each plot were counted before and 3 and 7 days after spray. Mortality and corrected population index based on the change in larval densities were calculated as follows:

$$\text{Mortality (\%)} = (1\ (T_a/T_b)) \times 100$$
$$\text{Corrected population index (\%)} = ((T_a \times C_b)/(T_b \times C_a)) \times 100$$

where 'T' and 'C' show the number of DBM larvae under treatment and control, respectively, and 'b' and 'a' show before and after spray, respectively. These data were also analyzed by analysis of variance. The degree of leaf damage was estimated at 3 or 7 days after the spray.

Evaluation of the effect of UV irradiation on insecticidal potency

Two kinds of experiments were conducted to examine the effect of UV irradiation on the efficacy of *Bt* preparations in the laboratory.

In the first experiment a thin layer of Toarow CT (Lot. 2060P; 10600 ± 670 IU/mg) was exposed to UV irradiation (UV lamp: 15 W). The irradiation time and the distance between a lamp and the sample were varied. One gram of the formulation was mixed with 10 ml of distilled water and 1 ml of the preparation was poured on a petri dish. The sample was dried at room temperature and 5 ml of distilled water was poured into each petri dish after exposure to UV and the insecticidal potency against silkworm was measured by the above method. The residual ratios of potency were obtained.

The second experiment was conducted on cabbage leaves (5 × 5 cm) dipped in the solutions of *Bt* preparation and held under UV irradiation. Samples were the same as those used in the field trials (equivalent to Toarow and Toarow CT). The concentrations of *Bt* solutions were 0 (control), 0.001 and 0.1% all with 0.03% of Triton X-100. The dried cabbage leaves after dipping were placed under the UV lamp at distances of 11 and 22 cm, respectively. After exposure to UV, the leaves were fed on the third-instar larvae of DBM and mortalities were recorded after 3 days.

Results and Discussion

We have developed several processes as shown in Fig. 1 to manufacture a dead-spore *Bt* formulation. *Bt* is an aerobic spore-forming bacteria that can be used to produce cell mass and crystal toxin depending on the media composition in culture (Mummigatti and Raghunathan 1990). We used a simple medium composed of glucose as a carbon source and corn steap liquor as a source of amino acids, minerals and vitamins. The medium was suitable for culturing *Bt*. We therefore investigated the effect of other conditions such as aeration and agitation on the productivity of cell mass and crystal toxin from this microbe.

As shown in Fig. 2, cell mass and insecticidal activity (proportional to the content of ICP) of culture broth increased with the increase of air flow rate and agitation speed, while insecticidal activity of culture broth became maximal at 12 l of working volume. This is supposedly due to inhibitory effect of excessive antifoamer (W = 14 l) in culture and the shortage of volumetric oxygen transfer coefficient, k_La (W = 10 l). Moreover, we scaled up fermentation stepwise to pilot-scale fermentation.

Determination of the endpoint of culture is important to achieve the maximal productivity of crystal toxin. From the trial and error experiments, it was descovered that culture broth within 24 hours after the release of 90% of spores from sporangia is suitable for this purpose.

We compared the efficacy of *Bt* preparation containing dead and live spores (equivalent to Toarow CT and Toarow, respectively) against DBM, both having the same insecticidal potency against silkworm, indoors and in a field. No significant differences in LC_{50} were observed between both preparations when we used cabbage leaf dipping method (P = 0.01) (Table 1). No significant differences were observed in mortality and corrected population index under the same dilutions in a field test (P = 0.05) (Table 2). In addition, no differences were observed in the degrees of leaf damage between the treatments by two *Bt* preparations.

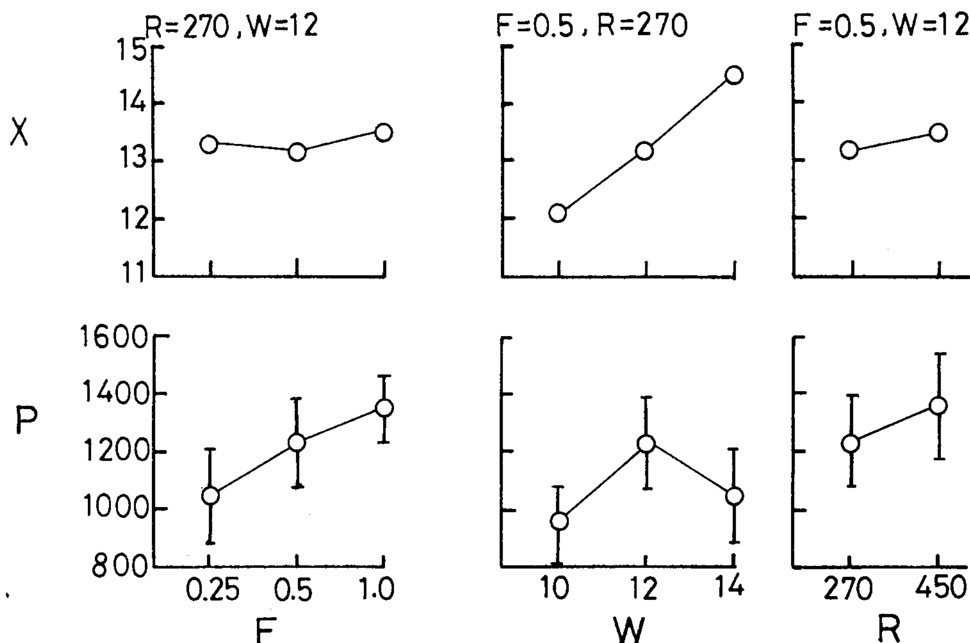

Fig. 2. Effects of aeration, agitation and working volume on productivities of cell mass and ICP in culture of *Bacillus thuringiensis* var *kurstaki* HD-1. Culture conditions are described in text. F, R and W show the flow rate of air (vvm), the agitation speed (rpm) of impeller and working volume (l), respectively. X and P show the maximal value of dry cell weight (g/l) in logarithmic growth phase and insecticidal potency against silkworm (KIU/ml) in finally harvested culture broth, respectively.

Table 1. Comparison of insecticidal potency against DBM between Toarow CT preparation and Toarow preparation with the same insecticidal potency against silkworm in a laboratory test.

Treatment	Insecticidal potency (LC_{50}, ppm) against DBM[a]			
	Exp. 1	Exp. 2	Exp. 3	Mean (±SD)
Toarow CT (dead spore)	19	15	16	17±1.7
Toarow (live spore)	18	16	18	17±0.9

[a]Third-instar larvae of DBM were used and LC_{50} was obtained from the mortality after 3 days. The insecticidal activity of Toarow CT and Toarow against silkworm was adjusted to 10000 IU/mg by the addition of adjuvants.

These results indicate that there is no difference in insecticidal potency against DBM between a dead and a live-spore *Bt* formulation. Similarly, Takaki (1975) reported that both Toarow and Toarow *Bt* (equal to Toarow CT) are effective against mugwort looper (*Ascotis selenaria* Denis et Schiffermüler), which is a pest of tea in Japan.

Table 2. Efficacy of *Bt* preparations containing dead or live spores against DBM in a field test.

Treatment	No. cabbage plants	No. larvae of DBM			Mortality (%)		Corrected population index (%)	
		before spray	3 days after spray	7 days after spray	3 days after spray	7 days after spray	3 days after spray	7 days after spray
Control	20	63	269	729	–	–	–	–
Toarow CT (× 1000)	20	53	8	24	85	55	3.5	3.9
Toarow (× 1000)	20	53	7	28	87	47	3.1	4.6
Toarow CT (× 2000)	20	55	29	138	47	-151	12.3	21.7
Toarow (× 2000)	20	54	43	159	20	-194	18.6	25.4

Mortality (%) and corrected population index (%) were calculated in the same manner as described in text. The mean larval density before spray among the treatments did not vary significantly. The field test was conducted in May 1990, and there was no rainfall during the test.

A gradual decrease of insecticidal activity in the field, due to inactivation of ICP by UV irradiation, is common in all *Bt* formulations. We, therefore, investigated the effect of UV irradiation on the efficacy of *Bt* formulations containing dead or live spores in laboratory tests. First, the relative potency of Toarow CT exposed to UV light under several conditions against silkworm was measured. Table 3 shows that the degree of insecticidal potency diminishes as the distance ('d') between a UV lamp and the sample increases. The half-life of potency was estimated at 9-10 hours at d = 46 cm. Second, we compared the relative potency of a dead-spore *Bt* preparation (Toarow CT) and a live-spore *Bt* preparation (Toarow) against DBM by using the cabbage leaves dipped in the preparations and exposed to UV. As shown in Table 4, the drastic decrease of potency was observed when the irradiation time ('t') was 2 hours. Also, the rate of decrease in insecticidal potency of Toarow CT seems to be less than the one of Toarow.

Ishiguro and Miyazono (1982) demonstrated the fate of viable *Bt* spores on cabbage leaves and a decrease in the pathogenicity to the silkworm in a glasshouse under different exposure conditions. Though the relationship between exposure to UV under the laboratory and the field in our experiment was not confirmed, the relative potency at t = 5 hours and at t = 10 hours (d = 46 cm) in our experiment correspond to the relative potency at t = 3 days and t = 7 days under exposure to UV light in their experiments, respectively. The exposure of cabbage leaves to UV light in our experiment was shortened to suppress chlorosis ($t \leq 2$ hours,

Table 3. Effect of UV irradiation[a] on efficacy of Toarow CT against silkworm in laboratory tests.

Distance (cm)	Relative ratio (%) of residual insecticidal potency against silkworm after hours								
	0	1	3	5	7	10	24	48	72
32	100	87	77	72	79	–	–	–	–
46	100	95	85	75	70	56	48	31	38
53	100	92	81	79	81	–	–	–	–

[a]UV irradiation was conducted as shown in text. [b]Distance between UV lamp and sample (cm).

Table 4. Effect of UV irradiation[a] on efficacy of various concentrations of *Bt* preparation against DBM.

Distance[b] (cm)	Mortality (%)[c] of larvae of DBM on treated cabbage leaves														
	$t^d = 0$	0.5	2	0	0.5	2	0	0.5	2	0	0.5	2	0	0.5	2
	Toarow CT 0.001%			Toarow 0.001%			Toarow CT 0.1%			Toarow 0.1%			Contol		
11	47	3	10	20	7	7	93	80	77	97	77	70	10	10	7
	50	13	10	40	13	10	100	90	90	100	100	90	10	10	7
22	47	13	7	20	3	7	93	73	83	97	93	73	7	0	0
	50	47	13	40	43	7	100	100	90	100	100	77	10	0	7

[a]UV irradiation was conducted as shown in text. [b]Distance between UV lamp and sample (cm). [c]Mortality (%) after 2 days in the upper row and after 3 days in the lower row. [d]t: the irradiation time (hours). Thirty 3rd-instar DBM larvae were placed on each cabbage leaf.

d≤22 cm). Our results on the mortality of DBM fed on the cabbage leaves dipped in *Bt* preparations and exposed to UV might suggest that the decrease in the number of spores due to UV irradiation relates to the mortality of DBM in the case of a live-spore *Bt* preparation.

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