Persistence of Natural and Genetically Engineered Insecticides Based on *Bacillus thuringiensis*[1, 2]

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ABSTRACT The effects of environmental factors on the persistence of formulations of *Bacillus thuringiensis* Berliner were investigated in a greenhouse study. The persistence of Dipel™, a conventional formulation of *B. thuringiensis*, was compared with that of MVP™, a commercial formulation consisting of *Pseudomonas fluorescens* Migula genetically engineered to express a δ-endotoxin gene of *B. thuringiensis* subsp. *kurstaki*. Sprayed foliage bioassayed with third instars of *Pseudoplusia includens* (Walker) indicated that overall persistence of Dipel™ was significantly better ($P < 0.05$) than that of MVP™, though the 2.5% difference probably was not meaningful from a practical standpoint. The two formulations had significantly ($P < 0.05$) better persistence on cotton than on soybean or tomato, though there was still > 25% bioassay mortality on all three plant species after 14 d. Sunlight and a combination of precipitation and ultraviolet light were most detrimental to the *B. thuringiensis* formulations, followed by precipitation only and ultraviolet light only. The formulations were most stable in the dark with no precipitation.

KEY WORDS *Bacillus thuringiensis*, microbial control, microbial persistence, *Pseudoplusia includens*, soybean looper.

The bacterium *Bacillus thuringiensis* Berliner is a safe and efficacious insecticidal agent, but certain of its characteristics can reduce this efficacy. One such characteristic is its poor persistence on vegetative surfaces. The entomocidal crystal δ-endotoxins of *B. thuringiensis* are subject to rapid inactivation by environmental factors such as sunlight, rain, and wind (Gelernter 1990b). Sunlight reportedly is the most detrimental factor (Pinnock et al. 1977); visible light, wavelengths above 400 nm, and ultraviolet light can be detrimental to *B. thuringiensis* (Griego and Spence 1978).

Various methods have been developed to increase the persistence of *B. thuringiensis*. The most common has been the use of spray adjuvants such as ultraviolet photostabilizers (Hostetter et al. 1975, Griego and Spence 1978, Morris 1983). Recently, a cell-encapsulation technique was developed (Gelernter 1990a). A δ-endotoxin gene of *B. thuringiensis* subsp. *kurstaki* was inserted into *Pseudomonas fluorescens* Migula. These recombinant-DNA bacteria, grown in

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culture, produce δ-endotoxin crystals at the end of the growth stage.However, unlike *B. thuringiensis,* the *P. fluorescens* cells do not lyse. Therefore, the crystal δ-endotoxin remains enclosed within the cell; the bacteria are then killed by heat and iodine. This cell-encapsulation process stabilizes the cell walls, thereby enclosing the crystal proteins. The insecticidal activity of this recombinant-DNA bacterium reportedly has greater environmental stability than *B. thuringiensis* formulations based on natural strains (Gelernter 1990b). However, the environmental factors involved in this increased persistence are not known.

The purpose of this study was to determine the effect of precipitation, ultraviolet light, and sunlight on the persistence of cell-encapsulated and conventional *B. thuringiensis* formulations on cotton, soybean, and tomato leaves.

Materials and Methods

Formulations. Two formulations based on *B. thuringiensis* were used in the experiment. The formulation Dipel™ (Abbott Laboratories, North Chicago, IL), based on the natural strain of *B. thuringiensis* subsp. *kurstaki*, was rated at 8,800 international units (IU)/mg. This strain contains the spore and two different δ-endotoxins produced by CryIA(a) and CryIA(c) genes. The experimental formulation MVP™ (Mycogen Corp., San Diego, CA) is a product of the CellCap™ technology based on a single δ-endotoxin gene of *B. thuringiensis* subsp. *kurstaki* inserted into *P. fluorescens,* the cells of which are killed at the end of the bacterial growth phase (Gelernter 1990b).

Insect rearing and plant culture. Larvae of the soybean looper, *Pseudoplusia includens* (Walker), were used in the bioassays. The insects were obtained from the ARS-USDA Southern Field Crop Insect Management Laboratory (Stoneville, MS). Larvae were reared in 300-ml paper cups with modified artificial diet (Burton 1969) containing brewer's yeast instead of torula yeast and a solution of 3.6 and 41.8% phosphoric and propionic acid, respectively, instead of 10% formalin. Pupae were sexed, then 25 pupae of each sex were placed in a 3.78-liter oviposition container, with cheesecloth as ovipositional substrate. Adults were provided with a 10% honey solution (Jensen et al. 1974). The insects were reared at ≈27°C, 50-70% relative humidity (RH), and a photoperiod of 14:10 (L:D) h. Three species of plants were used in the bioassay: cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), and tomato (*Lycopersicon esculentum* Miller). Plants were grown in 0.45-liter pots in the greenhouse until they reached the desired vegetative stage. Soybean plants were used at the V5 or V6 stage (Fehr and Caviness 1977). Cotton and tomato plants were used at the four- to five-leaf stage. An aqueous fertilizer solution was supplied weekly to the plants.

Application of formulations. The bacterial formulations were applied to plants with an adjustable spray pattern, hand-held sprayer (Model Spray Pal, Delta Industries, Sun Valley, CA). The sprayer was calibrated to deliver ≈0.75 ml per burst, though there was no control of the exact spray pressure. At a distance of 25 cm, two such bursts produced a homogeneous spray within a circular area of ≈15 cm diameter.

For the experiments, the two bacterial formulations were diluted in a 0.05% suspension of sticker-spreader, Triton-X-100™, in order to increase the adherence onto leaves. Preliminary experiments determined the concentration of each formulation on each plant species that would result in 90-to-100% mortality of third-instar *P. includens* on the day of application. Dipel™ was applied at 8.46 $\times 10^3$ IU/ml water and MVP™ at 0.01 × the original formulation.

The plants were sprayed in the greenhouse. Young leaves and leaves unevenly sprayed were immediately removed. The sprayed plants were allowed to dry for 1 h and then were allocated randomly to the experimental environmental regimens.

Experimental regimens. Treated plants were subjected to five environmental factors: control (no light or precipitation), precipitation only, ultraviolet light only, precipitation plus ultraviolet light, and sunlight only. It was necessary to run the experiment in the greenhouse rather than outdoors in order to control these variables. Two plants of each species were sprayed per treatment per replication. For "no light" treatments, plants were placed immediately after spraying in a booth with three contiguous compartments completely covered with black plastic, and then set in a greenhouse where the rest of the experiment was in progress. For the "precipitation" treatment, the plants similarly were placed in the dark, and the entire plant was exposed daily to pre-calibrated sprinkling of 34-mm of water for 15 min. Plants in the "ultraviolet light" treatment were exposed only to ultraviolet light from a 20-watt blacklight bulb (Model F20T12/BL, Philips Lighting Company, Somerset, NJ) emitting in a 100-360 nm range, suspended 20-30 cm above the plants. Plants in the "sunlight" treatment were exposed to full sunlight in the greenhouse. Plants in the control, ultraviolet light, and the sunlight treatments were watered only from the base of the plant. Two pre-calibrated hygrothermographs (Model 5020 HI-Q WEATHERtronics, West Sacramento, CA) were used to record the range of daily temperatures and relative humidity (RH) values during the experiment. One hygrothermograph was installed in the covered area allocated to plants kept in the dark. The second instrument was placed outside the covered booth, close to plants exposed to direct sunlight. Air temperature and RH were virtually identical inside and outside the covered booth; RH ranged from 24-100%, and the temperature reached 46°C.

Preliminary research established the effect of darkness on the growth and survival of experimental plants. Cotton leaves remained green and attached to the plants for 14 d, at which time some leaves began to yellow. Soybean and tomato plants began yellowing at 4-5 d; most of the leaves were lost from 7-10 d. The preliminary experiments indicated that *P. includens* larvae fed normally, without unusual mortality, on the plants kept in the dark, as long as the plants remained alive.

Bioassay. Leaf samples were collected from all 45 treatments (three plant species, two bacterial formulations plus an untreated control, five environmental regimens) immediately after treatment and 2, 5, 7, 10, and 14 d after plants were sprayed. For each sample, leaves were randomly collected from the top, middle, and bottom of each plant. The leaves then were laid, lower side down, in plastic petri dishes and transported to the laboratory.

Eight to 10 leaf disks were cut from each leaf. Leaf disks were cut with a 0.7-cm diameter cork borer and placed in 30-ml cups containing 2% agar for moisture. There were 25 disks per treatment per replication. One third instar of *P. includens* was transferred with a camel's-hair brush into each cup. The larvae were allowed to feed for 2 d, then they were transferred into cups containing the soybean looper artificial diet and observed an additional 12 d for mortality. Only larvae that ate 90% or more of the leaf disc, for a total of 20 insects per treatment per replication, were transferred to artificial diet and retained in the experiment. There were three replications per treatment.

Statistical analysis. The effects of environmental regimen, plant substrate, bacterial formulation, days after treatment, and the interactions were tested by the general linear models (GLM) procedure with Tukey's Studentized Range (HSD) test for comparison of means (SAS Institute 1985). The dependent variable was the mean percentage mortality corrected for mortality in untreated controls (Abbott 1925). Mortality in the untreated controls averaged 3.9% and ranged from 0-8.3%. Main treatment effects as well as all eleven 2-, 3-, and 4-way interactions were included in the GLM model.

Results

The environmental regimen, plant substrate, and formulation all affected persistence of mortality in bioassay insects (Tables 1, 2, Figs. 1, 2). Persistence of the *B. thuringiensis* formulations differed significantly ($P < 0.01$) among the five environmental regimens (Table 1). The sunlight and precipitation plus ultraviolet light treatments were most detrimental to Dipel™ and MVP™. The precipitation and ultraviolet light treatments were not as detrimental to the formulations as sunlight or the combined effect of precipitation plus ultraviolet light. Dipel™ had significantly ($P < 0.05$) better persistence than MVP™ (Table 1). However, the difference probably was not meaningful from a practical standpoint. The persistence of Dipel™ was approximately 5% higher on certain days (Fig. 2A). The *B. thuringiensis* formulations were more stable on cotton leaves than on tomato or soybean, with the greatest difference at 7-10 d after application (Fig. 2B). The number of days after treatment was highly significant ($P < 0.01$, Table 2), as would be expected in a study of environmental decay curves.

Certain interactions were significant (Table 2). None of the 3- or 4-factor interactions was significant ($P > 0.05$, Table 2). The only significant ($P < 0.05$) 2-factor interaction that did not involve days after treatment was the environmental regimen X plant substrate (Table 2).

Discussion

Persistence of the CellCap™ product MVP™ was significantly lower from a statistical standpoint than persistence of the formulation based on a natural strain of *B. thuringiensis* (Dipel™), though the overall difference of 2.5% (Table 1) was negligible from a practical standpoint. Thus, the CellCap™ technology did not increase persistence of insecticidal activity for *P. includens* feeding on cotton, soybean, or tomato foliage in a greenhouse setting. It is possible that the presence of a living spore and second δ-endotoxin (Dipel™) contributed to

Table 1. Persistence of two *Bacillus thuringiensis* formulations sprayed onto three different crop plants and subjected to five environmental regimens.

	Overall mean bioassay % mortality*
Environmental Regimen	
Dark, No Precipitation	75.4 a
Ultra Violet Light (UV)	70.8 b
Precipitation	70.2 b
Precipitation and UV	64.5 c
Sunlight	63.1 c
Plant Substrate	
Cotton	75.3 a
Tomato	67.0 b
Soybean	64.2 c
Formulation	
Dipel™	70.1 a
MVP™	67.6 b

Bioassay with third instars of *Pseudoplusia includens* for 14 d. *Means, within each experimental category, followed by the same letter are not significantly different ($P > 0.05$; Tukey's Studentized Range (HSD) test [SAS Institute 1985]). Three replications with 20 insects per treatment per replication.

increased persistence over that of a single δ-endotoxin (MVP™). These results support previous research indicating that spore-crystal aggregates can delay inactivation by ultraviolet, near ultraviolet, and visible light (Griego and Spence 1978).

The persistence of Dipel™ and MVP™ based on the current study was greater than previous estimates of persistence based on live spore counts. The half-life of spores ranges from 1 d (Ignoffo et al. 1974) to 2 d (Pinnock et al. 1974). The half-life in the current study was ≈10-11 d. Lynch et al. (1980) reported a linear decay of *B. thuringiensis* spores within 12 d. The MVP™ and Dipel™ formulations in the current study retained about 70% of their original activity 1 wk after application (Fig. 2A).

The persistence of Dipel™ and MVP™ in the current study also was greater than persistence determined by bioassay of insecticidal activity of *B. thuringiensis* on eastern red cedar (*Juniperus virginiana* L.), sorghum (*Sorghum vulgare* Pers.), and cotton (Hostetter et al. 1975, Gardner and Hornby 1987, Ali and Young 1993). On *J. virginiana,* Dipel™ lost 20% of its activity within 1 d and 95% within 7 d after application (Hostetter et al. 1975). The

Table 2. General linear models procedure indicating sources of variation in bioassay % mortality.

Source	df	SS	*F*	*P* > *F*
Environmental regimen	4	27098	80.4	0.0001
Plant substrate	2	5691	33.8	0.0001
Environ. * plant	8	5368	8.0	0.0001
Formulation	1	527	6.3	0.0128
Environ. * formul.	4	336	1.0	0.4086
Plant * formul.	2	98	0.6	0.5609
Environ. * plant * formul.	8	393	0.6	0.7915
Day after treatment	5	187773	445.9	0.0001
Environ. * day	20	7255	4.3	0.0001
Plant * day	10	5616	6.7	0.0001
Environ. * plant * day	31	2040	0.8	0.7946
Formul. * day	5	477	1.1	0.3423
Environ. * formul. * day	19	578	0.4	0.9944
Plant * formul. * day	10	1568	1.9	0.0498
Environ. * plant * formul. * day	31	797	0.3	0.9999

activity of Dipel™ on sorghum was reduced to 50% within 2 d and was totally lost in less than 1 wk. However, mortality on the day of application was less than 27% (Gardner and Hornby 1987). The half-life of *B. thuringiensis* applied to cotton leaves in the field was 1.5-3.1 d (Ali and Young 1993, Beegle et al. 1981). The longer persistence on cotton in the current study might be due to a greenhouse environment less harmful than outdoors, perhaps due to the passage of sunlight through the greenhouse glass.

Sunlight was the most detrimental environmental factor to *B. thuringiensis,* just as it has been in previous research (Ignoffo et al. 1974, Krieg 1975, Ignoffo et al. 1977, Pinnock et al. 1977, Dulmage and Aizawa 1982). Sunlight is more harmful than ultraviolet light because it contains a broader range of wavelengths, including ultraviolet, near ultraviolet, and visible light. All three types of light are deleterious to δ-endotoxins (Griego and Spence 1978).

Precipitation also was deleterious to the *B. thuringiensis* formulations (Table 1), which is in contrast to the results of Ishiguro and Miyazono (1982). The latter did not observe a significant inactivation of *B. thuringiensis* by artificial precipitation; however, their experiment was based on spore counts. In the current study, the bioassay method with *P. includens* monitored activity of the δ-endotoxin. Thus, the two studies together suggest that precipitation is detrimental to the spores, but not to the δ-endotoxin crystals, of *B. thuringiensis*. This conclusion would have to be tested with further research.

The differences in persistence on cotton, soybean, and tomato (Table 1; Fig. 2B) could be due to plant structure or perhaps to secondary factors such as plant compounds. Plant species with dense leaf pubescence or deep junction

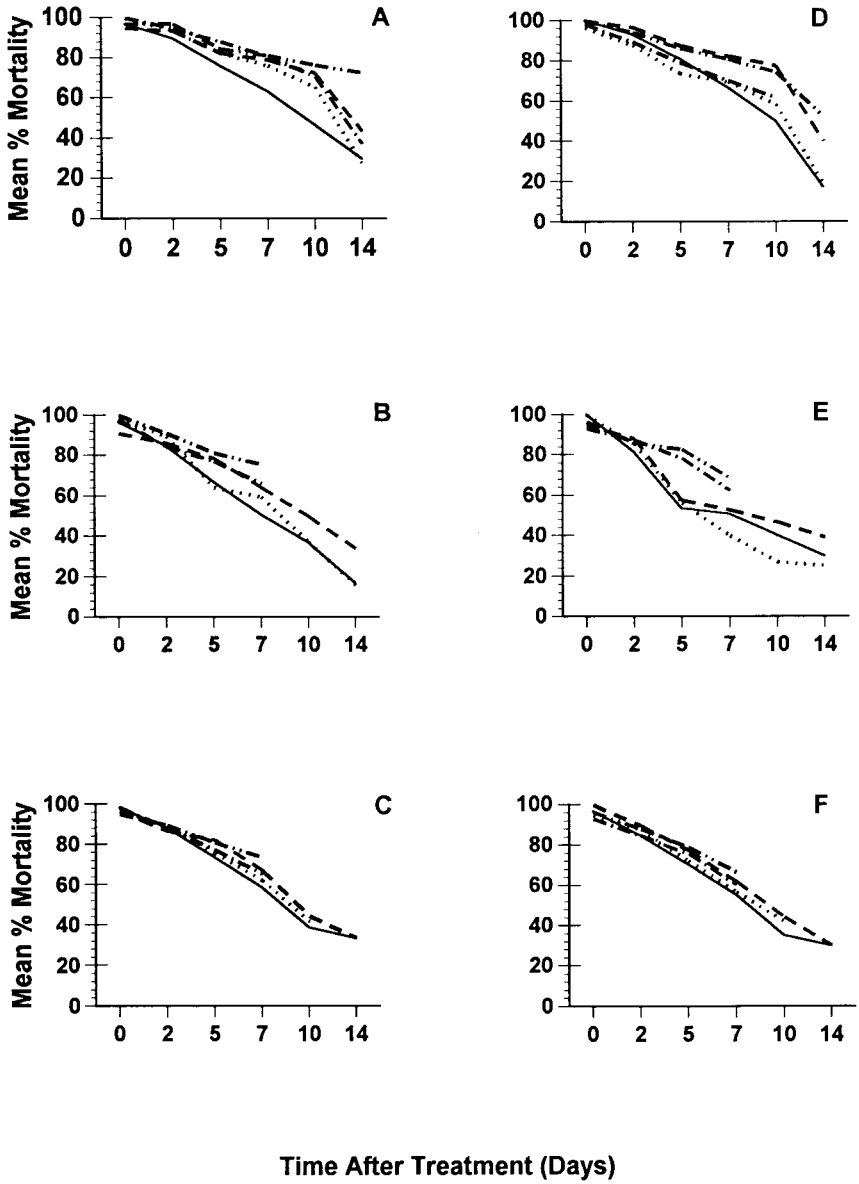

Fig. 1. Effect of environmental factors on persistence of Dipel™ on cotton (A), soybean (B), and tomato (C), and of MVP™ on cotton (D), soybean (E), and tomato (F), determined by percentage mortality in bioassays of sprayed foliage with third instars of *Pseudoplusia includens*. Environmental regimens included: darkness with no precipitation on foliage (– ·· –), darkness with 34 mm precipitation per day (– · –), ultraviolet (UV) light with no precipitation (– –), UV light with 34 mm precipitation per day (·· ·· ··), and sunlight with no precipitation (——).

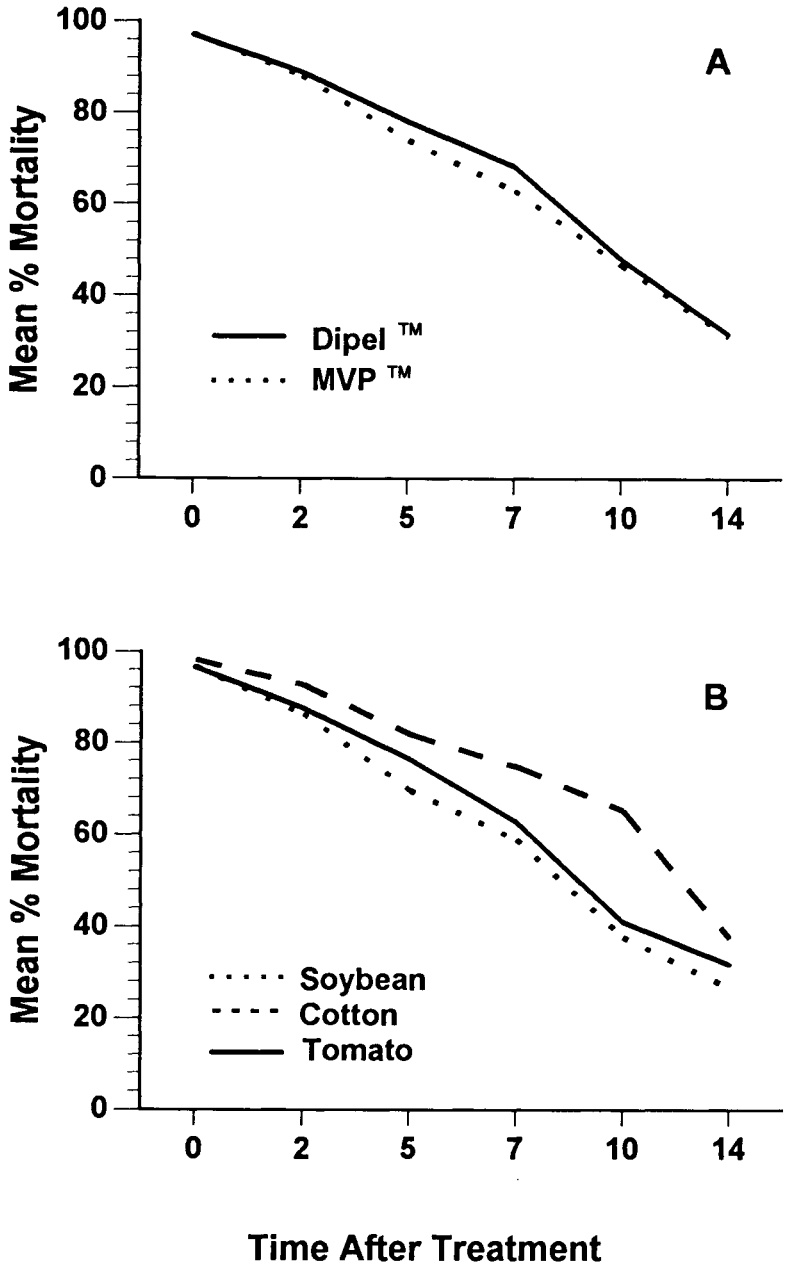

Fig. 2. Overall mean persistence of two formulations of *Bacillus thuringiensis* (A) on three crop plants (B), determined by percentage mortality in bioassays of sprayed foliage with third instars of *Pseudoplusia includens*.

lines separating epidermal cells might present shading areas for protection of δ-endotoxin of *B. thuringiensis*. The type of environment on the surface of the leaf can affect the survival of epiphytic bacteria (Blakeman 1982). Polyphenols, especially tannins, can precipitate proteins, inactivating them irreversibly (Lüthy et al. 1985). Delta-endotoxins are proteins and, therefore, could be inactivated by foliage rich in tannins, such as cotton. Lüthy et al. (1985) suggested that tannins released by digestive activity in larval gut might inactivate ingested δ-endotoxins before they reach the target site. The current results do not support the hypothesis of Lüthy et al. (1985), because persistence was greater on cotton, which is richer in tannin than soybean or tomato (Lüthy et al. 1985). Alternatively, the activity of *B. thuringiensis* might be enhanced by host plant tannins (Brewer and Anderson 1990). Tannins are defensive mechanisms of plants and can be detrimental to insects as well as microorganisms. The end result might depend on whether *B. thuringiensis* δ-endotoxin or the insect pest is more affected by the tannins (Brewer and Anderson 1990).

Thus, the overall purpose of the experiment, clarification of reason for improved persistence of cell-encapsulated δ-endotoxin over that of a conventional *B. thuringiensis* formulation, was negated due to the lack of improved persistence. Further research is necessary to determine why the cell-encapsulated products have improved environmental stability in some situations (Gelernter 1990b) but not in others.

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