Response of Starch-Encapsulated \textit{Bacillus thuringiensis} Containing Ultraviolet Screens to Sunlight

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\textbf{ABSTRACT} Formulations of \textit{Bacillus thuringiensis} Berliner spores and crystals, encapsulated together within a starch matrix containing no ultraviolet screens, lost all spore viability and insecticidal activity against the European corn borer, \textit{Ostrinia nubilalis} (Hübner), within 4 d. Encapsulated crystals and spores with Congo red or folic acid as screens exhibited moderate spore viability and retained at least 50\% of their original toxicity after 12 d. Congo red was the most effective protectant, followed by folic acid and para amino benzoic acid. Because \textit{Bacillus thuringiensis} is likely sensitive to the entire ultraviolet-component in sunlight, ability to provide broad-band ultraviolet protection is required to prolong insecticidal activity effectively.

\textbf{KEY WORDS} Insecta, \textit{Bacillus thuringiensis}, sunlight, encapsulation

\begin{abstract}
\textbf{SOLAR INACTIVATION of \textit{Bacillus thuringiensis}}
Berlin and other entomopathogens is a widely recognized phenomenon. Exposure for short periods (<24 h) to wavelengths below 500 nm inactivates spores, degrades protein structures of viral inclusion bodies, and may inactivate \textit{B. thuringiensis} crystalline toxins. The half-life for \textit{B. thuringiensis} has been estimated at 3.8 h when exposed to an ultraviolet (UV) source representative of the UV radiation in natural sunlight (Ignoffo et al. 1977). Numerous attempts have been made to develop protective measures against damaging UV radiation under field conditions, but success has been limited, suggesting that effects of solar radiation on entomopathogens are not sufficiently understood (Raun \& Jackson 1966, Ahmed et al. 1973, Morris 1983).

The response of \textit{B. thuringiensis} to solar radiation is complicated by differing responses of the spores and crystalline endotoxin. A variety of responses to the UV spectrum by \textit{B. thuringiensis} have been observed including absorption by spores at 270 nm (Morris 1983), inactivation of spores at 330 nm (Griego \& Spence 1978), production of peroxide compounds leading to inactivation of spores and possibly of crystals (Ignoffo \& Garcia 1978), production of photosensitive coproporphyrin compounds at 400–425 nm (Harms et al. 1986), and crystal inactivation caused by tryptophan destruction (Pozsgay et al. 1987).

The above studies suggest that although there may be differential responses of spores and crystals to solar radiation, exposure to any wavelength or combinations of wavelengths below 500 nm will inactivate \textit{B. thuringiensis} depending on exposure time and intensity levels. Therefore, methods capable of shielding \textit{B. thuringiensis} from the entire UV-energy component in sunlight (300–500 nm) should have the highest potential for extending insecticidal activity.

To be an effective control agent for the European corn borer, \textit{Ostrinia nubilalis} (Hübner), \textit{B. thuringiensis} must maintain insecticidal activity long enough to affect the maximum number of early instars in the first generation without repeated applications. In the Midwest, first-generation oviposition ranges from early June to early July, with the highest proportion of early instars occurring roughly in a 10–12-d interval in middle to late June (Showers et al. 1983). A granular formation enables effective delivery of the \textit{B. thuringiensis} into the whorls of the corn plant, which is the preferred habitat of the first-generation borers. Moreover, because \textit{B. thuringiensis} spores contribute to the overall insecticidal activity on the European corn borer (Mohd-Salleh \& Lewis 1982), formulations must protect spores as well as crystals. The objective of this research was to develop the means to extend the insecticidal activity of \textit{B. thuringiensis} for at least 12 d under field conditions with selected UV screens for the control of the European corn borer.

\textbf{Materials and Methods}

\textbf{Ultraviolet Screens.} The following materials were selected as experimental UV protectants based on their ability to provide protection to viruses under laboratory conditions (Shapiro et al. 1983; Shapiro 1985, 1989): laboratory grade Congo red
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Propagation of *B. thuringiensis*. *B. thuringiensis* HD-1 (NRRL B-3792) was propagated on a modified yeast-malt-glucose medium containing beer extract (0.5%), malt extract (0.5%), tryptone (0.5%), peptone (0.5%), glucose (0.5%), and potassium dihydrogen phosphate (0.1%). Fermentations were done using the method of Nickerson et al. (1974), which produced 100 g HD-1 (wet weight) of cell paste containing about 30% spores and crystals by weight after centrifugation. This paste was stored at 2°C until used.

Encapsulation Procedures. *B. thuringiensis* and UV screens were encapsulated in a starch matrix according to the method of Dunkle & Shasha (1988), except that before grinding in a blender, the gelatinous mass was placed at 2°C for 24 h, which promoted more complete association of amylopectin and amylose chains, and reduced the amount of pearl starch used from 50 to 30%. The following materials were used in the encapsulation system: pearl corn starch (CPC International, Englewood Cliffs, N.J.), pregelatinized starch (Mirage 463 starch, A. E. Staley Manufacturing, Decatur, Ill.), refined corn oil (Mazola, CPC International), and distilled water. Experimental UV protectants were incorporated at the initial gelatinization step as aqueous suspensions at either 0.1 or 1.0%. Granules passing 20-40 mesh (1,500-750 μm diameter) containing approximately 0.3% *B. thuringiensis* by weight were used in these experiments.

Sunlight Exposures. To expose experimental formulations of encapsulated *B. thuringiensis* directly to sunlight, cylindrical Plexiglas containers (1.9 cm by 7.0 cm diameter), each containing a 2-g sample, were placed 30 cm above ground level in an outdoor area that received unobstructed sunlight exposure from late May and through mid-August of 1987. These containers were open at the top and had nylon fabric (100 mesh) mounted on the bottoms to facilitate air movement and minimize heat build-up. A duplicate set of samples was exposed in the same manner except that a 125-mm thick, clear Plexiglas sheet was placed 10 cm above these samples to filter out that part of the solar spectrum below 400 nm. Subsamples (300 mg) were taken from each container at 4-d intervals and stored in darkness at 2°C until assayed. Controls consisted of duplicate samples stored in darkness at 2°C and samples containing UV screens without *B. thuringiensis* exposed to sunlight, while the treatment samples were receiving sunlight exposure. These exposure regimens were repeated three times. All radiation energy and intensity levels in the laboratory and outdoor exposure areas were measured with a Licor LI-1800 recording radiometer (Licor, Lincoln, Nebr.). A radiometric profile for the Plexiglas filter was obtained by placing the sensor under the plastic sheet and recording the resulting spectrum. Climatological data were obtained from the National Climatic Data Center (NOAA, Asheville, N.C.).

Assay Procedures. Samples from the direct sunlight exposure trials were assayed for insecticidal activity against neonate European corn borer larvae (Dunkle & Shasha 1988). The neonate larvae were allowed to feed on hydrated starch granules for 24 h, and surviving insects were transferred to artificial diet (No. 9078, BIOSERV, Frenchtown, N.J.). Mortality was recorded immediately after the 24-h exposure period (acute toxicity likely caused by gut paralysis and starvation) and again on the insects that survived the exposure period 4 d after transfer (delayed toxicity representing mortality due to combined effects of crystal toxicity and possibly septicaemia).

To determine the effects of sunlight on spore viability, a *B. thuringiensis* spore plating assay was developed. Small samples (100 mg) of encapsulated *B. thuringiensis* were treated with 5 ml of a 2% Termamyl 120 amylase solution for 2 h at 25°C. The suspension was mildly agitated for 20 s with a sonic cell disruptor and a 0.1-ml aliquot was removed and plated out by a three-fold serial dilution on a tryptic soy agar medium (Difco Laboratories, Detroit) (Ignoffo & Garcia 1978). Following incubation for 24 h at 30°C, colonies, each representing one viable *B. thuringiensis* spore, were counted. Formulations containing no *B. thuringiensis* served as controls to differentiate contaminants.

We used standard analysis of variance (ANOVA) techniques (SAS Institute 1982) to calculate means, confidence limits, and frequencies, to analyze interactions and plot data. Comparisons were made within ANOVAs by linear contrasts.

Results

Absorbances of UV Screens. Of the UV screens tested, PABA, a screen commonly used in suntan lotions, exhibited a narrow range of absorbance with peak absorbance at 275 nm extending to 320 nm (Fig.1). Folic acid exhibited a wider band of absorbance with the major peak at 284 nm, but also with moderate absorbance between 300 and 400 nm. Congo red had the broadest absorbance pattern with peaks at 235, 340, and 500 nm. More detailed information on the absorbance characteristics of these materials is available (Gurr 1971, 490; Sayre & Marlowe 1981; Shapiro 1985).

Climatological Data. Climatological patterns throughout these trials were characterized by warm to hot daytime temperatures and extended periods of sunlight unobstructed by clouds (Table 1). Daytime temperatures exceeded 32.2°C on 30% of the days throughout this period.

Sunlight Exposure. Although the overall sunlight spectrum between 300 and 1100 nm varied little among days or within a given day, intensity varied with time of day. Morning (0900 hours)
energy values ranged from 100 to 260 μmol/m² per second between 300 and 500 nm, whereas afternoon (1200 and 1500 hours) values ranged from 350 to 465 μmol/m² per second, depending on air quality, weather conditions, and other factors. The presence of cloud cover reduced intensity, although a proportionately larger amount of energy in the UV range (300-400 nm) compared with the visible range penetrated through the clouds.

Measurements also were taken of sunlight penetrating a 120-cm tall cornfield canopy on a sunny and a cloudy day (Table 2). The radiometer was placed between rows to measure maximum penetration. About 50-90% penetrated through the first 30 cm, whereas only about 20% penetrated to a depth of 60 cm. Shadow effects apparently caused comparatively lower light penetration under sunny conditions. Low levels of UV radiation were recorded at ground level.

The Plexiglas filter was effective in filtering out virtually all of the sun’s UV energy below 360 nm and approximately 30% of the UV energy between 360 and 400 nm (Fig. 2). The use of the filter allowed us to observe the effects of the higher range UV radiation on B. thuringiensis spores and crystals under field conditions.

Table 1. Climatological data for Peoria, Ill., June–August 1987 (NOAA, National Climatic Data Center, Asheville, N.C.)

<table>
<thead>
<tr>
<th>Month</th>
<th>Temptures</th>
<th>Sunlight</th>
<th>Rainfall in.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. max.</td>
<td>Avg. min.</td>
<td>Max.</td>
</tr>
<tr>
<td>June</td>
<td>39.6</td>
<td>17.1</td>
<td>37.2</td>
</tr>
<tr>
<td>July</td>
<td>32.1</td>
<td>20.1</td>
<td>37.3</td>
</tr>
<tr>
<td>August</td>
<td>28.8</td>
<td>17.5</td>
<td>37.8</td>
</tr>
<tr>
<td>Monthly avg.</td>
<td>30.2</td>
<td>18.2</td>
<td>37.8</td>
</tr>
</tbody>
</table>

**Spore Counts.** B. thuringiensis spores that were starch encapsulated but which did not contain any UV screen became inactivated rapidly by sunlight. Only a few spores were observed to germinate after 4 d of sunlight exposure, representing <1% of the original viability. Subsequent comparisons were therefore made only among experimental formulations containing a UV screen.

Spore viability of control samples that were not exposed to sunlight did not diminish over time within a trial or from trial to trial. Because only the initial difference in spore counts among the formulations was a significant source of variation (P < 0.01), the overall mean spore count for each formulation was used as a baseline to compare reductions in viability of corresponding treatments. Data are expressed as percent of original spore count (relative spore count).

Overall, the presence of a UV screen significantly enhanced survival of B. thuringiensis spores over the 12-d exposure interval (P < 0.01) (Fig. 3). When combined effects of UV screens on spore viability were plotted against time, the average reduction in spore viability was 3% per day from day 4 through day 12. However, there was a sharp drop-off in spore viability in all samples between days 0 and 4.

Spore viability remained higher in formulations containing screens at 1.0% compared with corresponding 0.1% levels (P < 0.01), indicating that ability to extend spore survival was related to the concentration of UV screen in the formulation (Fig. 4). Overall, Congo red and folic acid had similar effects and both were more effective at 1.0% than at 0.1% (P < 0.05). PABA at both levels was only slightly more effective than no screen (P < 0.01)
Fig. 2. Unfiltered (a) vs Plexiglas-filtered sunlight (b) between 300 and 500 nm.

and showed no significant differences between levels ($P > 0.10$).

Analyses were made comparing PABA, folic acid, and Congo red (each at 0.1%) to a formulation that contained all three screens at the 0.1% levels. The average relative spore count for the three screens formulated separately over the 12-d exposure period was 13.1%, and the average spore count for them combined in one formulation was 13.9%, which is a nonsignificant difference ($P > 0.10$). However, a mixture of PABA and folic acid each at 0.1% did offer improved protection ($P < 0.05$) over PABA or folic acid alone (19.2 versus 7.4%).

Unfiltered sunlight was generally more damaging to encapsulated spores than filtered sunlight. Spore counts of formulations containing a screen at the 1.0% level remained higher throughout the 12 d of exposure if exposed to filtered versus unfiltered sunlight (27.3 versus 17.5%, $P < 0.05$). At 0.1% levels, however, spore counts from exposure to filtered versus unfiltered sunlight were similar (17.0 versus 12.2%, $P > 0.10$).

**Insect Assays.** Encapsulated starch samples containing UV screens without *B. thuringiensis* that were stored at 2°C in darkness or exposed to sunlight did not kill any neonate European corn borers, indicating that neither the starch nor any of the screens was toxic.

Encapsulated *B. thuringiensis* formulated without UV screens exhibited no insecticidal activity after 4 d of continuous exposure to sunlight. In contrast, encapsulated *B. thuringiensis* formulated with Congo red (Fig. 5) exhibited an average acute toxicity of 29.5% and an average delayed toxicity of 63.0% throughout the 12 d of sunlight exposure (1.0 and 0.1% data combined, $P < 0.01$). *B. thuringiensis* formulated with folic acid had a similar, but somewhat lower, overall response with 16.3% acute toxicity and 49% delayed toxicity ($P < 0.01$). When level and day interactions combined over screens and trials were tested, results indicated that acute toxicity caused by the *B. thuringiensis* formulations containing 0.1% Congo red and folic acid were similar throughout the 12-d sunlight exposure period (Fig. 6). Acute toxicities of corresponding 1.0% formulations were higher, but appeared to decline after day 8. Delayed toxicity similarly appeared to decline after day 8; however, at the 0.1% level delayed toxicity declined between days 4 and 8. All formulations containing either Congo red or folic acid retained >50% of their original toxicity toward European corn borer after continuous exposure to sunlight for 12 d, when the combined effects of acute and delayed toxicity are considered.

Effects of unfiltered sunlight on acute toxicity of encapsulated *B. thuringiensis* containing Congo red or folic acid at either 0.1 or 1.0% were similar...
to filtered sunlight \( (P > 0.10) \). However, differential effects on delayed toxicity between Congo red and folic acid were observed. Delayed toxicity caused by folic acid at 0.1% was lower than the 1.0% folic acid for filtered and unfiltered sunlight (Table 3). Conversely, at the 0.1% level Congo red caused higher delayed toxicity from filtered sunlight (71.5 versus 42.5%), but higher delayed toxicity from unfiltered sunlight at the 1.0% level (77.8 versus 58.7%).

**Discussion**

The biological activity of *B. thuringiensis*, as measured by spore viability and toxicity to neonate European corn borers, can be extended greatly under natural conditions up to 12 d if encapsulated in starch along with broad-band UV absorbers. Encapsulation with starch alone did not provide protection. Formulations containing Congo red or folic acid exhibited significant spore survival and insecticidal activity after 12 d even though they were exposed directly to unobstructed sunlight during a period in which daytime temperatures exceeded 32.2°C on 30% of the days in the trials. Measurements of sunlight penetrating a corn canopy indicate that much of the UV radiation does not penetrate beyond 60 cm. Under normal field conditions, most of the encapsulated *B. thuringiensis* will be deposited in whorls and leaf axils and therefore may be exposed to less solar radiation than that provided in these experiments.

Although solar radiation below 360 nm was filtered out by Plexiglas, the remaining radiation was still damaging to *B. thuringiensis*. Spores were rapidly inactivated and insect toxicity was quickly lost by exposure to sunlight (filtered and unfiltered), when no UV screen was added to the encapsulated formulation. The sharp initial decline in spore viability of formulations containing screens was like-

**Table 3. Effect of filtered versus unfiltered sunlight on insecticidal activity (delayed toxicity) of encapsulated *B. thuringiensis* against neonate European corn borer (days and trials combined)**

<table>
<thead>
<tr>
<th>UV SCREEN</th>
<th>LEVEL</th>
<th>DAY</th>
<th>Mean % delayed toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>0.1</td>
<td>4</td>
<td>42.5a</td>
</tr>
<tr>
<td>FA</td>
<td>0.1</td>
<td>4</td>
<td>71.5c</td>
</tr>
<tr>
<td>CR</td>
<td>1.0</td>
<td>8</td>
<td>77.9b</td>
</tr>
<tr>
<td>FA</td>
<td>1.0</td>
<td>8</td>
<td>58.7d</td>
</tr>
<tr>
<td>CR</td>
<td>0.1</td>
<td>12</td>
<td>38.0e</td>
</tr>
<tr>
<td>FA</td>
<td>0.1</td>
<td>12</td>
<td>59.5f</td>
</tr>
<tr>
<td>CR</td>
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<td>12</td>
<td>38.7e</td>
</tr>
<tr>
<td>FA</td>
<td>1.0</td>
<td>12</td>
<td>58.5f</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different \( (P < 0.01) \).
ly due to destruction of the spores on the surface of the granules, where they receive less protection from the screens. The effect on spore viability did not appear to correlate with insecticidal activity (Fig. 5). Further studies are needed to determine if reduction in spore viability in formulations containing screens is related to granule size.

Broad-band screens were generally more effective than narrow-band screens. Congo red was the most effective, followed by folic acid then PABA. This correlates well with their absorption spectra (Fig. 1). When a narrow-band screen (PABA) was used with an intermediate band absorber (folic acid), the increased level of protection was additive but did not statistically exceed the level of protection provided by the broad-band absorber (Congo red). These data support the thesis that better protection is afforded by screens capable of broad-band UV absorption.

Formulations containing screens at the 1.0% level were more effective in extending spore viability and insecticidal activity than at corresponding 0.1% levels. Overall, insect mortalities at 24 h were similar; however, Congo red formulations showed superior extended insecticidal activity based on delayed toxicity. Folic acid formulations showed somewhat lower activities compared with Congo red formulations. Although it is possible that higher concentrations of these screens could give more protection, they also may reduce palatability of the formulations to the insects. We observed that larvae avoided samples containing >1% folic acid. If residual activity beyond two weeks is needed, higher concentrations of *B. thuringiensis* could be encapsulated to compensate. It is noteworthy that overall insecticidal activity remained above 50% original activity for the Congo red and folic acid formulations.

Under field conditions, insects will potentially be in contact with encapsulated *B. thuringiensis* for at least several days of their critical period of susceptibility during the first two to three instars in contrast with the 24 h of exposure in the assay, and this will possibly result in higher overall mortalities.

The differential effects of filtered versus unfiltered sunlight are best observed by comparing insect assay results (delayed toxicity) at the 0.1% levels. Formulations containing 0.1% folic acid performed remarkably similarly (38.0% for filtered versus 38.7% for unfiltered), indicating that folic acid is effective in absorbing UV radiation below 400 nm, with the reduced activity of the *B. thuringiensis* due to the radiation above 400 nm. In contrast, 0.1% Congo red formulations, although giving higher overall levels of protection compared with folic acid and showed much higher toxicity when exposed to filtered (71.5%) than to unfiltered (42.5%) sunlight, suggesting that Congo red is effective in absorbing UV radiation above 400 nm but is less efficient in absorbing UV radiation below 400 nm. These results correlate well with the absorption spectra for these two compounds (Fig. 1).

Results at the 1.0% levels for folic acid formulations were similar; however, Congo red at 1.0% showed higher activity when exposed to unfiltered sunlight (77.8%) than to filtered sunlight (58.7%). It is possible that reduced larval feeding occurred because of a photoreaction of the Congo red to a compound that made these formulations less palatable or to unexplained random variation, even though the data were consistent among trials. These results await further verification.

Starch-encapsulation coupled with broad-band UV screens such as Congo red provides protection from solar radiation that exceeds methods developed to date. Numerous UV screens have been evaluated in the laboratory and in the field for protection from solar radiation (e.g., Ignoffo & Batzer 1971, Bull et al. 1976, Shapiro et al. 1988; Shapiro 1985); however, biological activities of entomopathogens have rarely been extended over several days. In addition to the need for a UV screen(s) that can absorb the entire UV region, a medium is needed to evenly disperse the screen around individual spores and crystals. Starch encapsulation provides a simple and inexpensive method to disperse both entomopathogens and UV protectants.

Interestingly no insect toxicity was observed in samples in which there also was no spore viability, supporting the hypothesis that solar radiation inactivates the crystals as well as spores (Pozsgay et al. 1987). Mohd-Salleh & Lewis (1982) have shown that *B. thuringiensis* spores alone are not pathogenic to neonate and 6-d-old European corn borer, but act synergistically with crystals to achieve higher comparative mortalities. However, preliminary assays using the 24-h exposure method in addition to the tissue culture assay method of Johnson & Davidson (1984) indicate that purified (99%) *Bacillus thuringiensis* spores and purified crystals alone are toxic to neonate and second-instar European corn borers. Further studies to determine the responses of solar-irradiated and non-irradiated purified spores and crystals on early instar European corn borer are needed.

**Acknowledgment**

We thank Terry Neisen for advice and consultation on the statistical analyses, and Deborah Black for her technical assistance.

**References Cited**


Received for publication 17 August 1988; accepted 8 March 1989.