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Compatibility of Photoactive Dyes with Insect Biocontrol Agents

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Integrated pest management (IPM) programmes often look for more specific ways to control pests. Biological control agents, such as the bacterium, Bacillus thuringiensis *Berliner, and the fungus,* Beauveria bassiana *(Balsamo) Vuillemin, can control insects with minimal disturbance to the environment because of their host specificity and short half-lives. Often these agents alone cannot prevent yield loss or are too expensive. This study looked at the* in vitro *combination of these agents and photoactive dyes, especially phloxine B (red dye D&C 28), a Food and Drug Administration approved dye, with the intent to provide better insect control. Photoactive dyes are being tested for the control of many pest insects. Phloxine B and related xanthene dyes, eosin y, fluorescein and rose bengal inhibited the growth of both* B. thuringiensis *and* B. bassiana. *Phloxine B was the most inhibitory and fluorescein the least inhibitory dye for both microbes. The magnitude of inhibition increased with increasing concentration of dye and light intensity. Therefore, an adverse effect on the field performance of these biological control agents in combination with xanthene dyes would be expected.*

Keywords: *insect biocontrol,* Bacillus thuringiensis, Beauveria bassiana, *phloxine B, xanthene dyes*

INTRODUCTION

In recent years, the development of photoactive molecules as candidate pesticides has gained renewed vigor. These compounds become more active in sunlight rather than being inactivated quickly by sunlight. Several types of photoactive dyes have shown insecticidal action, but the class of compounds shown to be most effective against insects is the halogenated xanthenes (Heitz, 1995). Phloxine B is one of the most efficient pesticides of this class. The insecticidal activity of the xanthene dyes begins when the dye absorbs a photon of light. This photon raises the dye first to an excited singlet state and then to an

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excited triplet state. The excited dye molecule gives up this energy to ground state oxygen, producing oxygen in the excited triplet state. Finally, the excited oxygen reacts with the target substrate. This oxidation, and the resulting cellular damage, is believed to cause the death of the insect (Heitz, 1995). According to Heitz (1995), many insects are susceptible to photodynamic action by dyes.

Phloxine B or D&C red no. 28 is used in drugs and cosmetics, and has therefore undergone extensive toxicological testing by the Food and Drug Administration in the approval process. D&C red no. 27 and its disodium salt, D&C red no. 28, have been shown not to be carcinogenic in either rats or mice based on long-term dietary studies. The acceptable daily intake for humans has been set at 1.25 mg kg^{-1} /day (Lipman, 1995). Acute toxicity studies have been performed in laboratory rats, mice and dogs. This dye was approved by the Environmental Protection Agency for experimental use against the fruit fly (Moreno & Mangan, 1995), although the effects on non-target invertebrates as well as on soil microbes are not known.

Few of these dyes have been investigated for antimicrobial activity, especially in regard to microbes with which they might be combined in an integrated pest management (IPM) programme. One group of insecticidal photoactive compounds, the thiarubrines, has been shown to have light-activated antimicrobial activity against Gram-positive bacteria. This group also has *in vitro* antifungal activity that does not require light (Ellis *et al.*, 1995). Rose bengal, a xanthene dye, has been used as an inhibitor of fungal growth in media preparation (Martin, 1950), and is known to have increased toxicity towards fungi under continuous light. The antimicrobial activity of other photoactive dyes is not well known.

The effects of xanthene dyes on two microbial pesticides in commercial use were investigated. For these microbials, *Bacillus thuringiensis* Berliner and *Beauveria bassiana* (Balsamo) Vuillemin, an inhibition of germination or growth may affect field performance. The effect of *B. bassiana* on insects, including Colorado potato beetle, *Leptinotarsa decemlineata* (Say), requires the germination and growth of the fungus (Vey & Fargues, 1977). While the main toxicity of *B. thuringiensis* is in the crystal, spores present in many commercial preparations enhance insecticidal activity for certain insects (Heimpel & Angus, 1959). Many authors have proposed that the germination of spores and subsequent multiplication of bacteria are responsible for enhanced insect control.

MATERIALS AND METHODS

Strains and Media

The *B. thuringiensis* strains used were: NRRL B-14961, a non-toxic strain; NRRL B-14962, a mosquito-toxic strain; NRRL B-18197, a caterpillar-toxic strain; and NTEN3-2, a beetle-toxic strain. NTEN3-2 was isolated from Novodur (Abbott Labs, Chicago, IL). The *B. bassiana* strain, MBAS 3-1, was isolated from Mycotrol (Mycotech, Butte, MT).

B. thuringiensis strains were propagated on T3 agar (Travers *et al.*, 1987) for sporulation and various dilutions of L-agar (Miller, 1972). Half-strength L-agar was used routinely for the propagation and recovery of *B. thuringiensis* (recovery medium, RM). Quarter-strength L-agar was used to test for nutrient effects. *B. thuringiensis* was incubated at room temperature (ca. 25°C). Inhibition studies with *B. thuringiensis* were completed with vegetative cells and repeated with spores. When it was discovered that the strains of *B. thuringiensis* used showed no difference in inhibition with phloxine B, additional experiments were conducted with NTEN3-2 as a model for *B. thuringiensis* in general.

B. bassiana was cultivated on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) from conidia at 25°C.

Application of Dyes to Media and Inoculation of Microbes

Sterile Sensi Discs (Beckton Dickinson and Co., Cockeysville, MD, USA) were autoclaved, then soaked in various concentrations of dyes for 1 h. Excess dye solution was decanted

and the dye-impregnated disks were stored at 4°C in the dark until needed. Phloxine B and the related xanthene dyes, rose bengal, fluorescein and eosin yellow were purchased from the Sigma Corporation (St Louis). Assays were performed in polystyrene Petri dishes with internal diameters of 86 mm containing 20 ml of RM or other media as described above. *B. thuringiensis* (ca. 10^5 cells) or *B. bassiana* (ca. 10^4 conidia) suspensions were spread as a lawn. Disks were placed aseptically on the lawn at least 20 mm from the edge of the plate. For high concentrations of dye and incubation in the light, a single disk was placed in the center of the plate. For intermediate concentrations two disks were placed equidistant from each other. For low concentrations and incubations in the dark, three disks were spaced equidistant on the plate. Blank disks that had absorbed sterile water without dye were used for controls.

Assay of the Effect of Dye on Growth

Three replicate dishes for each dye treatment and controls were incubated at ca. 25°C under cool white fluorescent light. An identical set of Petri dishes was incubated in constant darkness. Light as total visible energy was measured with a light meter (Extech Instruments, Cincinnati, OH) in lux. Lux was used as an estimate of total visible energy because the maximum absorbance of phloxine B is at 540 nm. Varying light conditions were achieved by incubating plates at different distances from cool white fluorescent lights, which have a maximum spectral output around 540 nm (ISCO, 1982). Light conditions between 400 and 900 lux were obtained by incubating plates approximately 50 cm from the fluorescent light source. Light conditions of 4000–4500 lux were obtained by incubating plates 10 cm from the light source. Plates were exposed to light for 16 h during a 24-h period.

Comparison with Antibiotics

For *B. thuringiensis*, the inhibition of growth by phloxine B was compared with the antimicrobials ampicillin, tetracycline, doxycycline and erythromycin in a similar disk assay. Phloxine B and the antimicrobials were compared at therapeutic levels (1 μg ml^{-1}, 5 μg ml^{-1} and 50 μg ml^{-}). Antibiotic disks were stored in the refrigerator (4°C) in the dark.

Statistics

Diameters of zones of inhibition were measured with vernier calipers to the nearest 0.05 mm. For bacteria zones were measured at 24 h and for fungi at 48 h. Each zone reported was the mean of three measurements of zones of inhibition on different plates. Statistical analysis was performed using the MEAN, regression (REG) and general linear model (GLM) procedures from SAS (SAS Institute, 1988).

RESULTS

The growth of both *B. bassiana* and *B. thuringiensis* was inhibited by xanthene dyes, especially phloxine B. Experiments started at concentrations used for insect control (1% or 10 mg ml^{-1}) and concentrations of dyes were decreased to minimal inhibition concentrations (500 μg ml^{-1} in the dark). The inhibition of *B. thuringiensis* increased with the concentration of dye and amount of light, but was independent of strain, media and temperature. *B. bassiana* growth inhibition increased with the concentration of dye and increasing light.

Effects of Phloxine B on *B. thuringiensis*

The growth rates of four different strains of *B. thuringiensis* were similarly inhibited by concentrations of phloxine B ranging from 0.05 to 10 mg ml^{-1} (Table 1). Though the strains fell into two statistically distinct groups ($P < 0.0001$), less than a millimeter separated the zones of inhibition, making this statistical difference biologically inconsequential ($R^2 = 0.992$). This inhibition of growth was observed with incubation in both the light (400 lux, mean diameter of all zones for all strains = 26.27 ± 0.14) and the dark (10.2 ± 0.13),

TABLE 1. Inhibition of *B. thuringiensis* strains by phloxine B

Strain	Mean diameter (mm)[a]
NRRL B-18197	17.76 (0.2)[a]
NRRL B-14962	17.78 (0.2)
NTEN3-2	18.57 (0.2)
NRRL B-14961	18.86 (0.2)

[a]Diameter of zone of inhibition ± standard error (in parentheses).

though the zones of inhibition in the dark were always significantly smaller than those at the corresponding concentration in the light.

When the strains were grown on L-agar, half-strength L-agar and quarter-strength L-agar, there were no statistically significant differences in the diameters of the zones of inhibition observed. For NTEN3-2, the zones of inhibition for 1 mg ml^{-1} at 900 lux were: L-agar, 28.6 ± 1.1 mm; RM, 30.1 ± 2.2 mm; and 0.25 L-agar, 30.8 ± 1.6 mm. In addition, no significant differences were observed with different temperatures from 25°C to 35°C.

Due to the minimal differences among *B. thuringiensis* strains, a single strain, NTEN3-2, was used for the rest of the experimentation. These experiments were conducted using RM for the media and with incubation at 25°C.

Phloxine B caused an inhibition of NTEN3-2, using either vegetative cells or spores, that increased over a concentration range of 1 μg ml^{-1}–10 mg ml^{-1} (Figure 1) and with increasing light intensity from 900 to 4200 lux. The increase in zone of inhibition at 4200 lux was log-linear over this concentration range ($R^2 = 0.97$).

When vegetative cells were used, there was no growth within the zone of inhibition if the plates were subsequently incubated without light. If spores were used, then individual colonies appeared in the zone with further incubation in the dark.

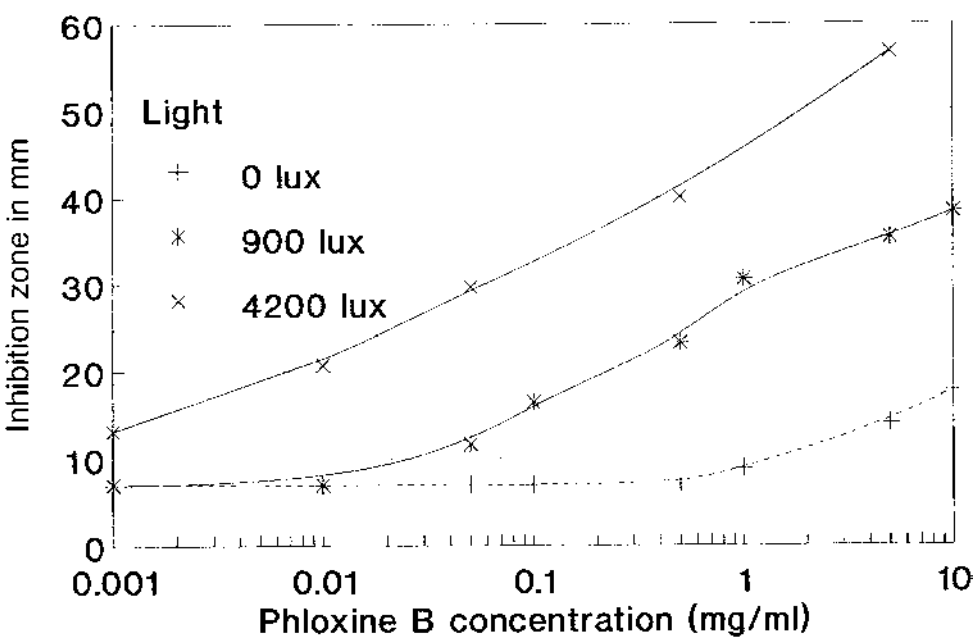

FIGURE 1. Inhibition of *B. thuringiensis* by phloxine B is dependent on concentration and light intensity. Curves were fitted using the curve trend function of Harvard Graphics (Software Publishing Corp., Fairfield, NJ).

TABLE 2. Inhibition of *B. bassiana* by xanthene dyes

Dye	Concentration (mg ml^{-1})	Diameter of zone of inhibition (mm) (standard error in parentheses)		
		0 lux	900 lux	4200 lux
Fluorescein	5.0	None	None	20.2 (1.9)
Eosin	0.05	None	None	8.7 (0.5)
	0.5	None	10.3 (1.5)	22.0 (0.4)
	5.0	14.2 (0.8)	43.6 (0.4)	48.7 (1.7)
Rose bengal	0.05	None	21.4 (0.5)	15.1 (0.9)
	0.5	9.2 (0.2)	41.6 (1.5)	26.6 (1.4)
	5.0	15.3 (2.7)	51.0 (1.0)	41.9 (0.2)
Phloxine B	0.5	None	20.6 (2.1)	27.5 (1.2)
	5.0	12.5 (0.4)	52.0 (1.0)	53.1 (1.0)

Effect of Xanthene Dyes on *B. bassiana* and *B. thuringiensis*

In the dark there was no inhibition of *B. bassiana* growth by phloxine B at concentrations up to 0.5 mg ml^{-1}. However, in the light (900 lux) growth was significantly inhibited ($P < 0.0001$) when measured in the same manner as *B. thuringiensis* (Table 2). Zones of inhibition measured greater than 52 mm in diameter at concentrations of 5 mg ml^{-1} at 4200 lux.

Generally, *B. bassiana* growth was inhibited less by other xanthene dyes. The growth of *B. bassiana* was inhibited only by fluorescein at the highest concentration (5 mg ml^{-1}) and higher light condition (4200 lux) tested. The zones of inhibition surrounding disks on *B. bassiana* lawns increased with both increasing concentrations of dye and increasing light. The inhibition pattern of *B. bassiana* to rose bengal was unusual in that the greatest inhibition of growth was under lower lighting (900 lux).

When tested against other xanthene dyes, *B. thuringiensis* growth was not inhibited when bacteria were incubated in the dark in concentrations of up to 5 mg ml^{-1}. The growth of *B. thuringiensis* was inhibited in the light as shown in Figure 2. Phloxine B was the most inhibitory dye and fluorescein was the least inhibitory dye over the range of concentrations studied (0.5–5 mg ml^{-1}). These concentrations of dyes are known to affect insects (Heitz, 1995).

Comparison of Antibiotics and Phloxine B

Amoxicillin at concentrations of 1, 10 and 50 μg ml^{-1} was not inhibitory against *B. thuringiensis* in the dark or light. Phloxine B at concentrations of 1, 10 and 50 μg ml^{-1} was also not inhibitory in the dark (Table 3). In the light at 4200 lux, the zones of inhibition caused by phloxine B were similar to erythromycin and doxycycline at the high concentrations and larger at the lower concentrations. Only erythromycin showed a slight increase in the zone of inhibition when the plates were incubated at 4200 lux.

DISCUSSION

Phloxine B and xanthene dyes, in general, were shown to inhibit the growth of the biological control agents, *B. thuringiensis* and *B. bassiana*. *B. thuringiensis* was inhibited in the light at concentrations comparable to three antibiotics tested. This inhibition was greater in the light than in the dark. The cool white fluorescent lights used throughout these experiments are recommended by Heitz (1995) as having the correct spectrum for the activation of

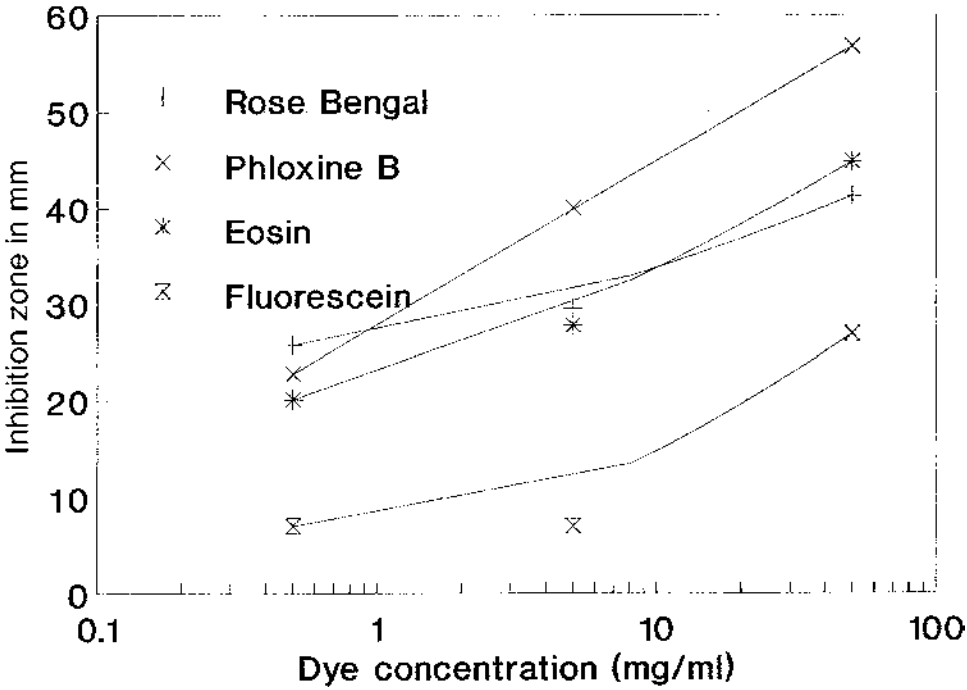

FIGURE 2. Inhibition of *B. thuringiensis* by xanthene dyes is dependent on both concentration and type of dye in light at 4200 lux. Curves were fitted using the curve trend function of Harvard Graphics.

TABLE 3. Inhibition of *B. thuringiensis* by phloxine B and antibiotics[a]

Antibiotic	Concentration (μg ml^{-1})	Mean diameter of zone of inhibition (mm) (standard error in parentheses)		
		0 lux	900 lux	4200 lux
Tetracycline	10	11.5 (0.3)	11.9 (0.6)	12.4 (0.7)
	50	19.4 (0.2)	18.7 (0.7)	18.9 (0.6)
Doxycycline	1	16.2 (0.2)	12.8 (0.7)	16.6 (0.6)
	10	22.8 (1.0)	21.7 (0.7)	24.0 (0.2)
	50	29.3 (0.2)	28.5 (0.1)	29.4 (0.2)
Erythromycin	1	None	8.2 (0.2)	11.2 (1.7)
	10	17.3 (2.8)	16.3 (0.9)	18.9 (0.3)
	50	23.2 (0.2)	24.3 (1.2)	27.3 (0.7)
Phloxine B	1	None	None	14.8 (0.8)
	10	None	None	24.0 (0.2)
	50	None	15.5 (2.5)	32.9 (1.0)

[a]*B. thuringiensis* growth was not inhibited by amoxicillin.

xanthene dyes. Rose bengal is the only dye in this group demonstrated to inhibit the growth of some fungi under certain light and media conditions (Martin, 1950; Pady *et al.*, 1960).

The concentration response in the inhibition assay for *B. thuringiensis* growth at 4200 lux was log-linear over a 10 000-fold range. At lower light intensity and in the dark, the response was also log-linear over the range of response from 1 to 10 mg ml^{-1}. As a result of this

linear relationship over a wide range, the inhibition of *B. thuringiensis* growth at different doses and light intensities, such as would occur in the field, should be predictable.

As spores act to enhance the field performance of *B. thuringiensis* (Heimpel & Angus, 1959), it would be predicted that phloxine B would adversely affect the performance of *B. thuringiensis* in the field. Preliminary field data showed that *B. thuringiensis* plus red dye provided only the level of control of Colorado potato beetle produced by phloxine B alone (by larval counts; P. A. W. Martin & R. F. W. Schroder, unpublished). This is surprising, because the majority of the toxicity to Colorado potato beetle larvae caused by *B. thuringiensis* is due to the crystal and not the spore. Further studies are in progress to investigate this phenomenon.

For *B. bassiana*, the greatest inhibition of growth was by phloxine B at 5 mg ml^{-1} in the light (4200 lux). This intensity is lower than the 70 000–150 000 lux that the present authors recorded in a Maryland field in July over the course of a day (data not shown). The fungus is more sensitive to phloxine B in the light than is *B. thuringiensis*, as the zones of inhibition are greater. As the inhibition of *B. bassiana* growth by phloxine B increased with light intensity, growth might not occur under all field conditions. It was not possible to recover *B. bassiana* from leaves within an hour after it was applied with phloxine B under field conditions, although *B. bassiana* was recovered under the same conditions when it was applied alone (P. A. W. Martin & R. F. W. Schroder, unpublished).

Given these data, these biological control agents and phloxine B are unlikely to be compatible in combination for enhanced insect control. However, they may be used in sequence for pest management as they have different modes of action. Phloxine B may also affect the toxicity of *B. thuringiensis* crystals. This could be a specific effect on the protein or its specific mode of action, rather than a general oxidation mechanism. Mutants which are able to grow in the presence of phloxine B might also enhance insect control.

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