Efficacy of Phloxine B as a Bactericidal Agent in Plants

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Agrobacterium tumefaciens cells were killed upon illumination when treated with submicromolar concentrations of phloxine B. Phloxine B could be distributed systemically through a plant by amending the water supply or delivered directly to specific locations through topical applications in 0.1% adjuvant (Kinetic HV). Sweet basil (*Ocimum basilicum* L.), jimsonweed (*Datura stramonium* L.), and soybean (G*lycine max* L.) were infected with *A. tumefaciens* to establish a marker for a systemic pathogenic bacteria. There was a >90% reduction in the plants' population of *A. tumefaciens* when watered daily with a 1 micromolar phloxine B solution. Furthermore, the plants treated with phloxine B were asymptomatic to the pathogenic challenge. Leaf tissue, collected from basil and soybean, also expressed a reduction in the population of *A. tumefaciens* by >90% when treated under greenhouse conditions for 24 h with 10 μM phloxine B in 0.1% Kinetic HV. The possible use of phloxine B as an agent to control bacterial diseases in plants is suggested.

Keywords: *Photodynamic action; singlet oxygen; phloxine B; xanthene dyes*

INTRODUCTION

Bacterial pathogens in plants, such as those that can cause blight or pustule (Kennedy and Tachibana, 1973), can greatly reduce harvest yields, impair the visual appeal of the plant, and endanger plant health. In an effort to gain insight into novel, safe, and environmentally sound measures to eradicate or control bacterial infections in plants, the bactericidal potential of photoactivated xanthene dyes, in particular the compound phloxine B, is being investigated. Halogenated xanthene dyes have been shown previously to have light-mediated herbicidal potential (Knox and Dodge, 1984, 1985a,b) but have been investigated mostly as insecticidal agents (Broome et al., 1975; Callahan et al., 1975). Over two dozen insect species (including flies, mosquitoes, ants, and cockroaches) have been documented as susceptible (Heitz, 1995). Phloxine B is an environmentally safe, water soluble dye that becomes lethal to cells only upon its uptake and exposure to light. Illumination provokes a series of photo-oxidation reactions that take place locally within the affected cell and which can lead ultimately to cell death. Photoactivation generates singlet oxygen, a short-lived toxic substance that attacks sites of unsaturation present in membranes, enzymes (proteins), and metabolites. Many toxic singlet oxygens can be generated from a single molecule of phloxine B before the dye finally decomposes. Although potentially useful as a bactericide and insecticide, the consumption of this dye has been shown to be harmless to humans and it is used commercially as a drug and cosmetic addictive (Lipman, 1995). The U.S. Food and Drug Administration has registered a daily allowable intake of 1.25 mg/kg of body weight for human consumption (Food and Drug Administration, 1982).

This paper reports on in vitro and in vivo experiments in which the use of micromolar concentrations of phloxine B exerts a bactericidal effect. The Gram-negative opportunistic bacterium *Agrobacterium tumefaciens* (E. F. Smith and Townsend) Conn was selected as the initial test organism. *A. tumefaciens* is the causal agent of crown gall, a serious worldwide problem for many plant species. Crown gall infects over 600 species from over 300 genera (De Cleene and De Ley, 1976). Individual *A. tumefacien*s isolates vary widely in their host specificity. Anderson and Moore (1979) found that of the 166 *A. tumefaciens* isolates tested from 26 host species and 11 plant families, no host was infected by all pathogenic strains. Jimsonweed (*Datura stramonium* L.) and tomato (*Lycopersicon esculentum* Miller) were the hosts infected by the largest number of pathogenic *A. tumefaciens* isolates (72 of 89). There are three recognized biovars of *A. tumefaciens* (biovars 1, 2, and 3), with biovar 3 limited to the grape host. The isolate FACH used in this study is a biovar 1, wide host range (Dong et al., l992), a highly infective strain of *A. tumefaciens* that has been shown to induce galling in jimsonweed (Diehl and Graves, 1991).

MATERIALS AND METHODS

Plant Growth Conditions. Jimsonweed was selected because it is a good host, easily germinated from seed in the greenhouse, and produces an obvious gall as a result of infection with the FACH strain of *A. tumefaciens.* Sweet basil (*O. basilicum* L.) was selected because of its ease of culture and its use in initial phloxine B studies. Sweet basil and jimsonweed were grown in a potting mix of peat moss and perlite (1:1) that had 29 kg of dolomitic lime/m^3 and 3.3 kg of Micromax fertilizer/m^3 (Scotts-Sierra Horticulture Products Co.) added. The plants were watered daily and fertilized weekly with a full-spectrum plant amendment. Phloxine B was administered to the plants by supplementing the water

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with the desired concentration of dye. Basil with heights of 10−12 cm was used in these studies. Soybean (*G. max* L.) was grown from seed in vermiculite. The soybean used in these studies was grown until two trifoliate leaf arrays were established. All plants were maintained in a greenhouse environment.

Bacterial Growth Conditions. The FACH strain (biovar 1) of *A. tumefaciens* was isolated by Dr. R. N. Goodman, University of Missouri, Columbia, MO, from *Vitis vinifera* L. and was cultured on nutrient glucose (2.5%) agar (NGA) or modified New and Kerr (NKS) (Burr and Katz, 1983; New and Kerr, 1971), a medium selective for all three *A. tumefaciens* biovars.

Bacterial inocula consisted of FACH cultures grown to an optical density (OD) of 0.1 at 640 nm (Moore et al., 1988) in 100 mL of nutrient glucose (2.5%) broth (NGB). Cultures were grown on a rotary shaker and maintained at 25 °C.

In Vitro Dose Response Profile. An inoculum of 1 mL (0.1 OD, 640 nm) was added to growth medium (100 mL of NGB) that ranged in phloxine B concentration from 0 to 5 μM. The flasks were placed in a completely randomized design on a rotary shaker thermostated at 25 °C and supplemented with continuous lighting (2700 lx) for a period of 24 h. After 24 h, a 1 mL aliquot was removed and serially diluted (up to 10^6 in increments of 100). An aliquot of 100 μL obtained from each dilution was spread onto NGA and NKS agar plates. The number of viable bacteria (colony forming units) present in the culture was quantified by determining the number of individual colonies produced and multiplying by the appropriate dilution factor. A minimum of four replicates was used to obtain each reported average value, with the variance reported as the experimental standard error.

Phloxine B Delivery Systems. Phloxine B was systemically distributed through a plant by amending the daily water supply with 1 μM phloxine B.

A series of commercially available adjuvants [Latron AG-98 (Rohm & Haas), Ortho X-77 Spreader (Universal Coops.), Ad-spray 80 (Setre Chemical Co.), Activator 90 (Loveland Industries), Kinetic HV (Setre Chemical Co.), and Maximizer (Loveland Industries)] was examined for the ability to introduce a halogenated xanthene dye through leaf tissue via a topical application in 0.1% adjuvant. The incorporation of dye was quantified by detecting the presence of [^{125}I]erythrosin B, a xanthene dye present in trace amounts, with a Packard Cobra II auto-gamma counter. [^{125}I]Erythrosin B (3.0 mCi/mL, 4.2 μM) was purchased as a custom synthesis product from ICN. The quantum yield and stability of erythrosin B are comparable to that of phloxine B, but it was more cost-effective to generate a radioactive trace compound by iodinating the parent compound, fluorescein, thus producing [^{125}I]erythrosin B, rather than performing a de novo (^{14}C) synthesis of phloxine B. Ten microliters of 10 μM phloxine B, 0.1% adjuvant, 10 mM HEPES (pH 7.5), and 0.06 μM [^{125}I]erythrosin B (9.1×10^5 cpm) were applied to the central leaf of the first trifoliate of the soybean. Control plants were treated in a similar fashion except with the exclusion of adjuvant. After 48 h, the soybean was sectioned into five samples: the treated leaf, the two associated leaves directly adjacent to the treated leaf, leaf material found above the treated site (the second trifoliate array), and root tissue and cotyledons that together constituted the material below the treated site. Unincorporated dye was removed by washing the treated leaf with two 5 mL aliquots of 50% methanol and blotting dry. A moistened cotton swab was then used to wash the treated area directly. The sectioned plant material was placed in a 12 × 75 mm plastic tube and assayed for ^{125}I. A minimum of three replicates (three individually potted plants per adjuvant trial) was performed for each adjuvant. The amount of dye incorporated with respect to time was determined for the adjuvant Kinetic HV. The same experimental protocol as detailed above was used except the leaf material was harvested at 2, 6, 12, 24, 36, and 48 h intervals. A minimum of seven replicates (seven plants per time point) was performed.

Plant Inoculation. A sterile 22 gauge needle was used to puncture the stem just below a leaf site and deliver 100 μL of inoculum (0.1 OD, 640 nm) of *A. tumefaciens* (1.6×10^5 colony forming units). A control set of plants was inoculated in a similar manner with growth medium only.

Phloxine B as an in Vivo Bacteriostat. Basil and jimsonweed plants were imbibed on a 1 μM solution of phloxine B 2 weeks prior to inoculation to distribute the dye systemically. Control plants were not exposed to dye. One week after being inoculated with *A. tumefaciens*, the inoculation site was excised and weighed. Bacteria, present in the excised tissue, were released into 5 mL of sterile water by grinding the plant material with a sterile mortar and pestle.

The mortar was rinsed with an additional 5 mL of sterile water, which was added to the first 5 mL. The number of viable bacteria per gram of plant tissue in the water samples was determined by colony numbers on NGA and NKS agar plates generated from a 100 μL aliquot. The samples were diluted as needed to obtain a quantifiable value. The NGA and NKS agar plates were maintained at 25 °C and the colonies counted after 14 and 48 h periods. Total bacteria, inclusive of both systemic bacteria and environmental bacteria residing on the tissue surface, were quantified with NGA plates. *A. tumefaciens* (FACH), a soil bacterium that does not normally reside on leaf tissue but spreads systemically after opportunistic infection, was used to infect plants so that systemic affects of the dye could be ascertained through monitoring the presence of *A. tumefaciens* when spread on NKS agar plates. Soybean and basil plants inoculated with *A. tumefaciens* were also treated with a topical application of 10 μM phloxine B, 0.1% adjuvant, and 10 mM HEPES (pH 7.5). Control plants did not receive dye. Dye was applied between 9 and 10 a.m. After 25 h, a leaf directly above the inoculation site was removed with sterile forceps and a 3.14 cm^2 disk excised and weighed. The surface of the disk was sterilized by soaking in 2% Clorox for 3 min and rinsing with sterile water. A mortar and pestle were used to release the bacteria present in the disk into 1 mL of sterile water. Bacteria were quantified as described previously. The reported value is the average determined from a minimum of 13 replicates.

Statistical Analysis. All experimental protocols required a completely randomized design to be followed. Data collected concerning the incorporation of dye with respect to adjuvant or time were analyzed by a one-way analysis of variance and the means separated by Fisher's projected LSD procedure. The Wilcoxon rank sums test for a two-sample test was performed to evaluate whether the difference in viable bacterial populations observed between control and treated samples was significant. A p value < 0.05 had to be reached to be considered significant. The recorded value was obtained as the average of all replicate trials with the variance recorded as the experimental standard error.

RESULTS

Photodynamic Effect of Phloxine B on *A. tumefaciens*. The dose response of bacteria mortality to phloxine B was dramatic when under continuous illumination. A 50% kill was observed with 0.05 μM dye, and 100% (no viable cells) was achieved at concentrations ≥ 0.1 μM (Figure 1).

Basil and jimsonweed could be watered with low levels of phloxine B (1−5 μM) without causing apparent harm to the plant. After a sustained 4 week treatment with 1 μM phloxine B, there was no detectable difference in dry weight, fresh weight, or protein or chlorophyll concentration in treated versus nontreated plants (data not shown). Symptomology of dye toxicity is the photobleaching of green foliage followed by severe desiccation (Rebeiz et al., 1991). These effects were not manifested at the low levels of dye used. However, the presence of the dye afforded these plants a bactericidal agent, as evidenced by a 99% reduction ($p < 0.05$) in the *A. tumefaciens* population (Table 1). The bacteri-

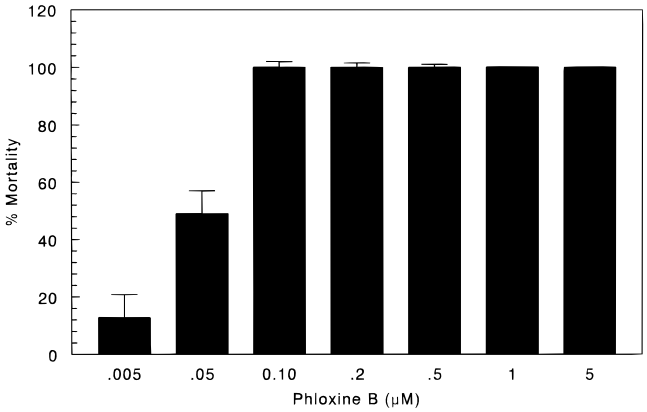

Figure 1. Demonstrated increase of *A. tumefaciens* (FACH) mortality with increasing concentrations of phloxine B after 24 h of illumination (2700 lx). The control cultures possessed 1.6×10^6 colony forming units/mL. The values reported are an average of four replicates with standard error bars.

Table 1. Reduction of *A. tumefaciens* Populations in Plants Maintained on 1 μM Phloxine B and Evaluated 1 Week after an Inoculation Challenge with *A. tumefaciens* (FACH)

	sweet basil		jimsonweed
	total bacteria[a]	*A. tumefaciens*[b]	*A. tumefaciens*[b]
control[c]	7.1×10^6	3.0×10^6	3.2×10^6
phloxine B	9.0×10^4	4.4×10^3	2.7×10^4
% inhibition	99	99	99

[a] Total viable bacteria (colony forming units) released per gram of tissue from the excised inoculation site were determined by dilution plating with three replications on nutrient glucose agar. [b] Total viable *A. tumefaciens* released per gram of tissue from the excised inoculation site was determined by dilution plating with three replications on modified New and Kerr selective medium. [c] A significant difference ($p < 0.05$) was present between all control samples and their corresponding phloxine B treated sample.

cidal effect was not discriminatory for *A. tumefaciens* in that the total bacterial count was reduced by the same level. Jimsonweed has previously been shown to form basal galls when infected with *A. tumefaciens* (Diehl and Graves, 1991) within a 2−3 week period. Sixty-five percent of the jimsonweed infected with *A. tumefaciens* (13 of 20) established a small basal gall. None of the phloxine B treated jimsonweed (0 of 25) were observed to form a gall after a 10 week observation period.

Adjuvant Facilitated Uptake of Phloxine B. Six commercially available adjuvants (which function primarily as nonionic surfactants) were tested for their ability to promote dye uptake. Amounts of 46−88% of a 10 μM phloxine B solution, applied in aliquots of 10 μL, were incorporated into leaf tissue when applied with 0.1% adjuvant (Table 2). AG-98 promoted the least amount of dye uptake, whereas X-77 generated the largest amount of incorporated dye. There was no significant difference among the other adjuvants tested (Ad-spray, Activator 90, Kinetic HV, and Maximizer). Kinetic HV (a gift from Dr. D. Shaw, Mississippi State University) was utilized as the adjuvant in all subsequent studies. When adjuvant was omitted, there was no incorporation of dye. Regardless of the adjuvant employed, ~97% of the dye remained localized in the area where applied (perhaps a function of the low level of dye used in this study). The uptake of dye appears to be relatively fast in that there is no significant difference between a 6 h application and a 48 h application made with 0.1% Kinetic HV (Table 3). The majority of the dye was incorporated in the first few hours after application. This is consistent with the mode of transport for these adjuvants, which require a wetted (noncrystalline) state to function efficiently.

Bactericidal Effects Provided by a Topical Application of Phloxine B. Plants challenged with an inoculation of *A. tumefaciens* had an in vivo bacterial population ≈100-fold that of the total bacteria (external and internal populations) associated with the unchallenged controls after 24 h (Table 4). Basil and soybean are therefore acting as viable hosts for *A. tumefaciens*. Bacterial populations were reduced by >90% in areas treated with a topical administration of 10 μM phloxine B in 0.1% Kinetic HV (Table 4). In all cases, the control and phloxine B treated data sets were significantly different ($p \leq 0.05$). The total population of bacteria associated with leaf material isolated 7 days after treatment showed no significant difference between control and phloxine B treated samples (data not shown).

DISCUSSION

The loss to commercial agriculture due to bacterial invasion is high. A loss of over $30 000 000 was suffered by farmers due to bacterial diseases of soybean in 1994 (Wrather et al., 1997). During this time, Florida lost almost one-fourth of its soybean production to plant disease (Pratt and Wrather, 1998). Field applications of antibiotics are not cost efficient for bacterial control. Phloxine B, a relatively inexpensive water soluble dye, possesses the capacity upon illumination to exert a photodynamic killing effect upon bacteria, for example, *A. tumefaciens*, and insects. This broad range of efficacy is testament to the indiscriminate nature by which halogenated xanthene dyes mediate the killing effect, through photocatalytic-like production of toxic singlet oxygen (Elstner, 1982; Knox and Dodge, 1985c; Spikes, 1977).

Dye was delivered systemically to the plant by amending the water supply or site-targeted by topically administering the dye in 0.1% adjuvant. The low levels of phloxine B used in this study (10 μM for topical applications and 1 μM as a water amendment) did not appear to harm the plants. Bacterial populations, however, were reduced by >90%, even when the plant had been directly challenged with a pathogen. Gall formation, a common characteristic of *A. tumefaciens* infection in jimsonweed, was not evident when the plant was maintained with a 1 μM phloxine B water regime. Furthermore, the reduction in bacteria population and incidence of galling was achieved under greenhouse conditions where continuous illumination was not present. Therefore, administration of phloxine B was able to reduce the pathogen population and symptomology of a pathogenic challenge. The reduction in bacteria was not permanent. Control levels of bacteria were restored within 7 days, resulting from the decomposition of the dye administered and termination of dye application. Additional incidences of gall formation, however, were not observed in plants previously challenged with *A. tumefaciens* once phloxine B was no longer administered. This indicates that, in this scenario, a pathogenic challenge by *A. tumefaciens* could not be mounted during the period when bacteria were re-establishing a control level value.

It is reasonable to assume that fungi and bacteria residing on external plant surfaces would also incorpo-

Table 2. Adjuvant-Facilitated Uptake of Halogenated Xanthene Dye through Soybean Leaf Tissue[a]

adjuvant	treated trifoliate leaf (cpm × 10^5)	associated trifoliate leaves (cpm × 10^3)	second trifoliate leaf array (cpm × 10^3)	roots and cotyledons (cpm × 10^4)	dye incorporated[b] (%)
AG-98	4.0 ± 0.2	6.1 ± 0.1	0.6 ± 0.3	1.1 ± 0.1	46 A
X-77	7.8 ± 0.2	8.5 ± 0.1	1.9 ± 0.4	1.2 ± 0.1	88 B
Ad-spray 80	6.9 ± 0.3	4.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	78 C
Activator 90	6.5 ± 0.3	7.2 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	74 C
Kinetic HV	5.7 ± 0.1	7.7 ± 0.02	0.9 ± 0.2	0.8 ± 0.1	65 C
Maximizer	6.0 ± 0.2	5.6 ± 0.2	2.0 ± 0.3	0.9 ± 0.1	68 C

[a] A 10 μL aliquot of 10 μM phloxine B (with tracer ^{125}I-labeled erythrosin B: 9.1 × 10^4 cpm) and 0.1% adjuvant were applied to the central leaf of the first trifoliate. After 48 h, the leaf was washed to remove unincorporated dye and the plant was sectioned into five groups to determine the uptake and dispersal of dye. The five groups consisted of the *treated* leaf, the two *associated* leaves adjacent to the treated leaf, leaves of the *second trifoliate* positioned above the treated leaf and below material that represents the combined data from *root tissue* and *cotyledons*. Samples were assayed for ^{125}I. Three replicates were performed for each adjuvant. The replicate average ± standard error was recorded. [b] Different letters within the column denote significant difference ($p < 0.05$).

Table 3. Time Course of Halogenated Xanthene Dye Incorporation into Soybean As Facilitated by 0.1% Kinetic HV Adjuvant[a]

time (h)	incorporated halogenated xanthene (%)	level of incorporated dye that remains in the treated leaf (%)
<0.01	1.2 ± 0.2 A	99
2	33 ± 3 B	98
6	62 ± 6 C	97
12	50 ± 4 C	97
24	59 ± 4 C	97
36	56 ± 5 C	97
48	60 ± 3 C	97

[a] A 10 μL aliquot of 10 μM phloxine B (with tracer ^{125}I-labeled erythrosin B: 9.1 × 10^4 cpm) and 0.1% adjuvant were applied to the central leaf of the first trifoliate. Soybean was harvested at the indicated time, washed to remove unincorporated dye, and sectioned to permit analysis of dye dispersal by quantifying the presence of ^{125}I. Seven replicates were performed for each time point except 48 h, which had 12 replicates. The replicate average ± standard error was recorded. [b] Different letters within the column denote significant difference ($p < 0.05$).

Table 4. Reduced Bacterial Presence in Pathogenically Challenged Soybean and Basil Leaves in Response to Treatment with 10 μM Phloxine B in 0.1% Kinetic HV

	control (× 10^3)	phloxine B treated (× 10^3)	inhibition (%)
unchallenged[a,c] (total bacteria/g of leaves)			
soybean	160 ± 40	6.7 ± 1.1	96
basil	290 ± 50	7.1 ± 2.1	98
challenged with *A. tumefaciens*[b] (*A. tumefaciens*/g of leaves)			
soybean	15000 ± 7000	3.5 ± 1.5	99
basil	26000 ± 4000	340 ± 216	99

[a] Fifty microliters of 10 μM phloxine B, 10 mM HEPES (pH 7.5), and 0.1% Kinetic HV was applied to the leaf of a soybean or basil plant. After 24 h, a 3.14 cm^2 disk was excised from the treated leaf and weighed. Surface and in vivo bacteria were released into 1 mL of sterile water and quantified by dilution plating on nutrient glucose agar. Seventeen replicates were performed. [b] Soybean and basil plants were treated in an analogous manner as stated above except the plants were injected with 100 μL of an *A. tumefaciens* (FACH) inoculum (0.1 OD, 640 nm). The ratio of in vivo bacteria present in control versus phloxine B treated leaves was quantified by dilution plating for *A. tumefaciens* on modified New and Kerr selective medium agar. Thirteen replicates were performed. The replicate average ± standard error was recorded. [c] A significant difference ($p < 0.05$) was present between all control samples and their corresponding phloxine B treated sample.

rate dye due to the action of the adjuvant (skin is an effective barrier to phloxine B but not in the presence of 0.1% Kinetic HV). These organisms would then also be sensitized to light.

Halogenated xanthene dyes may prove to be of benefit in searching for alternative bactericides that are environmentally sound. The human safety of phloxine B has been documented, which makes its potential use all the more attractive. Preliminary results show that pests (white flies and fall armyworm) which feed upon plant material treated with phloxine B also become sensitive to light (data not shown). Baiting the agricultural field with phloxine B treated foodstuffs would be a superior method of pest management, but an attractive second line of defense may be provided to plants after being treated with phloxine B. Over 100 phototoxins, which have been implicated in a variety of plant defense responses (including pathogenic attack), have been identified from higher plant tissues (Downum and Wen, 1995; Ellis et al., 1995; Hudson et al., 1995). Halogenated xanthene dyes may effectively augment the defensive arsenal of plants.

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