Recovery of D&C Red No. 28 from Potato Leaves[1]

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Abstract D&C Red No. 28 is a photoactive red dye that is insecticidal for a number of insect species. Unlike contact insecticides, D&C Red No. 28 must be consumed to be effective. Therefore, the red dye must adhere to leaves on which the insect feeds. During a field test of insecticidal activity of the red dye against the Colorado potato beetle (*Leptinotarsa decimlineata* L.), we measured the recovery of the red dye from potato leaves treated at rates of 70, 210, and 350 g per ha. When the dye was applied alone, we were able to recover over 91% of the red dye at 6 h post treatment and over 30% at one day. At 2 days post treatment, the limit of detection was reached (5 ng D&C Red No. 28/cm^2 leaf). In field studies, PEG 200 which improves dispersal, also improved adherence of red dye to leaves. In the laboratory, adjuvants such as PEG 200, Tween 80, and Gelva® also improved red dye adherence to leaves.

Key Words Phloxine B dye, xanthene dye, photoactive dye, Colorado potato beetle

In recent years, there has been renewed interest in the development of photoactive dyes as potential pesticides. These compounds become more active in sunlight, as opposed to many other pesticides which are quickly inactivated by sunlight. There are several types of photoactive dyes which have shown insecticidal activity, but the class of compounds demonstrated to be most effective against insects is the halogenated xanthenes (Heitz 1995). Phloxine B (D&C Red No. 28) is one of the most studied pesticides in this class. The insecticidal activity of the halogenated 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 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. Oxidation and resulting cellular damage is believed to cause the death of the insect (Heitz 1995).

Phloxine B, or D&C Red No. 28, is used in drugs and cosmetics, and therefore has undergone extensive toxicological testing by the Food and Drug Administration. D&C Red No. 27 and its disodium salt, D&C Red No. 28, have been shown to have no carcinogenic effects in either rats or mice based on long term dietary studies (Lipman 1995). The acceptable daily intake for humans has been set at 1.25 mg/kg/day (Lipman 1995). D&C Red No. 28 was approved by the Environmental Protection

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Agency for experimental use against the Mexican fruit fly, *Anastrepha ludens* (Loew) (Moreno and Mangan 1995).

D&C Red No. 28 has a reported half-life in sunlight of one hour (Bergsten 1995). This would be inadequate for an insecticide which must be ingested. In previous work, we demonstrated in cage studies that D&C Red No. 28 kills insects for several days (Schroder et al. 1998). In field trials, we studied the recovery of D&C Red No. 28 applied alone and with adjuvants. We wanted to determine if adjuvants, such as PEG 200, could affect the recovery of the dye under actual use conditions, with the purpose of extending the time that D&C Red No. 28 was insecticidal.

Materials and Methods

Thirty-three meter row plots of potatoes (*Solanum tuberosum* L. variety 'Superior') were planted in a random block design with 4 replications at U. Maryland Vegetable Farm, Salisbury, MD. D&C Red No. 28 (Warner Jenkinson, St. Louis, MO) was applied at rates of 70, 210, and 350 g/ha, resulting in concentrations of red dye in the finished spray of 0.149, 0.425 and 0.747 g/L, respectively. D&C Red No. 28 was applied alone and with 1% polyethylene glycol 200 (PEG 200, J. T. Baker, Phillipsburg, NJ) at each concentration for a total of 6 treatments. Leaf samples were collected within 1 h after treatment and 6 h post-treatment because D&C Red No. 28 was reported to degrade quickly in the light (Bergsten 1995), and at 1 d and 2 d. Five leaves were collected at each sampling interval for a total of 20 leaves per treatment including an untreated control. Light intensity per steradian per m^2 in the field was measured with an Extech (Ben Meadows, Cincinnati, OH) light meter in lux.

Additional plots were treated with D&C Red No. 28, in combination with *Beauveria bassiana* (Balsamo) Vuillemin as wettable powder and liquid concentrate formulations (Mycotrol™, Mycotech, Butte, MT). These complete formulations, which incorporate *B. bassiana,* contained adjuvants that aid in dispersal, adherence, and UV protection. In laboratory studies, D&C Red No. 28 inhibited the growth of *B. bassiana* in the light (Martin et al. 1998, Mischke et al. 1998). Therefore, as expected *B. bassiana* was not recovered from these initial treatments (data not shown). These treatments were considered as complete formulations that may affect D&C Red No. 28 recovery from leaves. These plots were treated at the rate of 210 g D&C Red No. 28 per ha.

Individual leaves were collected, placed in Whirl Pak® bags (NASCO, Fort Atkinson, WI), and stored at 4°C until processed. The area of the leaf samples was determined using a leaf area meter (LiCor, Lincoln, NE). Five ml of deionized water were added to each bag containing a leaf. The bag was placed in a Stomacher™ blender (Techmar, Cincinnati, OH) and processed on the low setting for 30 s. Higher settings or longer times resulted in damage to the leaf which interfered with the fluorometric readings. The liquid was decanted into a 4-ml quartz cuvette and the fluorescence of D&C Red No. 28 was measured in a Shimadzu RF-5000U scanning fluorometer (Shimadzu Instruments, Columbia, MD). The wavelengths used for excitation were 300 to 600 nm. Emission was measured at 560 nm. Excitation peaks for D&C Red No. 28 occurred at 307 nm, 346 nm, 425 nm, and 540 nm. Concentrations of D&C Red No. 28 at 0.15 μg/ml to 5 μg/ml produced a linear response ($r^2 > 0.98$, relative fluorescence vs. concentration) that was 10× more sensitive than the response using spectrophotometric measurements. The absorbance maximum of D&C Red No. 28 varied from 538 nm to 558 nm in this concentration range (data not shown). Concentrations of D&C Red No. 28 recovered from leaves were determined by comparison to a standard curve generated the same day the samples were analyzed. Recovery was calculated as nanograms D&C Red No. 28 per cm^2 of leaf surface.

Counts of large Colorado potato beetle (*Leptinotarsa decimlineata* L.) larvae (third and fourth instar) were made 4 d after application. Counts were recorded as larvae per 10 stems.

Laboratory experiments were conducted to determine the effect of other adjuvants on D&C Red No. 28 recovery. PEG 200, Tween 80 (Difco Laboratories Detroit, MI), and Gelva® (Monsanto, St. Louis, MO) were used at 1% of the final solution. Three mid-size potato leaves were treated with solutions of D&C Red No. 28 alone or D&C Red No. 28 with 1% adjuvant. Concentrations of 50, 500, 1000, and 5000 µg/ml of D&C Red No. 28 were used to simulate field application concentrations, for a total of 12 leaves per treatment. The leaves were stirred gently on a magnetic stirrer for 120 s in 100 ml solutions of red dye alone or red dye with adjuvants, and allowed to dry on racks. Dry leaves were placed in Whirl Paks and refrigerated overnight. These leaves were then processed as described above. To determine efficiency of recovery from leaves, a known amount of dye was placed on the leaves and processed as described above.

Statistical analyses were performed using SAS procedures for regression for standard curve calculations and analysis of variance for differences among treatments (PROC REG, PROC MIXED, SAS Institute 1997). The REG procedure was used to calculate D&C Red No. 28 concentration from standard curves. For analysis of field data, the MIXED procedure with repeated measures was used; D&C Red No. 28 concentration and the presence or absence of adjuvants were fixed effects. To analyze the effects of four different formulations on field recovery of D&C Red No. 28, the Mixed procedure was used with formulation as a fixed effect. For laboratory analysis of recovery of D&C Red No. 28 from leaves we used the Mixed procedure with fixed effects of treatment and concentration. For all analyses, variance grouping was used because of heterogeneous variances.

Results

Recovery of red dye under field conditions. Because the activity of D&C Red No. 28 against insects is dependent on illuminance (Foundren and Heitz 1978, Schroder and Martin, unpubl. data), readings in lux were collected. The light readings varied from 79,000 lux at the time of application (9 AM) to a maximum of 131,500 lux 1 h after initial sampling (12 noon) to 82,400 lux 6 h after initial sampling. Between the 6 h post treatment sample and the 1 d post treatment sample, there was a brief shower (rainfall less than 0.5 cm) which may have affected recovery. At the time the next two samples were collected, the illuminance measurements were 78,800 lux at 1 d; and 76,000 lux at 2 d, similar to the illuminance at time of application.

While D&C Red No. 28 alone in water had excitation peaks at 307 nm, 346 nm, 425 nm and 540 nm when fluorescence emission was measured at 560 nm, these peaks sometimes shifted as much as 5 nm when D&C Red No. 28 was recovered from leaves. Most of the variability was within the 5 nm bandwidth used for detection.

At the highest application rate of D&C Red No. 28 (350 g/ha), we recovered significantly more red dye from leaves when the red dye was applied alone than when the red dye was applied with PEG 200 ($F = 6.63$; df = 111; $P < 0.001$). Because some red dye was still visible on these leaves, this suggests that the addition of PEG made the red dye more difficult to remove from leaves (Fig. 1). At 6 h post treatment 91.9% of the initial amount of red dye applied was recovered from leaves treated with D&C Red No. 28 alone, and 83% of the initial amount of red dye applied was recovered from leaves treated with D&C Red No. 28 + 1% PEG 200. From the 6 h post treatment sample to the 1 d sample there was an additional decline of 66% in red dye recovery in the treatment with red dye alone compared to a 50% decline in the recovery of red dye in the D&C Red No. 28 + 1% PEG 200 treatment. During this time interval, there was intermittent rainfall.

Recovery of red dye was more variable from leaves treated with D&C Red No. 28 alone. By 2 d post-treatment, the limit of detection by this method, approximately 5 ng/cm^2 of leaf surface, had been reached for D&C Red No. 28 + 1% PEG 200 with no red dye visible on the leaves. The red dye alone was still recovered at more than 100 ng/cm^2 of leaf surface, comparable to the initial recovery of the 210 g/ha treatment.

The initial recovery of D&C Red No. 28 for the medium (210 g/ha) and the low (70 g/ha) application rates were not statistically different with and without PEG 200 (medium, $F = -.035$; df = 75; $P = 0.7272$; low, $F = -0.51$; df = 90.7; $P = 0.609$). There was

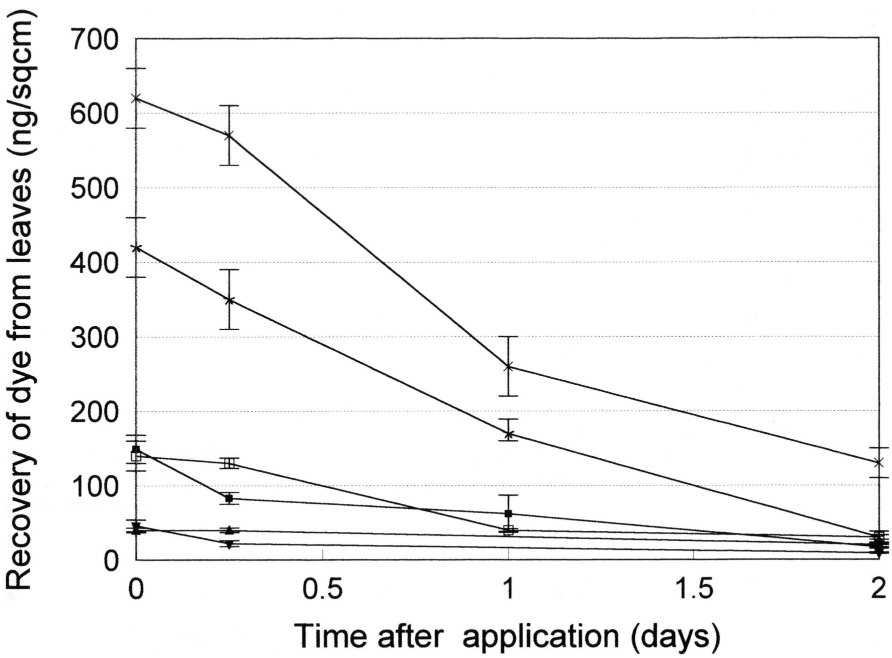

Fig. 1. Recovery of D&C Red No. 28 from field collected potato leaves per square centimeter of surface. X - D&C Red No. 28 applied at 350 g/ha, ✻ - D&C Red No. 28 + 1% PEG 200 applied at 350 g/ha, □ - D&C Red No. 28 applied at 210 g/ha, ■ - D&C Red No. 28 + 1% PEG 200 applied at 210 g/ha, ▲ - D&C Red No. 28 applied at 70 g/ha, ▼ - D&C Red No. 28 + 1% PEG 200 applied at 70 g/ha. The points represent the mean of 20 samples.

no significant decline in recovery of red dye from either of these treatments at 6 h post treatment when D&C Red No. 28 was applied alone. However, the recovery of the red dye from leaves treated with D&C Red No. 28 + 1% PEG 200 declined by 50% at 6 h post treatment.

The recoveries of red dye from treatments of D&C Red No. 28 with the commercial Mycotrol formulations (both wettable powder and liquid concentrate) were more consistent than the recovery of D&C Red No. 28 alone or D&C Red No. 28 + 1% PEG 200 (Fig. 2). However, recovery of D&C Red No. 28 in combination with both commercial formulations also declined with time. For the red dye applied alone, the recovery declined 74% overnight, where the decline in the red dye + 1% PEG was 26%, suggesting that the red dye + 1% PEG did not wash off to the same extent when it rained.

Insect counts. Counts of large larvae (third and fourth instar) 4 d after application are shown in Table 1. Insect numbers were high with 23 large larvae per 10 stems in the controls. Larval counts per 10 stems ranged from a high of 51.75 on the 210 g of D&C No. 28 + Mycotrol liquid concentrate treatment to a low of 6.75 on the 210 g of

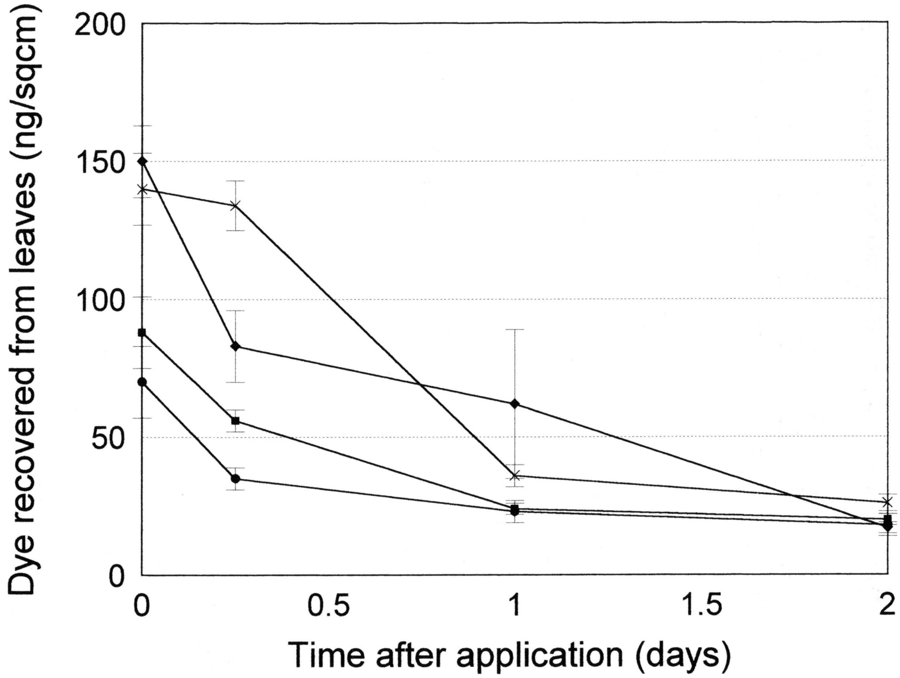

Fig. 2. Recovery of D&C Red No. 28 from field collected potato leaves per square centimeter of surface with various adjuvants applied at a rate of 210 g/ha. X - D&C Red No. 28 applied alone, ♦ - D&C Red No. 28 + 1% PEG 200, ● - D&C Red No. 28 + liquid formulation of *B. bassiana,* ■ - D&C Red No. 28 + powder formulation of *B. bassiana.* The points represent the mean of 20 samples.

Table 1. Counts of large Colorado potato beetle larvae (third instar) after application of red dye

Dye concn	Additive	Mean (SE)
Control	—	23.0 (12.5) AB
70 g	none	8.0 (5.8) A
210 g	none	30.8 (21.4) AB
350 g	none	13.0 (4.7) AB
70 g	1% PEG	9.8 (5.52) A
210 g	1% PEG	26.3 (3.9) AB
350 g	1% PEG	32.6 (21.6) AB
210 g	liquid Mycotrol	51.8 (30.8) BC
210 g	WP Mycotrol	6.8 (2.8) A

Counts were made 4 days after application. Values followed by the same letter are not statistically different $P = 0.05$.

D&C No. 28 + Mycotrol wettable powder treatment. The insect counts also exhibited high variability.

Recovery of red dye under laboratory conditions. Because less red dye was recovered from leaves that were treated with D&C Red No. 28 + 1% PEG 200 compared to the control leaves treated with D&C Red No. 28 alone, we conducted a replicated laboratory experiment to determine the effect of different adjuvants on red dye recovery without the complications of degradation from sunlight or wash off from rain. The concentrations chosen bracketed the concentrations used in the field. The recovery of red dye was linear over concentrations as calculated using linear regression. The r^2 value for the control with red dye alone was 0.97. The r^2 values for the adjuvants were: Gelva - 0.87, PEG 200 - 0.86, and Tween 80 - 0.51. In general, we recovered less red dye from all treatments with adjuvants than the controls with red dye alone (Fig. 3). This is comparable to what we found on the field-collected leaves. There were significant differences in recovery from leaves treated with different concentrations of D&C Red No. 28. Recovery was more consistent for all of the adjuvants, but varied 5-fold for the controls. There were no significant differences in recovery among the adjuvants. The recovery from leaves treated with a known amount of dye averaged 86%.

Discussion

In the field, D&C Red No. 28 was recovered from potato leaves up to 2 d after application. The half-life of the D&C Red No. 28 at all concentrations was between 6 h and 24 h in bright sunlight, which may have included some wash-off by rain. More measurements in this time frame would be necessary for a more accurate calculation. This is contrary to reports of a half-life of 1 h (Bergsten 1995). The addition of PEG 200, which aided the dispersal of the D&C Red No. 28 on the leaf, affected the initial recovery of the red dye only at high concentrations (>500 ng/cm^2). PEG 200 improved

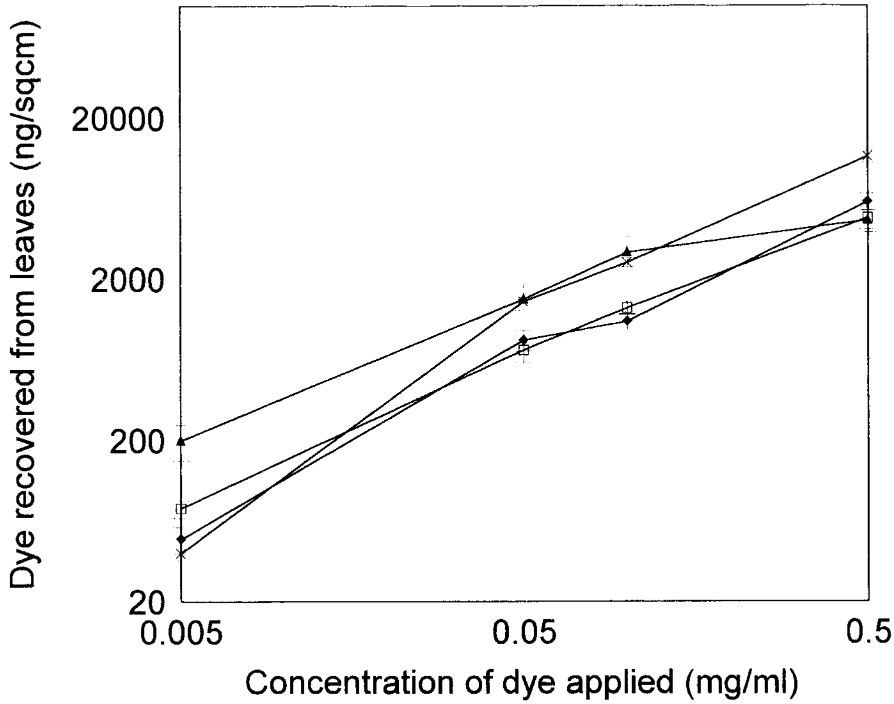

Fig. 3. Recovery of D&C Red No. 28 from potato leaves per square centimeter of surface dipped in various concentrations of D&C Red No. 28 and adjuvants. X - D&C Red No. 28 alone, ♦ - D&C Red No. 28 + 1% PEG 200, ▲ - D&C Red No. 28 + 1% Tween 80, □ - D&C Red No. 28 + 1% Gelva. The points represent the mean of three samples.

the adherence of the red dye to the leaf over a longer period at higher concentrations than at lower concentrations. At lower concentrations (<100 ng/cm^2) recovery was similar for D&C Red No. 28 alone and in combination with PEG 200. The addition of the commercial formulations of Mycotrol also decreased recovery of the D&C Red No. 28 because of the presence of adjuvants in these formulations.

Field degradation of chemical insecticides usually occurs in the presence of ultraviolet light. Adjuvants can increase the life of chemical insecticides by absorbing UV light. The complete formulations, which had adjuvants with potential UV protection, did not increase the amount of red dye recovered over time. PEG 200 does not absorb UV light at the same wavelengths the red dye absorbs UV light, at concentrations up to 10% (Martin, unpubl. data). Perhaps other additives which absorbs UV light at these wavelengths (307 nm, 346 nm, and 425 nm) may extend the time of recovery and thus enhance insecticidal activity of the D&C Red No. 28.

The insect counts were quite variable. The number of third-instar larvae did not decrease with increasing concentrations of D&C Red No. 28. This may be due to avoidance of the larvae of higher concentrations of D&C Red No. 28 and uneven application on the leaves. With red dye, the distribution on leaves can be readily observed and was not evenly spread on all leaves. But, at least in some cases insect counts were reduced to 1/3 of the control indicating that different formulations may improve control. Dead and moribund larvae were observed when collecting leaves for recovery of dye. Insects must consume D&C Red No. 28 and be exposed to high level sunlight to die. This multi-step process may also account for some of the variability of insect counts which may have avoided exposure to direct sunlight.

Inability to recover a chemical, such as D&C Red No. 28, may also be due to other environmental conditions, such as rain. In the field experiments, the presence of the PEG 200 or either of the formulated products resulted in a decrease in the amount of loss of red dye after the overnight rainfall, compared to D&C Red No. 28 applied alone (Fig. 2).

This method of extracting red dye from leaves, yields greater than 85% recovery after overnight storage and can be used for the comparative studies shown here. Recovery from leaves is a linear function of the initial concentration. Studies are in progress investigating the differences in quantitative recovery of formulated D&C Red No. 28 from leaves based on time, type of adjuvant, and half-life determination under field conditions. Quantitative recovery of D&C Red No. 28 from soil has been obtained using supercritical fluid extraction and HPLC (Alcantara et al. 1997). The procedure described here is simpler for such recovery studies from leaves.

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