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Effects of Optical Brighteners Used in Biopesticide Formulations on the Behavior of Pollinators

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A patent has been granted for the formulation of baculoviruses with stilbene-derived optical brighteners, a group of compounds that absorb ultraviolet (UV) radiation and emit visible blue wavelengths. These compounds are being extensively tested for control of forest-feeding lepidopterous insects in North America; optical brighteners may thus become a common ingredient in commercial baculovirus formulations in the near future. Many flower species use UV signals to attract insects and to direct them to the nectaries. We examined a possible consequence of field applications of optical brighteners: their effects on the ability of pollinators to find and handle flowers. In field studies carried out in Mexico and the United Kingdom on three different flower species, application of dilute (0.1% or 1%) concentrations of the optical brightener Tinopal CBS reduced recruitment of bees to flowers. Bees that approached flowers were less likely to land and feed on flowers treated with Tinopal than on controls. On one plant species, *Trifolium repens*, the time taken for bees to handle inflorescences was longer following applications of Tinopal. It seems that this optical brightener may both reduce recruitment of insects to flowers and interfere with their ability to locate rewards. Field-scale applications could reduce pollination of crops, weeds, and wildflowers and adversely affect bee populations. These possibilities should be examined in more detail before widespread applications of these compounds to the environment are made.

Key Words: Bombus; Apis mellifera; **Apoidea; Tinopal CBS; foraging; pollination.**

INTRODUCTION

Baculoviruses are biocontrol agents of lepidopteran pests and have advantages over conventional synthetic insecticides because they are specific to Lepidoptera and thus pose little or no threat to nontarget organisms (Miller, 1997). With the growing interest in integrated approaches to pest management, baculoviruses are being increasingly adopted. A number of baculoviruses are currently manufactured on a commercial scale and applied to large areas of crops; for example, *Heliothis/Helicoverpa* nucleopolyhedrovirus (NPV) in the United States (Gemstar, Thermo Trilogy Corp., Columbia, MD), *Anticarsia gemmatalis* NPV in Brazil (Moscardi, 1999), and *Spodoptera exempta* and *S. littoralis* NPV in Egypt and Kenya (McKinley *et al.*, 1989), among others.

One limitation of baculovirus applications is that the virus can be rapidly deactivated by exposure to ultraviolet (UV) light. Thus formulations that protect the virus by acting as a sunscreen are desirable. In this capacity, optical (fluorescent) brighteners are being investigated. Optical brighteners are widely used in paints, fabrics, detergents, paper, and plastics, wherein they enhance the apparent whiteness of the product by absorbing UV radiation and emitting light in the blue portion of the visible spectrum. They have shown great potential as protective agents for a number of biological insecticides (Shapiro, 1992; Nickle and Shapiro, 1992; Inglis *et al.*, 1995).

By chance it was discovered that optical brighteners provide an additional benefit; mixtures of baculovirus and optical brighteners have substantially enhanced infectivity in bioassays conducted with or without the degrading effect of UV (Hamm and Shapiro, 1992; Dougherty *et al.*, 1996). It appears that they change the pH of the midgut and also block the sloughing of primary infected midgut cells, leading to a higher probability of establishment of infection in larvae simultaneously fed virus and optical brightener compared with conspecifics fed virus alone (Sheppard *et al.*, 1994; Washburn *et al.*, 1998). Several optical brighteners are known to interfere with chitin fibrillogenesis, and because chitin is an important component of the insect gut's peritrophic membrane, these substances may increase the permeability of this structure that normally acts as a defensive barrier to microbial invasion

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(Brandt *et al.*, 1978; Elorza *et al.*, 1983). The inclusion of optical brighteners in formulations may substantially increase the viability of using baculoviruses for biocontrol, since higher pest mortality can be achieved with lower applications of virus. The few field studies of virus applied with optical brighteners indicate that virus-induced pest mortality can be significantly enhanced over simple, unformulated virus applications (Hamm *et al.*, 1994; Thorpe *et al.*, 1999).

The types of optical brighteners of the greatest interest for their interactions with baculoviruses belong to a group of disulfonic acid substituted stilbenes, of which the most studied is Tinopal LPW (Fluorescent Brightener 28, Calcofluor white M2R). The use of stilbene-derived optical brighteners as a component of biological insecticide formulations is the subject of a patent (U.S. Patent No. 5,124,149).

Although optical brighteners are widely used for domestic and industrial applications, they have not been sprayed operationally in the environment. If they become widely adopted for use with bioinsecticide-based control systems, they could be applied to vegetation over large areas. Nothing is known of the potential consequences. One possible effect may be on the behavior of pollinators, since foraging insects use visual cues in the UV to locate many species of flowers (Chittka *et al.*, 1994). Flowers may also provide UV nectar guides to aid the insect in locating nectaries (Jones and Buchmann, 1974). If by applying optical brighteners, the reflected UV portion of the spectrum is altered, then this may affect the ability of insects to find or recognize flowers and their ability to handle them efficiently. This could have consequences for the pollination of crops sprayed with optical brighteners, for the pollination of wildflowers contaminated by spray drift, and for the conservation of pollinating insects.

We describe a pilot study intended to address these possibilities by examining whether flowers treated with typical field application rates of an optical brightener are less acceptable and attractive to foraging bees. We examine both long-range attraction to patches of flowers treated with brightener and also the response of bees to individual flowers within a patch. Tinopal CBS was selected for this study as it is currently being evaluated as an enhancer for a baculovirus bioinsecticide of *Spodoptera frugiperda* (J.E. Smith) in Mexico (Martínez *et al.*, 2000).

METHODS

Attraction of Bees to Patches of Trifolium repens (UK)

This experiment was conducted on foraging bees visiting *T. repens* L. (Fabaceae) growing in dense stands in a meadow near Southampton (UK) during June 1999. One- by one-meter patches of flowers were treated with 0.1% Tinopal CBS solution (CIBA Speciality Chemicals Holding Inc., Basle, Switzerland) in distilled water (plus 2 drops/l Farman Blue wetting agent) or with water and wetting agent only. Solutions were applied with a hand-held sprayer at a rate of approximately 20 ml/m^2. Patches were then covered with netting for 40 min to exclude insects while the applications dried. Subsequently, the netting was removed and the patch observed for 20 min. The number and species of foraging bees recruited to the patch were recorded, along with the duration of their stay. For each bee, the handling time per inflorescence was recorded for the first 10 inflorescences that it visited. This was the time from when the bee first touched the inflorescence until it departed. This was repeated for all bees that visited each of 10 replicate patches of each treatment.

The numbers of bees attracted per 20 min were analyzed by one-way analysis of variance on ln $(x + 1)$ transformed data (using all bee species combined). A mean duration of stay within the patch was calculated for each replicate and the means were analyzed by one-way analysis of variance. For each bee recruited, the mean handling time per inflorescence was calculated and similarly analyzed.

Attraction to and Acceptability of Inflorescences of Bidens pilosa to Foraging Insects (Mexico)

This experiment was conducted in Tapachula, Chiapas, Mexico, in October 1999. *B. pilosa* L. (Asteraceae) produces large clumps of several hundred inflorescences and attracts a range of nectivorous insects, particularly bees and butterflies. Pairs of inflorescences were selected at random from within a patch. One inflorescence was treated with distilled water (plus 2 drops of Triton X-100 wetting agent/liter), and the other with Tinopal CBS solution plus wetting agent. Solutions were applied with a hand-held sprayer to runoff. Two separate experiments were performed, using 0.1% and 1% Tinopal CBS, each time paired with controls. Forty replicate pairs of flowers were used in each experiment. The inflorescences were observed for 2 min. All insects that approached within 5 cm of the flower and the number that landed and probed for nectar were recorded. Insects were broadly grouped as bees (including honeybees *Apis mellifera* L., Megachilidae, and various small species that were not identified) or butterflies. Simultaneous observation of paired inflorescences enabled us to control for the considerable variation that occurs in insect activity as weather conditions change.

The numbers of insects that approached within 5 cm of inflorescences were ln + 1 transformed and analyzed using a paired t test. The overall proportion of these insects that landed and probed for nectar was examined using a χ^2 test with Yates' correction. Butterflies and bees were analyzed separately.

Acceptability of Symphytum officinale Florets at Close Range (UK)

S. officinale L. (Boraginaceae) has been previously used to examine the factors bumblebees (*Bombus* spp.) use in choosing which individual flowers to visit (Goulson *et al.*, 1998; Stout *et al.*, 1998). When feeding with their head inside a flower, they are not disturbed by movements nearby, so that test flowers can be placed in an adjacent position. Acceptance or rejection of these flowers can then be scored; acceptance involves landing on the flower and probing for nectar. The response is classed as rejection if the bee approaches the flower, sometimes briefly touches the flower with legs or antennae, but fails to alight or to probe for nectar (Goulson *et al.*, 1998).

Test flowers were treated with 0.1% Tinopal CBS or water as above and offered to foraging bees of three different species; *Bombus terrestris* L., *B. pascuorum* L., and *B. pratorum* L. (Hymenoptera: Apoidea). Acceptance or rejection of flowers was recorded. This was repeated with 49 treated flowers and 58 control flowers. This experiment was carried out in Southampton (UK) during June, 1999. Data were analyzed in GLIM with binomial errors according to treatment and bee species (Crawley, 1991).

RESULTS

Attraction of Bees to Patches of T. repens (UK)

Because visitation rates by bees were low, all species were combined for analysis. Four different species were recorded, *B. terrestris*, *B. pascuorum*, *B. lapidarius* L., and *Apis mellifera*. Overall, there was no significant difference in the number of bees attracted to patches of flowers treated with Tinopal CBS compared to control patches sprayed with water ($F = 1.87$, $df = 1{,}19$, $P > 0.05$), although more bees were recorded in control patches (means ± SE; 1.7 + 2.0 for patches treated with Tinopal CBS compared to 2.5 ± 1.4 for controls). Similarly, treatment had no statistically significant influence upon the time that these bees remained within each patch ($F = 0.70$, $df = 1{,}16$, $P > 0.05$), although bees spent on average nearly twice as long in plots treated with optical brightener (means ± SE; 96.7 ± 74.2 s for controls versus 175.8 ± 273.5 s for Tinopal CBS-treated patches). However, the handling time per inflorescence of bees in plots treated with Tinopal CBS was significantly longer than that in control plots ($F = 2.58$, $df = 1{,}34$, $P < 0.05$) (means ± SE; 3.67 ± 0.81 s for control patches versus 4.50 ± 0.90 s for Tinopal CBS-treated patches). The presence of optical brightener appears to result in an increase in handling time of approximately 0.8 s per inflorescence.

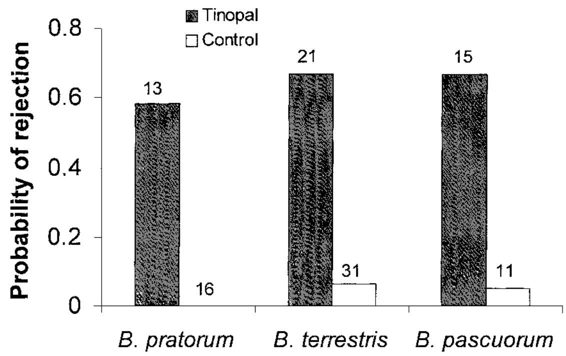

FIG. 1. The probabilities of rejection of flowers of *Symphytum officinale* treated with 0.1% Tinopal CBS versus controls, when encountered by three bee species, *Bombus terrestris*, *B. pascuorum*, and *B. pratorum*, are shown. Sample sizes are indicated above bars.

Attraction of Bees to Inflorescences of B. pilosa (Mexico)

Significantly more bees were attracted to control inflorescences than to those treated with 1% Tinopal CBS ($t = 4.30$, $df = 39$, $P < 0.001$, means ± SE; 1.6 ± 0.4 bees/2 min for controls and 1.2 ± 0.4 for Tinopal CBS). There was a smaller but still significant difference resulting from the application of 0.1% Tinopal CBS compared to controls ($t = 2.87$, $df = 39$, $P < 0.01$, means ± SE; 1.0 ± 0.3 bees/2 min for controls and 0.7 ± 0.3 for Tinopal CBS). Of those bees that approached inflorescences, significantly fewer were likely to land and probe for nectar on flowers treated with 1% Tinopal (3.4%) compared to controls (12.0%) ($\chi^2 = 5.72$, $df = 1$, $P < 0.05$). There was no significant difference in the proportion of insects that landed between flowers treated with 0.1% Tinopal CBS (13.0%) and controls (18.8%) ($\chi^2 = 0.45$, $df = 1$, $P > 0.05$).

Butterfly visits were comparatively scarce ($N = 16$), and all butterflies which approached within 5 cm landed and fed from flowers. Numbers were too low for meaningful analysis.

Acceptability of S. officinale Florets at Close Range (UK)

The rejection rate of flowers treated with Tinopal CBS (64.6%) was significantly higher than that of control flowers (5.1%) ($\chi^2 = 47.7$, $df = 1$, $P < 0.001$). There were no differences among the three bee species in the likelihood of their rejecting flowers ($\chi^2 = 0.99$, $df = 2$, $P > 0.05$), and there was no significant interaction among bee species and treatment ($\chi^2 = 1.46$, $df = 2$, $P > 0.05$) (Fig. 1).

DISCUSSION

Individual inflorescences of *B. pilosa* attracted significantly fewer bees when treated with either 0.1% or 1% Tinopal CBS, and there appeared to be a greater effect at the higher concentration. Foraging bumblebees of three different species were far more likely to reject flowers of *S. officinale* treated with optical brightener than control flowers. The handling time of bees when foraging on *T. repens* was longer when the flowers were contaminated with Tinopal CBS, suggesting a reduced efficiency of pollinator foraging. Clearly, dilute solutions of optical brightener on flowers can have a marked influence on the behavior of insect pollinators.

These results are not unexpected. Bees rely heavily on visual cues (among others) when foraging and are very selective as to which flowers they visit (reviewed in Goulson, 1999). Entering a flower takes time, and flowers vary greatly (both within and between species) as to how much reward they contain (Wetherwax, 1986; Real and Rathcke, 1988; Cresswell, 1990; Waser and Mitchell, 1990). Bees learn associations between color and reward and thus are able to concentrate their foraging efforts on more rewarding flowers (Menzel and Muller, 1996; Goulson, 1999). For example, bees are able to distinguish and preferentially select the most rewarding age classes of flowers (e.g., Müller, 1883; Ludwig, 1885, 1887; Thomson *et al.*, 1982; Weiss, 1995) or the most rewarding sex of flowers in monoecious and dioecious species (e.g., Delph and Lively, 1992; Shykoff and Bucheli, 1995). Optical brightener on the petals of flowers will alter their color, although to the human eye the concentrations used gave no discernible effect. UV reflectance will presumably be reduced, and emission of blue light increased. This may reduce the apparency of the flower or patch of flowers to foraging insects and may also reduce the contrast between flowers and foliage, which would explain the reduced number of insects approaching *B. pilosa* when treated with optical brightener. It will certainly make individual flowers appear different than other flowers of their species. Learning by pollinators results in a marked fidelity to flower types that have previously provided a reward, a phenomenon known as flower constancy (a term first used by Plateau, 1901, defined by Waser, 1986). Any individual flower that is different is thus likely to be overlooked. Of course, if all of the flowers in a large area were treated with optical brightener (as might be the case if included in a biopesticide formulation), this effect may not occur since the insects could learn that this "new" flower type was rewarding. Our studies were both small in scale and short in duration, and the effects of treating large areas, perhaps for long periods, might be quite different.

In addition to attracting pollinators, petals often have a secondary function of guiding insects to the nectaries. Nectar guides, lines of contrasting color, indicate to an insect that has arrived at the flower exactly where it needs to probe to obtain nectar (Jones and Buchmann, 1974). These lines are sometimes visible to the human eye, but are often lines of UV reflectance. Our finding that Tinopal CBS on *T. repens* increases handling time suggests that the brightener may be obscuring nectar guides and so making it more difficult for the bees to handle the flowers. The effect on pollination of the plant is less obvious; prolonged handling times might actually improve pollination, although if bees have difficulty locating rewards, then they may be incorrectly positioned to transfer pollen. It is likely that the outcome may vary between plant species.

We cannot be certain that the effects we observed are entirely due to the influence of the optical brightener on visual cues. It is possible that in addition to visual effects, Tinopal CBS alters the odor or texture of flowers. This too might reduce their acceptability to bees.

Some baculoviral pesticides are applied on a very large scale. For example, applications against gypsy moth are aerially applied over large areas of forest in North America and more than 1 million ha of soya is treated with NPV in Brazil (Moscardi, 1999). Inclusion of optical brighteners in baculovirus formulations may have important environmental consequences. If the spray is being applied to a crop that benefits from insect pollination (for example, most fruit crops), then clearly pollination could be disrupted. Conversely, benefits may be obtained through reduced pollination and seed set of crop weeds. Contamination of wildflowers, either via spray drift from crops or when formulations are applied to seminatural habitats, such as forests, could result in negative impacts upon both flower and pollinator populations.

The environmental benefits of use of baculovirus pesticides compared to conventional pesticides are great. Optical brighteners appear to offer a means of rendering baculovirus pesticides more efficient and could lead to their increased adoption as control agents against a range of crop pests. However, we suggest that more detailed studies of these possible undesirable side effects, preferably conducted on a larger scale, are needed before they are widely applied in the environment.

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REFERENCES

Brandt, C. R., Adang, M. J., and Spence, K. D. S. 1978. The paratrophic membrane: Ultrastuctural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. *J. Invertebr. Pathol.* **32,** 12–24.

Chittka, L., Shmida, A., Troje, N., and Menzel, R. 1994. Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. *Vision Res.* **34,** 1489–1508.

Crawley, M. J. 1991. "GLIM for Ecologists." Blackwell, Oxford.

Cresswell, J. E. 1990. How and why do nectar-foraging bumblebees initiate movements between inflorescences of wild bergamot *Monarda fistulosa* (Lamiaceae). *Oecologia* **82,** 450–460.

Delph, L. F., and Lively, C. M. 1992. Pollinator visitation, floral display, and nectar production of the sexual morphs of a gynodioecious shrub. *Oikos* **63,** 161–170.

Dougherty, E. M., Guthrie K. P., and Shapiro, M. 1996. Optical brighteners provide baculovirus activity enhancement and UV radiation protection. *Biol. Control* **7,** 71–74.

Elorza, M. V., Rico, H., and Sentandrau, R. 1983. Calcofluor white alters the assembly of chitin fibrils in *Sachromyces cerevisae* and *Candida albicans* cells. *J. Gen. Microbiol.* **129,** 1577–1582.

Goulson, D. 1999. Foraging strategies for gathering nectar and pollen in insects. *Perspect. Plant Ecol., Evol., System.* **2,** 185–209.

Goulson, D., Hawson, S. A., and Stout, J. C. 1998. Foraging bumblebees avoid flowers already visited by conspecifics or by other bumblebee species. *Anim. Behav.* **55,** 199–206.

Hamm, J. J., and Shapiro, M. 1992. Infectivity of fall armyworm (Lepidoptera: Noctuidae) nuclear polyhedrosis virus enhanced by a fluorescent brightener. *J. Econ. Entomol.* **85,** 2149–2152.

Hamm, J. J., Chandler, L. D., and Sumner, H. R. 1994. Field tests with a fluorescent brightener to enhance infectivity of fall armyworm (Lepidoptera: Noctuidae) nuclear polyhedrosis virus. *Fla. Entomol.* **77,** 425–437.

Inglis, G. D., Goettel, M. S., and Johnson, D. L. 1995. Influence of ultraviolet light protectants on persistence of the entomopathogenic fungus *Beauveria bassiana*. *Biol. Control* **5,** 581–590.

Jones, C. E., and Buchmann, S. L. 1974. Ultraviolet floral patterns as functional orientation cues in hymenopterous pollination systems. *Anim. Behav.* **22,** 481–485.

Ludwig, F. 1885. Die biologische Bedeutung des Farbenwechsels mancher Blumen. *Biol. Centralblatt* **4,** 196.

Ludwig, F. 1887. Einige neue Falle von Farbenwechsel in verbluhenden Blüthenstanden. *Biol. Centralblatt* **6,** 1–3.

Martínez, A. M., Goulson, D., Chapman, J. W., Caballero, P., Cave, R. D., and Williams, T. 2000. Is it feasible to use optical brightener technology with a baculovirus bioinsecticide for resource-poor maize farmers in Mesoamerica? *Biol. Control* **17,** 174–181.

McKinley, D. J., Moawad, G., Jones, K. A., Grzywacz, D., and Turner, C. 1989. The development of nuclear polyhedrosis virus for the control of *Spodoptera littoralis* (Boisd.) in cotton. *In* "Pest Management in Cotton" (M. B. Green and de B. Lyon, Eds.), pp. 93–100. Ellis Horwood, New York.

Menzel, R., and Müller, U. 1996. Learning and memory in honeybees: From behaviour to neural substrates. *Annu. Rev. Neurosci.* **19,** 379–404.

Miller, L. K. 1997. "The Baculoviruses." Plenum, New York.

Moscardi, F. 1999. Assessment of the application of baculoviruses for control of Lepidoptera. *Annu. Rev. Entomol.* **44,** 257–289.

Müller, H. 1883. The effect of the change of colour in the flowers of *Pulmonaria officinalis* upon its fertilizers. *Nature* **28,** 81.

Nickle, W. R., and Shapiro, M. 1992. Use of a stilbene brightener, Tinopal LPW, as a radiation protectant for *Steinernema carpocapsae*. *J. Nematol.* **24,** 371–373.

Plateau, F. 1901. Observations sur le phénomène de la constance chez quelques hyménoptères. *Ann. Soc. Entomol. Belgique* **45,** 56–83.

Real, L., and Rathcke, B. J. 1988. Patterns of individual variability of floral resources. *Ecology* **69,** 728–735.

Shapiro, M. 1992. Use of optical brighteners as radiation protectants for gypsy moth (Lepidoptera: Lymantridae) nuclear polyhedrosis virus. *J. Econ. Entomol.* **85,** 1682–1686.

Sheppard, C. A., Shapiro, M., and Vaughn, J. L. 1994. Reduction of midgut luminal pH in gypsy moth larvae (*Lymantria dispar* L.) following ingestion of nuclear or cytoplasmic polyhedrosis virus/fluorescent brightener on natural and artificial diets. *Biol. Control* **4,** 412–420.

Shykoff, J. A., and Bucheli, E. 1995. Pollinator visitation patterns, floral rewards and the probability of transmission of *Microbotryum violaceum*, a venereal disease of plants. *J. Ecol.* **83,** 189–198.

Stout, J. C., Goulson, D., and Allen, J. A. 1998. Repellent scent marking of flowers by a guild of foraging bumblebees (*Bombus* spp.). *Behav. Ecol. Sociobiol.* **43,** 317–326.

Thomson, J. D., Maddison, W. P., and Plowright, R. C. 1982. Behaviour of bumblebee pollinators on *Aralia hispida* Vent. (Araliaceae). *Oecologia* **54,** 326–336.

Thorpe, K. W., Cook, S. P., Webb, R. E., Podgwaite J. D., and Reardon, R. C. 1999. Aerial application of the viral enhancer Blankophor BBH with reduced rates of gypsy moth (Lepidoptera: Lymantriidae) nucleopolyhedrovirus. *Biol. Control* **16,** 209–216.

Waser, N. M. 1986. Flower constancy: Definition, cause and measurement. *Am. Nat.* **127,** 593–603.

Waser, N. M., and Mitchell, R. J. 1990. Nectar standing crops in *Delphinium nelsonii* flowers: Spatial autocorrelation among plants? *Ecology* **71,** 116–123.

Washburn, J. O., Kirkparick, B. A., Haas-Stapelton, E., and Volkman, L. E. 1998. Evidence that the stilbene-derived optical brightener M2R enhances *Autographa californica* M nucleopolyhedrovirus infection of *Trichoplusia ni* and *Heliothis virescens* by preventing sloughing of infected midgut epithelial cells. *Biol. Control* **11,** 58–69.

Weiss, M. R. 1995. Associative color learning in a nymphalid butterfly. *Ecol. Entomol.* **20,** 298–301.

Wetherwax, P. B. 1986. Why do honeybees reject certain flowers? *Oecologia* **69,** 567–570.