Interactions in Entomology: Enhanced Infectivity of Entomopathogenic Viruses by Fluorescent Brighteners[1]

John J. Hamm

Insect Biology and Population Management Research Laboratory, U.S. Department of Agriculture, Agriculture Research Service, P.O. Box 748, Tifton, GA 31793-0748 USA

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Abstract Fluorescent brighteners or optical brighteners, chemicals that absorb energy from ultraviolet light (UV) and emit it as visible light, have been studied as UV protectants for entomopathogens to extend their effectiveness in the field as biocontrol agents. Some stilbene fluorescent brighteners have been demonstrated both to provide some protection from UV degradation and to enhance infectivity independently of the UV protection for several entomopathogenic viruses. Although the mode of action of this enhanced infectivity is not fully understood, enhanced infectivity of 10 nucleopolyhedroviruses and a granulovirus (Baculoviridae), a cypovirus (Reoviridae), an iridovirus (Iridoviridae), and an entomopoxvirus (Poxviridae) has been demonstrated. The enhanced infectivity produced by the fluorescent brighteners generally resulted in earlier mortality and in some cases extended infectivity of the virus to older instars. In a few cases the host range was extended, i.e., viruses which were not infective for a particular species under normal conditions were infective with the addition of a fluorescent brightener. Thus, if proper formulations can be made, the fluorescent brighteners have the potential to increase the effectiveness of certain entomopathogenic viruses for biocontrol of certain lepidopterous pests of forest and agricultural crops.

Key Words *Lymantria dispar, Spodoptera frugiperda, Spodoptera exigua, Helicoverpa zea, Heliothis virescens, Pseudoplusia includens, Trichoplusia ni, Anticarsia gemmatalis, Plutella xylostella,* nucleopolyhedrovirus, granulovirus, cypovirus, iridovirus, entomopoxvirus, fluorescent brightener, optical brightener, biocontrol, UV protection, enhanced infection.

Some of the factors that have inhibited commercial development of entomopathogenic viruses for control of insect pests have been: (1) degree of host specificity, (2) time required to kill the pest, (3) cost of production of the virus, and (4) rapid inactivation of the virus by sunlight or UV light. Many insect viruses are highly specific and will infect only a single species or a few closely-related species of pests (Gröner 1986). Certainly, a virus that is highly effective against several species of pests, even within the same family, would have a larger market potential than a species-specific virus. Many viruses require 3 to 8 days after being ingested to produce an infection and kill the host, during which time the pest continues to damage the crop. A virus which kills the pest sooner would provide more protection for the crop. However, if the virus has time to replicate so that it contaminates the crop when the insect dies, it has the potential for establishing epizootics in the pest population. This potential to initiate epizootics could be lost if the virus is engineered to kill the host before the virus has

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time to replicate. The cost of the virus needed for a commercial product could be reduced if the amount of virus needed were reduced through increased infectivity. Also, the persistence of the virus on the crop could be increased if appropriate UV protectants could be found and formulated with the virus. Some progress is being made on all these fronts.

Two broad spectrum nucleopolyhedroviruses (NPVs) which are being considered for commercialization are the NPV of alfalfa looper, *Autographa californica* (Speyer), (AcMNPV) and the NPV of celery looper, *Anagrapha falcifera* (Kirby), (AfMNPV). While these viruses are closely related and have overlapping host ranges, there are some important differences. Whereas, the potency of AcMNPV against corn earworm, *Helicoverpa zea* (Boddie), is low (Vail et al. 1978, Vail and Collier 1982), the potency of AfMNPV against *H. zea* is relatively high (Hostetter and Puttler 1991). Although several species of lepidopteran pests have been listed as susceptible to one or the other or both of these viruses, some species are much less susceptible, i.e., have much higher median lethal concentrations (LC_{50}s) than other species.

Shapiro (1992) tested some diamiostilbenedisulfonic acid derivatives as UV protectants for the gypsy moth NPV (LdMNPV) against gypsy moth, *Lymantria dispar* (L.) and demonstrated that 17 of 23 compounds gave significant protection under laboratory conditions. A number of these derivatives are used in laundry detergents to make fabrics appear brighter, chiefly by absorbing energy from ultraviolet light and emitting it as visible light. These materials are, thus, commonly known as fluorescent whitening agents, fluorescent brighteners or optical brighteners. He discovered, however, that independent of their action as UV protectants, some of these materials strongly enhanced the potency of the virus (Shapiro and Robertson 1992). Shapiro's work led to considerable research on enhancement of virus infection by fluorescent brighteners which is the subject of this paper.

Laboratory tests. In laboratory bioassays, feeding of LdMNPV in a 1% concentration of selected optical brighteners reduced the LC_{50} from 18,000 polyhedral inclusion bodies (PIB)/ml to values between 10 (Phorwite RKH®, Mobay Chemical Corp., Union, NJ) and 44 (Leucophor BSB®, Sandoz Chemical Corp., Charlotte, NC) PIB/ml. These brighteners also reduced the median lethal time (LT_{50}) at every virus concentration tested. At the highest virus concentration (1×10^6 PIB/ml), LT_{50}s (time required to produce 50% mortality) were reduced from 13.9 to 7.1 days. The addition of Tinopal LPW® (Sigma Chemical Co., St. Louis, MO) to the virus also enhanced mortality among mature (fourth instar) larvae. Thus, the magnitude of reduction in LC_{50}s (up to 1800 fold) plus the reduction in LT_{50}s in gypsy moth larvae indicated that selected brighteners greatly enhanced the virulence of the gypsy moth NPV.

Laboratory tests with the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) NPV (SfMNPV) against 3-day-old fall armyworm larvae showed that 0.1% Tinopal LPW greatly enhanced infectivity of the SfMNPV. LC_{50}s in PIB/cup ranged from 185 to 4,910 when tested in water and from 0.0162 to 3.36 when tested in 0.1% Tinopal LPW (Hamm and Shapiro 1992). LC_{90}s ranged from 13,800 to 305,000 PIB/cup in water and from 25.4 to 5,720 PIB/cup for virus in 0.1% Tinopal LPW. Because of the unique level of enhancement of viral infectivity for two important lepidopteran pests (the gypsy moth, a forest pest and the fall armyworm, an agricultural pest) by this fluorescent brightener, a patent for the use of fluorescent brighteners in biological control was awarded 23 June 1992 (Shapiro et al. 1992). Preliminary tests also showed enhanced infectivity of the fall armyworm granulovirus (SfGV) for fall armyworm and

the corn earworm, *Helicoverpa zea* (Boddie) iridovirus (HzIV) for corn earworm (Shapiro et al. 1992).

Shapiro and Dougherty (1994) showed that infectivity of the gypsy moth cytoplasmic polyhedrosis virus (CPV) was enhanced by the addition of a selected stilbene optical brightener (Phorwite AR®, Mobay Chemical Corp., Union, NJ) at 1%. The LC_{50} was reduced 864-fold, from 1.2×10^5 to 1.4×10^2 PIB per ml. The brightener also reduced the LT_{50} from the gypsy moth CPV from 13.2 to 8.4 days (at a concentration of 1.6×10^6 PIB per ml). They also demonstrated that Phorwite AR could extend the host range of two viruses which normally did not infect gypsy moth larvae. The AcMNPV and an entomopoxvirus from *Amsacta moorei* Butler (Lepidoptera: Arctiidae) killed more than 50% of gypsy moth larvae at concentrations of 10^7 and 10^6 PIB per ml, respectively. No mortality was observed at these concentrations of virus without the brightener.

Farrar et al. (1995) tested Blankophor BBH (Bayer Corp., Pittsburgh, PA) both in a tank-mix preparation and in an experimental wettable powder formulation of LdMNPV. Blankophor BBH in the tank-mix and wettable powder formulation at a concentration of 1% (wt/vol) reduced the LC_{50}s by 42- and 214-fold, respectively. Blankophor BBH was also a moderate feeding deterrent to gypsy moth larvae which could limit its efficacy as an enhancer of the virus. However, the addition of molasses to Blankophor BBH at least partially overcame the feeding deterrence.

Zou and Young (1994) conducted laboratory tests on first, second, third, and fourth instars of four species of noctuid pests using two levels of Tinopal LPW. The higher rate of Tinopal was 1% = 0.14 µg/mm^2 of diet surface. The soybean looper, *Pseudoplusia includens* (Walker), showed a significant increase in mortality due to the singly enveloped NPV of *P. includens* (PiSNPV) at the higher rate of Tinopal in all four instars and was the only species to show enhanced mortality in the fourth instar. The singly enveloped HzSNPV, showed significantly enhanced mortality at the higher level of Tinopal against first and third-instar *H. zea* and against first and second-instar tobacco budworm, *Heliothis virescens* (F.). The multiply-embedded NPV of beet armyworm, *Spodoptera exigua* (Hübner) (SeMNPV), showed enhanced mortality against first and second-instar beet armyworm. Zou and Young (1996) conducted additional tests with PiSNPV using a slightly different fluorescent brightener, Blankophor BBH (Blankophor BBH is the same as Tinopal LPW but at pH 6.9 instead of 9.5 for Tinopal LPW (Shapiro, per. commun.). The addition of 0.1% Blankophor BBH to the viral suspension on semisynthetic diet reduced the LC_{50} from 970 PIB/mm^2 to 0.0625 PIB/mm^2 for second instars. Blankophor BBH at 0.08% reduced the LT_{50} from 15.9 to 6.4 days.

Shapiro and Vaughn (1995) tested five different NPVs in water and in 1% Tinopal LPW against second-instar corn earworm. The HzSNPV was the most active virus tested (LC_{50} = 130 PIB/30-ml cup) followed by AfMNPV (LC_{50} = 2100 PIB/cup), the MNPV from *Helicoverpa armigera* (HaMNPV; LC_{50} = 4,800 PIB/cup), the MNPV form wax moth, *Galleria mellonella* (L.) (GmMNPV; LC_{50} = 6,500 PIB/cup), and the AcMNPV (LC_{50} = 7,500 PIB per cup). The addition of Tinopal LPW reduced the LC_{50}s for HzSNPV by 8.7-fold, for AfMNPV by 13.1-fold, for HaMNPV by 25.3-fold, for GmMNPV by 2.1-fold, and for AcMNPV by 50.0-fold. Although Tinopal LPW significantly decreased the LT_{50}s, it did not reduce the LT_{50}s for the less virulent viruses to equal the LT_{50} for HzSNPV without the brightener.

Hamm and Chandler (1996) tested the effect of Tinopal LPW on the infectivity of SPOD-X® (Crop Genetics International Columbus, MD), a commercial formulation of

the beet armyworm NPV, against six colonies of beet armyworm established from field collections in Georgia, Alabama and Mississippi. The LC_{50} was lower in all bioassays with SPOD-X in 0.25% Tinopal LPW than with SPOD-X in water, although in three of 12 bioassays the difference was not significant based on overlapping 95% confidence intervals. The mean LC_{50} for SPOD-X in water was 376 PIB/cup compared with a mean LC_{50} of 30 PIB/cup for SPOD-X in 0.25% Tinopal LPW. The LC_{50} for SPOD-X in water was not significantly different between the laboratory colony and 5 of 6 field-collected colonies when bioassayed concurrently. One field-collected colony was significantly less susceptible to SeMNPV than the laboratory colony. There was no significant difference in LC_{50} for SPOD-X in 0.25% Tinopal LPW between the laboratory colony and any of the field-collected colonies. Thus, Tinopal LPW enhanced the infectivity of SPOD-X for beet armyworm larvae and reduced the variability of response for the beet armyworm colonies to the virus.

Vail et al. (1996) tested the effects of Tinopal LPW on infectivity of AfMNPV against neonate tobacco budworm, corn earworm, beet armyworm, and cabbage looper, *Trichoplusia ni* (Hübner), on diet. Levels of enhancement as determined by LC_{50} values varied from 2.9- to 13.6-fold; whereas, levels of LC_{95} values ranged from 3.7- to 16-fold, depending on the species. The greatest enhancement occurred among cabbage looper larvae. LT_{50} values were significantly affected, particularly when lower concentrations of polyhedra were fed. Tinopal LPW reduced LT_{50} values up to 2.1-fold among the species tested. The optimum concentrations for Tinopal LPW enhancement were between 0.25 and 1% (wt/vol). Choice tests between water- and 1% Tinopal LPW-treated diet showed that larvae preferred water-treated diet.

Farrar and Ridgway (1997) tested the potency of AfMNPV against second instars of corn earworm, beet armyworm, and diamondback moth, *Plutella xylostella* (L.). Potency was highest against the corn earworm, least against the diamondback moth, and intermediate against the beet armyworm. The addition of Blankophor BBH to AfMNPV increased the potency of the virus against all insect species tested. However, the relative differences in potency among host species was not greatly affected. Mortality increased as the concentration of Blankophor BBH was increased from 1 to 5 μg/μl but did not increase as the concentration was increased from 5 to 10 μg/μl; which may indicate a feeding deterrence at higher concentrations.

Fuxa and Richter (1998) selected a population of velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, for resistance to the *A. gemmatalis* nuclear polyhedrosis virus (AgNPV). The fluorescent brightener, Tinopal LPW, increased the susceptibility of resistant and susceptible insects by 24- and 58-fold, respectively, resulting in an increase of the resistance ratio from 4.7- to 11.2-fold. However, the LC_{50} of AgNPV with the fluorescent brightener against resistant insects (628 POB/ml) was less than the LC_{50} of AgNPV without brightener against susceptible insects (3243 POB/ml).

Field tests. Webb et al. (1994a) reported the results of optical brighteners on the efficacy of gypsy moth NPV in forest plots with high and low levels of natural NPV. Doses of a standard formulation of Gypcheck® (U.S. Forestry Service, Washington, DC) (gypsy moth NPV) containing the sunscreen Orzan® (ITT Rayonier Inc., Seattle, WA) and a sticker were evaluated against gypsy moth, along with aqueous formulations of Gypcheck in which Orzan was replaced by stilbene disulfonic acid optical brighteners, Phorwite AR in 1991 or Blankophor BBH in 1992. The treatments were applied to gypsy-moth-infested forest plots where preliminary studies had demonstrated high (1991) or low (1992) levels of natural gypsy moth NPV. The 1991 treatments containing Phorwite AR had significantly higher levels of gypsy moth larval

mortality and significantly reduced LT_{50}s compared with equivalent treatments containing Orzan. The Phorwite AR increased the mortality of gypsy moths (caused by natural virus) to that of the high dose of applied virus without Phorwite AR. In 1992, all treatments containing Blankophor BBH with the low dose of virus had levels of gypsy moth larval mortality equal to or higher than the standard formulation with the higher dose of virus, significantly higher levels of gypsy moth larval mortality than the standard formulation with the lower dose of virus, and significantly reduced LT_{50}s compared with either of the treatments containing Orzan. Natural gypsy moth NPV levels remained low in the control plots, and Blankophor BBH applied alone significantly increased the mortality of gypsy moths.

Webb et al. (1994b) tested the efficacy of Gypcheck in combination with Blankophor BBH® against third and fourth-instar gypsy moths on three oak trees in an infested woodlot. The plots treated with Gypcheck and Blankophor BBH had significantly more larval mortality and significantly lower LT_{50} values than did plots treated with Gypcheck alone. Additionally, Blankophor BBH applied alone appeared to interact with native virus present in the field plots and significantly increased larval mortality compared with untreated plots. The 98% mortality of third and fourth instars resulting from treatment with Gypcheck and Blankophor BBH compared with only 63% for the standard Gypcheck makes the combination a candidate for use by arborists for homeowners. It also expands the window for application from only first and second instars to include third and fourth instars which would substantially ease current time constraints in treatment programs.

Vail et al. (1993) conducted small field plot tests with AfMNPV and Blankophor BBH for control of lepidopterous pests of cotton, including bollworm, tobacco budworm, beet armyworm, and cabbage looper. Tests at Shafter, CA, indicated that Blankophor BBH reduced the loss of activity of the virus at the two highest applications tested. Time to 50% loss of original activity was extended from 5.5 to 11.5 days. Tests at Maricopa, AZ, confirmed that all four species could be infected under field conditions. The addition of a bait (COAX®, Agri Solutions, San Marcos, CA) increased the percentage of infection of bollworm larvae with all applications rates of AfMNPV. At Stoneville MS, AfMNPV performed as well as two other baculoviruses known to infect tobacco budworm (HzSNPV and AcMNPV). However, the addition of either baits or brighteners to sprays did not provide a clear pattern as to the relative merits of either adjuvant.

Hamm et al. (1994) tested Tinopal LPW with the fall armyworm NPV against fall armyworm in whorl-stage corn. The fluorescent brightener interacted significantly with virus concentration and with water volume to increase fall armyworm larval mortality. There was no increase in mortality due to NPV as the percent fluorescent brightener increased beyond 1%. In the higher volumes of water, 0.25% fluorescent brightener resulted in the highest percentage mortality due to NPV. *Cotesia marginiventris* (Cress.) was the most abundant parasitoid recovered from fall armyworm in these tests and, as the percentage mortality due to NPV increased, the percentage mortality due to parasitoids and ascovirus decreased. Thus, the total mortality was not affected as greatly as the percentage mortality due to NPV by changes in water volume or fluorescent brightener concentration. The reduction in mortality due to parasitoids did not appear to be a direct effect of the fluorescent brightener on the parasitoids. However, increased infectivity of the NPV and earlier mortality from NPV associated with the fluorescent brightener resulted in more host larvae dying of NPV before the parasitoids could complete development.

A field test on a natural population of *P. includens* larvae of mixed ages showed that 0.3% and 1.0% Blankophor BBH significantly enhanced mortality due to PiSNPV (Zou and Young 1996).

Mode of action. The mode of action of fluorescent brighteners in enhancing infectivity of viruses is not well understood. Adams et al. (1994) conducted a histopathological study of the midgut of gypsy moth larvae infected with LdMNPV plus Tinopal LPW. They showed early and heavy infection of the midgut epithelium when the LdMNPV and Tinopal LPW were used together. Release of these extracellular virions could spread the infection more rapidly to the susceptible tissues that are involved with the secondary cycle of infection and may account for earlier larval mortality than in those larvae fed the LdMNPV alone. This differs from LdMNPV alone which does not undergo the typical primary cycle of infection in the larval midgut. Apparently virions released from the polyhedra in the alkaline midgut lumen pass through the midgut to the blood cells where replication and the first production of extracellular virions occurs (Shields 1985).

Sheppard and Shapiro (1994) showed that feeding gypsy moth larvae a combination of LdMNPV and Tinopal LPW resulted in a drop in pH in the lumen of the midgut from the normal alkaline range (approximately 10.0-10.5) to values near neutrality and a cessation of larval feeding. Gypsy moth larvae reared on red oak foliage that had been treated with Tinopal LPW and LdMNPV exhibited changes in gut appearance and midgut luminal pH similar to those observed in larvae reared on artificial diet (Sheppard et al. 1994). They also demonstrated that a cytoplasmic polyhedrosis virus (LdCPV) which infects midgut epithelial cells produced an enhanced infection and enhanced drop in midgut luminal pH when combined with Tinopal LPW.

Dougherty et al. (1995) demonstrated that Tinopal LPW (= Calcofluor white M2R®, Sigma Chemical Co., St. Louis, MO) was effective only if it was present with the virus at the time of ingestion and that virions derived from dissolved polyhedra were more stable in the presence of Tinopal LPW. They also showed that the extracellular phenotype of the virus which is normally not infectious *per os* is infectious in the presence of Tinopal LPW. The data indicate that the virions are not permanently affected by the brightener but rather the polyhedra-derived virions and the brightener interact reversibly to achieve enhancement of viral efficacy. They suggest the possibility that increased efficacy may be mediated by cellular processes; that is, the Tinopal LPW and the virus never interact, rather the Tinopal LPW alters cellular processes to allow the virus to express itself in a more efficient manner.

Washburn et al. (1998) studied the effects of Tinopal (M2R) on infectivity of AcMNPV for *T. ni* and *H. virescens* when ingesting virus at different times after molting. M2R significantly reduced the time to death by AcMNPV for both hosts. The time to death was not significantly affected by the time after molting when inoculated. In contrast, mortality levels of intermolt *H. virescens* treated 12 to 18 h into the fourth instar with AcMNPV and 1% M2R were elevated an average of 33%, indicating that the fluorescent brightener counteracted developmental resistance. The fluorescent brightener appeared to enhance AcMNPV pathogenesis by blocking the sloughing of infected primary target cells in the midgut, resulting in an increase in the number of primary target cells infected, an acceleration in the onset of systemic infections in the tracheal epidermis, and an increased percentage mortality. They suggest that if host species respond to initial infection by sloughing midgut cells, and if M2R does block

this response, fluorescent brighteners should improve baculovirus efficacy under field conditions.

Shapiro and Argauer (1995) showed that the activity of Tinopal LPW as an enhancer for the gypsy moth NPV was not adversely affected by pH ranges from 3.0 to 10.4, temperatures of 121°C for 5 min., or exposure to UV (254, 302, and 360 nm) for periods up to 7 days. Argauer and Shapiro (1997) compared eight structurally-related stilbene optical brighteners as enhancers for LdMNPV. Five of the eight brighteners acted as activity enhancers. The most effective brighteners reduced LC_{50}s from 800- to 1,300-fold. LT_{50}s were influenced by some brighteners but not by others. All eight brighteners exhibited fluorescence, which was concentration dependent. In general, the brighteners exhibiting the greatest viral enhancement tended to exhibit the greatest fluorescence, and those exhibiting the least viral enhancement tended to exhibit the least fluorescence. Although pH of the brighteners ranged from 7 to 10, no correlation was found between pH and activity enhancement. Shapiro and Argauer (1997) tested components of Tinopal LPW (i.e., triazines, sulfonic acids, and stilbenes) to determine whether these components could act as enhancers. Most of the components tested showed no enhancement, and none of the derivatives was as active as Tinopal LPW.

The fluorescent brightener Tinopal LPW also has been shown to provide some UV protection for the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemins (Inglis et al. 1994) and the entomopathogenic nematode *Steinernema carpocapsae* Weiser (Nickle and Shapiro 1992, 1994). With these insect control agents the fluorescent brightener did not produce an enhancing effect beyond the UV protection as it did for some of the entomopathogenic viruses. However, not all insect viruses appear to be enhanced by fluorescent brighteners. Preliminary tests indicated that Tinopal LPW does not enhance infectivity of the *S. frugiperda* ascovirus for *S. frugiperda* or the *H. zea* gonad-specific virus for *H. zea* (Hamm, unpub. data).

Thus, while the mode of action for the enhanced infectivity of some insect pathogenic viruses by certain fluorescent brighteners is not fully understood, enhanced infectivity of 10 different nucleopolyhedroviruses and a granulovirus (Baculoviridae), a cypovirus = cytoplasmic polyhedrosis virus (Reoviridae), an iridovirus (Iridoviridae) and an entomopoxvirus (Poxviridae) has been demonstrated. The enhanced infectivity of certain insect pathogenic viruses produced by the fluorescent brighteners generally resulted in earlier mortality and in some cases extended infectivity of the virus to older instars, two factors that could make baculoviruses more acceptable as microbial control agents. In some cases viruses which are not infective for a particular species under normal conditions were infective with the addition of a fluorescent brightener. This could be particularly important for commercialization of the broad spectrum baculoviruses (i.e., AcMNPV and AfMNPV) if the use of a fluorescent brightener can bring the infectivity of these viruses to a practical level for more species of pests.

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