Optical brighteners – Pathogenicity enhancers of entomopathogenic viruses

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Optical brighteners are chemicals, which are used in laundry detergents to make fabrics appear brighter, chiefly by absorbing energy from ultraviolet (UV) light and emitting it as visible light. They have been reported as UV protectants for entomopathogens to extend their effectiveness in the field as microbial agents. Some brighteners have been demonstrated not only as UV protectants, but have also shown the potential to enhance virus efficacy against forest and agricultural insect pests. The enhanced infectivity produced by optical brighteners generally resulted not only in increased larval mortality (reduced LD_{50} values), but also hastened larval death (reduced LT_{50} values). Moreover, in some cases, extended infectivity of the virus to older larvae has also been observed. In a few cases the host range was extended, i.e. viruses, not infective for a particular species under normal conditions, were infective with the addition of an optical brightener. This article outlines the major features of optical brighteners as potential enhancers of viral pathogenicity and their utility in pest management.

MICROBIAL insecticides are becoming increasingly attractive as alternative pesticides. In particular, a family of insect-specific viruses, the baculoviridae, has long been recognized as an environmentally safe potential alternative to chemical pesticides as the viruses are highly host-specific, non-pathogenic to beneficial insects and other non-target organisms, including mammals[1–5], thus making them attractive candidates for integrated pest management (IPM). Other advantages of baculoviruses for pest control include a lack of toxic residues, allowing growers to treat their crops even shortly before harvest and unlikelihood development of stable resistance[6,7]. Despite these advantages, their practical application as microbial pesticides has not been fully exploited. Among the various limiting factors, some of the important ones that inhibit their commercial development as a microbial pesticide include: (i) narrow host range, (ii) slow speed to kill the pest, (iii) technical and economical difficulties in commercial production, and (iv) rapid inactivation of the virus by sunlight or ultra violet (UV) light. With few exceptions, most of the insect viruses are highly host-specific and infect only a single species or a few closely related species[8]. Their narrow host range, discussed as an advantage above, may be considered a disadvantage by some growers who often tend to favour agents that control several pests; and certainly, a virus that is highly effective against several species of pests would have a larger market potential than a species-specific virus[9]. Many viruses require 3 to 8 days after being ingested to produce an infection and kill the host[10], during which time the pest continues to damage the crop. A virus, which kills the pest sooner would provide more protection to the crops. Economic production of virus in sufficient quantities is another limiting factor, because viruses can replicate only in living cells. Therefore, it is necessary to produce viral insecticides either in host insect larvae (*in vivo*) or in susceptible cell culture (*in vitro*)[11]. *In vitro* systems are the preferred option for recombinant baculovirus production, but the techniques have not yet been perfected for large-scale production of either recombinant or natural isolates[12]. It is believed that an *in vitro*-produced viral pesticide may not be commercially feasible because of the high cost of culture media. Commercial media in today's market are designed for the production of high-value recombinant proteins and range in price from US $ 10.00 to 40.00 per litre. Such costs do not militate against commercial production of high-value therapeutic proteins, but are certainly cost-prohibitive for agricultural application[13]. Various analyses have concluded that the price of cost-effective media must be less than US $ 10.00 per litre, and the media must be capable of supporting virus yields sufficient to treat at least one acre of cropland per litre of medium. *In vivo* baculovirus production is easier; however the process of optimization is critical to its economic success. Even for non-commercial ventures, optimization of virus yields is crucial to minimizing costs. *In vivo* virus production largely depends upon the age of the larvae at the time of treatment, dose of the virus inoculum and incubation period of the virus[14–18]. Thus production of virus for field use is currently more

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economically feasible in developing countries where inexpensive labour is available and insecticides are expensive. The cost of commercial virus production could be further brought down if the amount of virus needed is reduced through increased infectivity. The lack of field persistence of baculoviruses is another serious impediment to their use for pest control, as they are rapidly inactivated by sunlight. In particular, UV radiation between 280 and 320 nm is detrimental to all microorganisms[19–21]. Generally, most viruses present on foliage are inactivated within a few days after application. Small numbers of polyhedra situated at protected sites may persist longer[22], whereas those exposed to full sunlight typically lose viability within only a few hours[23]. In soil, viruses were found to retain their biological activity for periods of more than 10 years[22]. Some earlier findings have shown optical brighteners as effective UV protectants against nucleopolyhedrovirus (NPV) of *Orgyia pseudotsugata* (McCunnough)[24], *Spodoptera frugiperda* (Smith)[25], *Lymantria dispar* (L.)[26–28], *Pseudoplusia includens* (Walker)[29], *Anagrapha falcifera* (Kirby)[30], and *Spodoptera litura* (Fabricius)[31], when incorporated into inoculum formulation. The rational of this approach was that the persistence of viable virus would be prolonged because an optical brightener coating on the polyhedra would absorb long-wavelength UV light and thereby provide protection from inactivation. The persistence of the virus on the crop could be increased if appropriate UV protectants could be found and formulated with the virus. Though some progress has been made on all these aspects, available information is scanty, fragmentary and some aspects are even unexplored. In view of the above, an attempt has been made to review the documented information on this topic in order to prepare a case for future studies. The aim of this article is to outline the major features of the optical brighteners as potential enhancers of viral pathogenicity under laboratory as well as field conditions and to examine the mechanism behind the increased pathogenicity of viruses.

Laboratory tests

Shapiro[26] tested some diaminostilbenedisulfonic acid derivatives as UV protectants for the gypsy moth NPV (LdMNPV) against gypsy moth, *Lymantria dispar* (L.) and demonstrated that 17 out of 23 compounds gave significant protection under laboratory conditions. His work not only showed optical brighteners as UV protectants, but also revealed that some of these brighteners strongly enhanced the potency of the virus[27]. In laboratory bioassays, feeding of LdMNPV in 1% concentration of selected optical brighteners reduced the LC_{50} (concentration resulting in 50% mortality) from 18,000 polyhedral inclusion body (PIB)/ml to values between 10 (Phorwite RKH) and 44 (Leucophor BSB) PIB/ml. These brighteners also reduced the LT_{50} (time required to produce 50% mortality) at every virus concentration tested. The addition of Tinopal LPW to the virus also enhanced mortality among mature (4th instars) larvae. Thus, the magnitude of reduction in LC_{50} values (up to 1800-fold) plus the reduction in LT_{50} values in gypsy moth larvae indicated that selected brighteners greatly enhanced the virulence of the gypsy moth NPV. Similarly, fall armyworm, *Spodoptera frugiperda* NPV (SfMNPV) showed enhanced infectivity against 3-day-old fall armyworm larvae when tested in 0.1% Tinopal LPW[25]. Because of the unique level of enhancement of viral infectivity for two important lepidopteran pests by these optical brighteners, a patent for the use of optical brighteners in biological control was awarded on 23 June 1992 (ref. 32). Zou and Young[33] found that the addition of Tinopal LPW 1% with the NPV of soybean looper, *Pseudoplusia includens* (PiSNPV) caused significantly enhanced mortality in all four instars of *P. includens* larvae tested, whereas *Helicoverpa zea* nucleopolyhedrovirus (HzSNPV) showed enhanced mortality only against first and third instars larvae of *H. zea* and against first and second instars of the tobacco budworm, *Heliothis virescens* (F.). The NPV of beet armyworm, *Spodoptera exigua* (Hubner) (SeMNPV), showed enhanced mortality against first and second instars of beet armyworm. Zou and Young[29] conducted additional tests with PiSNPV using slightly different optical brightener, Blankophor BBH (same as Tinopal LPW, but at pH 6.9 instead of 9.5 for Tinopal LPW) and found that the addition of 0.1% Blankophor BBH to the viral suspension reduced the LC_{50} from 970 to 0.0625 PIB/mm^2 for second instars, whereas LT_{50} reduced from 15.9 to 6.4 days by the addition of 0.08% Blankophor BBH. The infectivity of gypsy moth cytoplasmic polyhedrosis virus (CPV) was found to be enhanced by addition of Phorwite AR at 1%, with reduction in LC_{50} by 864-fold[28]. The brighteners also reduced the LT_{50} for the gypsy moth CPV from 13.2 to 8.4 days at a concentration of 1.6×10^6 PIB/ml. They also noticed that Phorwite AR could extend the host range of two viruses, which normally did not infect gypsy moth larvae. The *Autographa californica* nucleopolyhedrovirus (AcMNPV) and an entomopoxvirus from *Amsacta moorei* Butler killed more than 50% of gypsy moth larvae at concentrations of 10^7 and 10^6 PIB/ml respectively. No mortality was observed at these concentrations of virus without the brightener. Shapiro and Vaughn[34] tested five different NPVs–HzSNPV, AfMNPV, *Helicoverpa armigera* (HaMNPV), *Galleria mellonella* (L.) (GmMNPV) and the AcMNPV diluted in 1% Tinopal LPW against second instar *H. zea*. The reduction in LC_{50} by 8.7-fold in HzSNPV, 13.1-fold in AfMNPV, 25.3-fold in HaMNPV, 2.1-fold in GmMNPV and 50.0-fold in AcMNPV was observed in the virus due to the addition of Tinopal LPW 1%. Farrar *et al.*[35] found that Blankophor BBH 1% both in a tank mix preparation and in an experimental wettable powder

formulation of LdMNPV reduced LC_{50} by 42- and 214-fold respectively. However, as Blankophor BBH acts as a moderate feeding deterrent to gypsy moth larvae, the addition of molasses to this brightener was advocated to overcome the feeding deterrence. Hamm and Chandler[36] reported 12 times reduction in LC_{50} for SPOD-X (a commercial formulation of beet armyworm NPV), when diluted in 0.25% Tinopal LPW. Vail *et al.*[30] tested the effects of Tinopal LPW on infectivity of AfMNPV against neonates of tobacco budworm, beet armyworm, corn earworm and cabbage looper, *Trichoplusia ni* (Hubner) on diet. Levels of enhancement as determined by LC_{50} values varied from 2.9 to 13.6-fold, depending on the species. The optimum concentrations for Tinopal LPW enhancement were between 0.25 and 1% (w/v). Similarly, Farrar and Ridgway[37] found that the addition of Blankophor BBH to AfMNPV increased the potency of the virus against corn earworm, diamondback moth and beet armyworm. 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. This may indicate a feeding deterrence at higher concentrations. Fuxa and Richter[38] selected a population of velvetbean caterpillar, *Anticarsia gemmatalis*, which was resistant to the *A. gemmatalis* nucleopolyhedrovirus (AgNPV). Tinopal LPW increased the susceptibility of resistant and susceptible insects by 24- and 58-fold respectively. Moreover, the LC_{50} of AgNPV with the brightener against resistant insects was 5.16 times less than the susceptible insects without brightener. Li and Otvos[39] recognized five brighteners, viz. Blankophor RKH, Blankophor BBH, Blankophor P167, Blankophor HRS and Tinopal LPW, which enhanced the viral activity of western spruce budworm, *Choristoneura fumiferana* (Clemens) nucleopolyhedrovirus (CfMNPV) against fourth instar *C. occidentalis* Freeman. The enhancement of viral activity ranged from 1.76 to 13.08 times, with a reduction in killing time by 30–72%. The optimum concentration of brighteners for effective enhancement was found to be 1%. In an additional experiment, Li and Otvos[40] tested four brighteners (Blankophor HRS, Blankophor P167, Blankophor RKH and Tinopal LPW) against non-diapausing laboratory and diapausing field strains of the same insect, and found that the non-diapausing strain was 2.7 times as susceptible to the virus as the field population in terms of LD_{50} (dose at which 50% of the tested larvae died). When 1% concentration of optical brightener was added to the virus, lethal doses for both strains were significantly reduced, indicating that all four brighteners acted as activity enhancers for CfMNPV. In terms of LD_{50}, brightener-enhanced viral activity against the field strain was more (7.6–11.0 times) than the laboratory strains (2.5–3.5 times), and the LT_{50} was reduced by 39–60% for field strain and by 43–57% for the laboratory strain by the addition of 1% brightener. On the basis of these results they suggested that the nondiapausing laboratory strain of *C. occidentalis* might be used for viral studies with optical brighteners, as the nondiapausing laboratory colony can provide a convenient source of insects for research. Also, this strain can be reared continuously and it is difficult to collect a large number of the same instars at the same time from the field. Monobrullah and Nagata[31] tested seven optical brighteners using diet disc bioassays in the laboratory to determine their effects on the protection and activity of *Spodoptera litura* (Fabricius) nucleopolyhedrovirus (SlMNPV) against fourth instar *S. litura* larvae. They found that all brighteners tested were effective UV protectants, whereas five brighteners (Blankophor BBH, Blankophor RKH, Blankophor P167, Blankophor HRS and Tinopal LPW) enhanced viral activity by 5.53 to 11.08 times. Viral activity was found to increase with the concentration of brighteners. LT_{50} was reduced by 24–27% at 1% concentration of brighteners. Monobrullah and Nagata[41] recorded no mortality in older larvae of *S. litura* against SlMNPV, even though 4.8×10^7 PIB/larva were fed orally as against the 100% mortality in susceptible stage. But 27% of mortality was found at the same dose of virus when diluted in 1% Blankophor HRS[31]. Thus optical brighteners also expand the window for application against older larvae, which generally show resistance against viruses.

Field tests

Webb *et al.*[42] evaluated a standard formulation of Gypcheck (gypsy moth NPV) containing the sunscreen Orzan and a sticker against gypsy moth, along with aqueous formulation of Gypcheck in which Orzan was replaced by Phorwite AR in 1991 and Blankophor BBH in 1992. In 1991, treatments containing Phorwite AR gave significantly higher levels of gypsy moth larval mortality and significantly reduced LT_{50} values compared with equivalent treatments containing Orzan. In 1992, all treatment 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. Webb *et al.*[43] conducted additional experiments to check the efficacy of Gypcheck in combination with Blankophor BBH against third and fourth instar gypsy moth on oak trees. Plots treated with Gypcheck containing Blankophor BBH had significantly more larval mortality and lower LT_{50} values compared to those treated with Gypcheck alone. 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 more suitable for the management of gypsy moth. It also opens a pathway for application from only first and second instars to third and fourth instars, which would substantially ease current time constraints in treatment programmes. Vail

et al.[44] conducted field tests at different locations with AfMNPV and Blankophor BBH for control of lepidopteran pests of cotton, including tobacco budworm, cabbage looper, beet armyworm and bollworm. Results showed that Blankophor BBH reduced the loss of activity of the virus at the two highest applications tested. Time to lose 50% of original activity was extended from 5.5 to 11.5 days. Hamm *et al.*[45] tested Tinopal LPW with the fall armyworm NPV against fall armyworm in whorl-stage corn. The 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 concentration of brightener increased beyond 1%. In the higher volume of water, 0.25% 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 mortality due to NPV increased, the per cent mortality due to parasitoids and ascovirus decreased. Thus, the total mortality was not affected as much as the per cent mortality due to NPV by changes in water volume or by brightener concentration. The reduction in mortality due to parasitoids did not appear to be a direct effect of the brightener on the parasitoids. However, increased infectivity of the NPV and earlier mortality from NPV associated with the brightener resulted in more host larval dying of NPV before the parasitoids could complete development. Zou and Young[29] conducted a field test on a natural population of *P. includens* larvae of mixed ages and showed that 0.3 and 1% Blankophor BBH significantly enhanced mortality due to PiSNPV. Cunningham *et al.*[46] evaluated the feasibility of reducing the dosages of NPV against *L. dispar* by using Blankophor BBH as pathogenicity enhancer and found that 1% Blankophor BBH in conjunction with 1/10th dosages of virus gave similar results compared to the full dosages of virus without Blankophor BBH.

Mode of action

Although the enhancing effects of optical brighteners are well documented for several baculoviruses and their hosts, little is known about the mechanism of enhancement. The first documented evidence providing a clue for the basis of the enhancing activity comes from a study of Tinopal LPW (M2R) effect on LdMNPV pathogenesis in *L. dispar*[47]. They showed that in the presence of M2R, LdMNPV progeny virions were produced by midgut epithelial cells. This might have spread the infection more rapidly to the susceptible tissues that are involved in the secondary cycle of infection and thus account for early larval mortality in comparison to those larvae, which were fed with LdMNPV alone. The larvae, which were fed LdMNPV alone, did not undergo the typical primary cycle of infection in the larval midgut. Dougherty *et al.*[48] showed that M2R at 1% concentration was sufficient to enhance the mortality and hasten the death of larvae when fed orally with baculoviruses. Moreover, enhanced viral performance of LdMNPV was observed specifically when M2R was present concurrently with virus in the lumen of the larval midgut, while M2R and other optical brighteners apparently were not directly toxic to larval lepidopterans[49] and other beneficial insects[1,2]. Shapiro and Argauer[50] showed that the activity of Tinopal LPW as an enhancer for the gypsy moth NPV was not adversely affected by temperature at 121°C for 5 min or exposure to UV (254, 302 and 360 nm) for a period up to 7 days. Argauer and Shapiro[51] compared eight structurally related optical brighteners as enhancers for LdMNPV. Five of the eight brighteners acted as activity enhancers. The most effective brighteners reduced LC_{50} values from 800 to 1300-fold and all eight brighteners were concentration-dependent. Shapiro and Argauer[52] tested components of Tinopal LPW (i.e. triazines, sulphonic acids and stilbenes) to determine whether these compounds could act as enhancers. Most of the components tested showed no enhancement and none of the derivatives was as active as Tinopal LPW. Washburn *et al.*[53] used a reporter gene recombinant of *Autographa californica* MNPV (AcMNPV-*hsp70/lacZ*) to investigate the enhancing effect of M2R on pathogenesis, mortality and time to death in larvae of *T. ni* and *H. virescens* inoculated as newly moulted fourth instars (4°s) and at various hours after moulting (e.g. 16 h – 4^{16}). For both hosts, M2R significantly reduced the time to death by AcMNPV. In 4°-inoculated larvae, M2R accelerated infection of tracheal cells, but overall the effects on pathogenesis were minor and no significant increase in mortality over control (without M2R) was observed. Among infected M2R-treated 4^{16} *H. virescens*, there were significantly more foci per insect compared to controls and first lacZ expression was detected in the midgut epithelium at 6 h post-inoculation (hpi). In contrast, no lacZ signals were detected in the midgut epithelia of control insects at any time point and first lacZ expression was observed in the tracheal system at 14 hpi. When they compared virus-induced mortality in *H. virescens* inoculated at various times during the fourth instar, they found that the M2R treatment enhanced mortality levels progressively for larvae inoculated later during the instar. Therefore, they suggested that in *T. ni* and *H. virescens*, optical brightener M2R enhanced 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 per cent mortality. They suggested that if host species respond to initial infection by sloughing midgut cells, and if M2R does block this response, optical brighteners should improve baculovirus efficacy under field condition. Therefore, these results suggests that M2R does not

enhance the ability of midgut cells to become infected, but instead blocks sloughing of infected primary target cells in the midgut, thereby countering developmental resistance and increasing mortality[53–55]. Hoover *et al.*[56] observed that the mortalities of *H. virescens* larvae challenged with a reporter gene construct of *Autographa californica* nucleopolyhedrovirus (AcMNPV-*hsp70/lacZ*) and when fed either lettuce or artificial diet were 2.5-fold higher than that of cotton-fed insects. This decrease in susceptibility on cotton was observed following oral but not intrahaemocoelic inoculation of virus, and it was negatively correlated with levels of foliar peroxidase. The rates of development of both infected and uninfected larvae also were correlated negatively with levels of foliar peroxidase, and hence, were significantly lower for cotton-fed insects. However, when an optical brightener, Calcofluor White M2R was included in inoculum and administered orally to larvae, mortality levels were equivalent regardless of diet. Therefore, they suggested that sloughing of infected midgut cells occurred at a higher rate in insects that fed on cotton compared to the other two diets, and that midgut cell sloughing is the mechanism whereby susceptibility to mortal infection by AcMNPV-*hsp70/lacZ* decreased on cotton. Monobrullah and Nagata[31] reported that the addition of optical brighteners at 1% concentration to SlMNPV not only increased larval mortality in *S. litura*, but also hastened larval death. They also conducted an experiment with five different dilutions of SlMNPV ranging from 9.6×10^8 to 9.6×10^4 PIB/ml diluted in 1% Blankophor HRS and incubated for 1 h to determine whether the brightener acted at the chemical or physical level on the virus prior to ingestion by the larvae or not. The results revealed that PIB diluted in 1% Blankophor HRS was 11.8 time more active than PIB diluted in distilled water. However, PIB incubated for 1 h in 1% Blankophor HRS followed by three distilled water rinses were no more active than PIB diluted in distilled water. Therefore, the brighteners apparently do not chemically or physically alter either the polyhedral inclusion body before the virions enter the midgut of the insect. The increase in virulence of SlMNPV due to addition of optical brightener might be due to a peculiar mode of action. Several optical brighteners are known to interfere with cellulose[57] and chitin fibrillogenesis[58]. The peritrophic membrane is a continuous tube that encases the food in the midgut of lepidopteran larvae and is composed of chitin microfibrils, which presumably protect midgut cells from abrasion by food particles and serve as a barrier for the invasion of microorganisms, including insect viruses[59–62]. Therefore, they concluded that optical brighteners act synergistically with SlMNPV and ultimately disintegrate the larval peritrophic membrane. Thus the damaged peritrophic membrane permits a large number of virions to pass into the ectoperitrophic space and ultimately to invade susceptible cells and replicate, thus accounting for the observed increase in viral potency. The optical brightener Tinopal LPW has also shown to provide some UV protection for the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemins[63] and the entomopathogenic nematode *Steinernema carpocapsae* Weiser[64]. But with these insect control agents, the optical brightener did not produce an enhancing effect beyond the UV protection as it did for the entomopathogenic viruses. While the mode of action for enhanced infectivity of insect pathogenic viruses by certain optical brighteners is not fully understood, enhanced infectivity of several viruses has been demonstrated and reviewed in this article. The enhanced infectivity of these insect pathogenic viruses produced by the optical brighteners generally resulted in earlier larval mortality (reduced LT_{50}), increased infectivity (reduced LD_{50}) and in some cases extended infectivity of the virus to older instars. Therefore, these factors could certainly 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 an optical brightener. This could be particularly important for commercialization of the broad-spectrum baculoviruses, if the use of optical brighteners can bring the infectivity of these viruses to a practical level for more species of pests.

Conclusion

The role of optical brighteners in relation to insect-pest management is yet to be fully explored. The selection and use of optical brighteners and then incorporation with the virus may be an important economical decision and intelligent choices need to be made. Continuous research is needed to identify whether the degree of enhancement of pest control or the reduction in quantity of virus needed to achieve effective control, justify the additional cost of incorporation of an optical brightener. Moreover, the mechanism of action of optical brighteners in relation to increased viral pathogenicity is not fully understood. It would be interesting to determine the mechanism by which baculoviruses penetrate the peritrophic membrane in association with optical brighteners. This will be important not only for a greater understanding of viral infection in insects, but will also give an insight into the mechanisms of other viral pathogens. Therefore, detailed histopathological and biochemical studies of peritrophic membranes are required to fully determine the effect of optical brighteners as pathogenicity enhancers of viruses.

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