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BIOLOGICAL CONTROL

Insecticidal Activity of the Granulosis Virus in Combination with Neem Products and Talc Powder Against the Potato Tuberworm *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae)

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Abstract
The potato tuberworm *Phthorimaea operculella* (Zeller) is an important agricultural pest that causes significant economic losses to potato growers worldwide. The addition of an effective method of biological control for the potato tuberworm is greatly needed, and is currently unavailable in Brazil. The granulosis virus (Baculoviridae) is a promising biological control agent to protect post-harvest potatoes and in storage from the potato tuberworm. However, the control measure must be economically feasible. Liquid suspensions of a granulosis virus applied alone or in mixture with two commercial neem oil-based products (DalNeem™ and NeemAzal™), and a dry powder formulation of viral granules were evaluated for control of potato tuberworm larvae by treating potato tubers under laboratory conditions. High larval mortality (86.7%) was achieved when DalNeem and virus were applied together at 4 mg of azadirachtin/L and 10^4 occlusion bodies (OBs)/mL, respectively. This combination resulted in ≥50% efficacy in relation to their counterparts alone. Conversely, NeemAzal did not enhance virus effectiveness against larvae of the potato tuberworm. The talc-based virus formulation was used for dusting seed tubers at different concentrations and resulted in 100% larval mortality at 5×10^8 OBs/g. Formulated and unformulated virus provided 50% mortality at 166 OBs/g and at 5.0×10^5 OBs/mL, respectively. As a result, talc-based virus formulation had a better control efficiency on potato tuberworm than the aqueous virus suspension. The granulosis virus combined with DalNeem at low rates or formulated with talc powder is a viable option to control the potato tuberworm under storage conditions.

Introduction

The potato tuberworm, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), is an important economic pest of potato crops and stored potatoes, causing high yield losses in warm climates worldwide (Radcliffe 1982, Hanafi 1999). This pest, originating in South America, has spread to almost all potato-producing areas throughout the world (von Arx *et al* 1990, Rondon 2010). The main economic damage occurs towards the end of the growing season, when moths lay eggs on exposed tubers. Secondary damage by potato tuberworm re-infestation as well as pathogen inoculation during larvae entry also affect potatoes during storage and are considered a problem (Trivedi & Rajagopal 1992, Kroschel *et al* 1996). Foliar damage to potato crops usually does not result in significant yield loss, but infested tubers and storage losses may reduce marketability as much as 100%, especially in nonrefrigerated systems (Rondon 2010). There are several different methods for controlling potato tuberworm pests (Hanafi 1999, Rondon 2010).

In Brazil, there are no biological products currently available for use against the potato tuberworm. The

granulosis virus (GV) (Baculoviridae) is a promising biological control agent that could be used in integrated pest management programs for controlling this lepidopteran pest. Granulosis virus has been successfully used as a biopesticide for the control of potato tuberworm under field conditions and at small scale, nonrefrigerated rustic storages in several developing areas of the world (Wraight *et al* 2007). Furthermore, its use can help to mitigate insecticide resistance and reduce secondary pest outbreaks. Granulosis viruses have little negative impact on nontarget organisms and the environment (Gröner 1986). However, granulosis viruses have some disadvantages that have limited their commercial use as bioinsecticides, including a narrow host range and slow action against target insects.

The combined use of insect pathogens and stressors (e.g., botanical agents) has received some interest in recent years. Typically, such combinations are used as an attempt to improve the virulence of the pathogen by inducing mortality at low doses or by accelerating the development of the disease (Murugan & Jeyabalan 1998). Efficacy of baculoviruses has been increased when used in combination with other agents such as stilbene-derived optical brighteners, chitinase, boric acid, and botanical extracts (Shapiro & Bell 1982, Shapiro *et al* 1987, Shapiro & Robertson 1992, Murugan *et al* 1998). Synergistic or additive effects at low doses of botanical insecticides and viruses have been demonstrated by several studies. For example, previous studies have shown increased mortality of lepidopteran larvae by the combined use of nucleopolyhedrosis virus and azadirachtin (Nathan *et al* 2005, Nathan & Kalaivani 2006). Azadirachtin A, a tetranotriterpenoid molecule, is the main active ingredient in most commercial neem formulations. It has a strong negative effect at low concentrations on feeding, growth, molting, and reproduction of more than 400 insect species (Mordue Luntz *et al* 1998). In laboratory trials, the combined use of azadirachtin and the gypsy moth nucleopolyhedrosis virus caused significantly faster mortality of gypsy moth larvae when compared to larvae that were fed either with only azadirachtin or the virus (Cook *et al* 1996). Both azadirachtin and baculoviruses affect insect physiology by altering ecdysteroid and juvenile hormone titers increasing larval duration and delaying molting (O'Reilly & Miller 1989, Dhadialla *et al* 1998). Studies evaluating the combined use of azadirachtin and baculoviruses have not yet reported any antagonistic interactions neither effects of the combined use of azadirachtin and granulosis viruses for potato tuberworm.

Insect baculoviruses are generally highly sensitive to ultraviolet (UV) radiation, extreme pH levels, and high temperatures (Morris 1971). The effects of abiotic conditions in the field due to prolonged exposure can be reduced through the use of adjuvants in formulation, resulting in increased viral persistence. Talc dust (magnesium silicate), a dry inert carrier, can be formulated with a virus in a dustable or wettable powder and increase its effectiveness. Besides, it is cheap, easy to obtain, provides UV protection, and can have negative effects on insect behavior and physiology (Helson 1942, Korunic 1998). A dry powder formulation of granulosis virus containing occlusion bodies (OBs) incorporated into talc has been successfully developed and marketed by the International Potato Center in Lima, Peru, as a nontoxic method to protect tubers in nonrefrigerated storage facilities (Lagnaoui *et al* 1997). Talc-based formulations have also been tested for potato tuberworm control in nonrefrigerated storages (Alcázar *et al* 1992b, Arthurs *et al* 2008) and in field trials (Salah & Aalbu 1992) with some success. However, the median lethal concentration of virus talc-based formulation in comparison with aqueous virus suspensions remains undetermined.

Our study investigated whether potato tuberworm control with granulosis virus was improved by the addition of azadirachtin. In addition, granulosis virus formulated with talc was evaluated for effectiveness in controlling the potato tuberworm during storage.

Material and Methods

Insect rearing and virus preparation

Healthy potato tuberworm neonates were obtained from a laboratory colony maintained for 3 years at the "Laboratório de Patologia e Controle Microbiano de Insetos, Departamento de Entomologia e Acarologia, ESALQ/USP", following Mascarin *et al* (2010). Insect rearing was conducted in an aseptic room with controlled environmental conditions (25±1°C, RH ≥50% and 12 h daily photoperiod).

The granulosis virus used in this study was originally isolated in 2006 from a laboratory colony of potato tuberworm larvae and identified under scanning electron microscopy. Fresh virus-filled potato tuberworm cadavers were obtained using the egg dip and tuber dip methods for virus propagation (adapted from Alcázar *et al* 1992a, Sporleder *et al* 2005). Batches of potatoes or potato tuberworm eggs were dipped for 2 and 1 min, respectively, into a virus suspension consisting of 10 virus-infected fourth instars diluted in 1 L distilled water (dH_2O)+0.1% Tween® 20 (polyoxyethylene sorbitan monolaurate; Oxiteno S/A, São Paulo, Brazil). One hundred virus-treated eggs were inoculated on 200 g of tubers and maintained in plastic containers lined with cardboard to provide pupal shelter at 25±1°C. The virus-infected larvae were collected at third or fourth instars (2–3 weeks old), lyophilized, and stored at −40°C until needed. The original virus suspension was partially purified by the following procedure: virus-filled

cadavers, mostly diseased last instars were thoroughly macerated in 18.2% (*w*/*v*) Tris buffer solution (pH=7.4) with 0.1% (*w*/*v*) of sodium lauryl sulfate (Sigma Chemical Co., St. Louis, USA). This suspension was filtered through fine-mesh fabric (*voile*) to remove insect debris; the filtrate was transferred to tubes, centrifuged at 150×*g* (=1,000 rpm) for 3 min, and the pellet was discarded. The concentration of the virus suspension was then determined using a Petroff-Hausser counting chamber (Hausser Scientific, Pennsylvania, USA), and adjusted to concentrations ranging from 1×10^4 to 5×10^8 OBs/mL in sterile dH_2O for testing.

Neem insecticides and granulosis virus formulation

Neem products consisted of two oil-based formulations (emulsifiable concentrate) of azadirachtin commercialized as DalNeem™ (Dalquim Indústria e Comércio Ltda., Itajaí, SC, Brazil) and NeemAzal™-T/S (Trifolio-M GmbH, Lahnau, Germany), containing 859 and 10,000 mg/L of azadirachtin A, respectively, as the principal active ingredient. DalNeem™ also contained 736 mg/L of azadirachtin B. Each neem formulation was diluted with dH_2O to obtain azadirachtin A concentrations of 4 and 8 mg/L.

The inert carrier used for granulosis virus formulation in dry powder (dustable) was talc, which was comprised primarily of magnesium silicate hydroxide $[Mg_3(OH)_2(Si_2O_5)_2]$ (Mineração São Judas Ltda., Itararé, SP, Brazil) with particle diameters measuring between 1.9 and 2.3 µm. The talc was autoclaved for 20 min at 120°C and cooled before being mixed with the virus suspensions. Briefly, granulosis virus-infected potato tuberworms (~6.6×10^9 OBs/larva, determined in Mascarin *et al* 2010) were macerated in Tris virus suspension and used as stock inoculum to prepare subsequent dry powder virus treatments. Talc was added to various virus–suspension (w/v) concentrations at the same ratio, while being continuously stirred, and then dried in a metallic tray under a laminar flow chamber with airstream (26±1°C) before use.

Interactive effects between granulosis virus and neem products

Surface-treated tubers were prepared using the tuber dip method by dipping pesticide-free tubers in aqueous treatments for 5 min. Due to the large numbers of treatments and insects required for each bioassay, DalNeem and NeemAzal were tested independently and concentrations adjusted based on the azadirachtin A. Both products were tested in nine treatments: dH_2O (control), granulosis virus (1×10^4 and 1×10^5 OBs/mL) alone, neem products (4.0 and 8.0 mg of azadirachtin A/L) alone, and the four paired combinations between the individual virus and neem treatments (i.e., virus 1×10^4+neem 4.0, virus 1×10^4+neem 8.0, virus 1×10^5+neem 4.0, and virus 1×10^5+neem 8.0; Table 1). These experiments simulated a tank mixture with two control agents against potato tuberworm.

Eight tubers were used for each of the nine treatments. But only four tubers (~150 g) were treated by time using a 300-mL suspension, and then allowed to air dry. Each treatment consisted of four replicates, each one containing two tubers (70±5.0 g) placed in 470-mL plastic cups with a piece of cardboard at the bottom as a pupation shelter. Tubers from each replicate were inoculated with 30 neonates of the potato tuberworm (<12 h old) after potato treatment (n=120 per treatment) and maintained at controlled conditions (24±0.5°C; 55±5% RH; 12 h photophase).

Symptoms of virus-infected larvae and dead larvae due to azadirachtin, previously described by Reed (1969) and Kumar *et al* (2008), were helpful in larval mortality evaluation. The combined treatments caused both larval blackening and oozing of the body contents. Larval mortality was recorded after 21 days posttreatment, when larvae in controls started to pupate. Larvae that were unable to move and feed were considered dead. The proportion of larval mortality was calculated by dividing the number of dead larvae by the total number of larvae introduced into each cup (i.e., 30). Larvae collected with typical symptoms of viral disease were recorded as recovery of symptomatic infected larvae, referred to as percentage of infected larvae, which was calculated by dividing the number of virus-infected larvae recovered by the total number of larvae introduced into the cups (i.e., 30). Although most infected larvae were found inside the pupation shelter, all tubers were dissected, using a fine needle and scalpel, to ensure all larvae were accounted for.

Dry powder formulation and aqueous suspension bioassays

Two bioassays with tubers were independently conducted to evaluate the ability of the granulosis virus to infect and cause mortality to tuberworm larvae. The first bioassay assessed the effectiveness of the virus treatment as a dustable powder formulation, while the second evaluated the efficacy of an aqueous (crude) virus suspension. In both cases, untreated tubers were used as controls.

The dry powder treatments were prepared by formulating virus suspensions on talc at a 1:1 ratio. The mixture was vigorously agitated for at least 10 min to achieve a homogenous product. To prepare the desired virus concentrations, serial dilutions with dH_2O and the virus stock suspension were thoroughly mixed with talc powder. Talc–virus preparations were air-dried in a laminar flow chamber for 12 h at 26±1°C. After drying, mixtures were grounded to a fine powder using a mortar and pestle and used immediately to treat tubers. Six treatments were

Table 1 Larval mortality (in percent, ±SE) of *Phthorimaea operculella* after 3 weeks exposure to single and combined treatments of granulosis virus with DalNeem or NeemAzal (24±0.5°C; 55±5% RH; 12 h photophase).

Virus (OBs/mL)[a]	Observed mortality (%)[b]					
	DalNeem (mg/L)[c]			NeemAzal (mg/L)[c]		
	0	4.0	8.0	0	4.0	8.0
0	18.3±8.9 Bb	57.5±6.0 Ba	80.8±2.8 Aa	16.7±1.9 Bb	42.2±5.9 Aab	59.2±3.7 Aa
10^4	51.7±3.5 Ab	86.7±3.6 Aa	92.5±1.1 Aa	50.8±7.6 Aa	54.4±5.9 Aa	40.8±5.2 Aa
10^5	70.1±4.6 Aa	74.4±6.2 ABa	80.0±5.1 Aa	75.0±4.4 Aa	53.3±8.5 Aa	57.5±8.7 Aa

[a] Treatments consisted of two concentrations of aqueous virus suspension or oil-based neem formulations applied alone or as paired combinations (mixtures).

[b] Means (±standard error) followed by the same uppercase letters within columns, and lowercase letters within rows are not significantly different by Tukey–Kramer test ($P>0.05$).

[c] Concentrations of neem products were standardized based on milligrams per liter of azadirachtin A.

tested: untreated larvae (blank control), talc without virus (negative control), and four different concentrations of virus formulated with talc, 5×10^2, 5×10^4, 5×10^6, and 5×10^8 OBs/g. Dusting consisted of placing tubers (approximately 300±5.0 g) in a 4.5-L sealed plastic bag with 120 g of each dry powder formulation, followed by gentle shaking the bags for 1 min.

Virulence of the aqueous virus suspension (also called crude virus as standard treatments) was tested by dipping tubers for 2 min in aqueous suspensions of granulosis virus at equivalent concentrations of those tested before (5×10^2, 5×10^4, 5×10^6, and 5×10^8 OBs/mL). Control consisted of tubers dipped in sterile dH_2O.

For both bioassays, potatoes were inoculated with neonates of the potato tuberworm after treatment of the tubers. Batches of 20 neonates (<12 h old) were inoculated on 50±5.0 g of potato tuber placed in 470-mL plastic cups, and then incubated at controlled conditions (24±0.5°C; 55±5% RH; 12 h photophase). Six replicates were used, with a total of 120 larvae/treatment. Three weeks later, larval mortality (in percent) was determined based on the number of dead larvae compared to the total number of larvae introduced.

Data analyses

Each experiment was designed as a completely random design and carried out at different dates. All statistical analyses were performed in SAS program version 9.2 (SAS Institute Inc. 2008). Statistical analyses were performed independently for each bioassay. Before analysis, residuals of data sets were checked for normality assumptions using Shapiro–Wilk and Brown–Forsythe tests. In order to determine whether granulosis virus and neem products had significant effects on larval mortality and whether the interaction between the two (virus×neem) was significant, data sets were subjected to two-way analysis of variance (ANOVA) with virus and neem as the model main effects using PROC GLM. When interactions were significant, differences between treatment means were determined by Tukey–Kramer test ($\alpha=0.05$) as the option selected for the LS means statement; otherwise, means were compared separately within each main factor. As for the variable "percentage of infected larvae recovered", data sets from each trial were submitted to one-way ANOVA and means from combined treatments and virus alone were compared for each neem product separately by the Tukey's honestly significant difference test (Tukey's HSD, $\alpha=0.05$).

Synergistic, additive, or antagonistic interactions of the virus combined with neem treatments were determined using a chi-square (χ^2) test (Finney 1971). Analysis of virus interactions and the two neem products were conducted independently. For calculations, the corrected percent mortality was used before data analysis (Püntener 1981). The formula $M_e=M_{aza}+M_{gv}\times[(100-M_{aza})/100]$ (MacVay *et al* 1977) was employed here to determine the expected mortality if these two agents acted independently from each other, where M_e is the percentage expected mortality, M_{gv} is the percentage observed mortality produced by the virus alone, and M_{aza} is the percentage observed mortality produced by the stressor agent alone (neem). Results from a chi-square test, $\chi^2=(M_{gv+aza}-M_e)^2/M_e$, where M_{gv+aza} is the observed mortality for the combination and M_e is the expected value were compared to the chi-squared table value (χ^2 table=3.84) for $df=1$ ($\alpha=0.05$). If table value exceeded calculated value, it would be concluded that the observed mortality for the combination of agents was within the range expected for additive effects. If the calculated value exceeded the table value, there would be reasons to expect a synergistic or antagonistic result between the two agents.

Relationships between larval mortality and log concentration for formulated and unformulated (aqueous suspension)

granulosis virus were determined by binomial model with a complementary log–log link function (Gompertz model, Mascarin *et al* 2010). This model fitted well these two data sets due to low deviance values (Pearson's chi-square/degree of freedom), which was provided by the chi-square goodness-of-fit test performed in PROC PROBIT statement of SAS 9.2 (Collett 1991). Differences between median lethal concentration values (LC_{50}) were determined by overlapping fiducial limits. To determine whether slopes were different, a comparison of the regression coefficients was performed using the PROC PROBIT with a test of parallelism (H_o, slopes are equal). Furthermore, in order to verify whether pathogen concentration significantly affected larval mortality, a type III analysis of effects based on Wald chi-square (α= 0.05) was performed separately for formulated and unformulated virus.

Results

Interactive effects between granulosis virus and neem products against the potato tuberworm

Average mortality of larvae from untreated tubers (control) was 18.3% and 16.7% for virus×DalNeem and virus×NeemAzal bioassays, respectively. The mortality of larval treated with either concentrations of granulosis virus alone differed significantly from the controls for both experiments (F=22.5; df=2, 25; P<0.0001 and F=8.1; df=2, 25; P<0.0021, respectively; Table 1). However, larval mortality was not significantly different between the two virus concentrations tested (P>0.05).

DalNeem applied alone on tubers at the high rate (8.0 mg of azadirachtin A/L) provided greater mortality than the control (F=22.48; df=2, 25; P<0.0001), whereas mortality was not increased between the low and high concentrations of NeemAzal (F=0.25; df=2, 25; P< 0.7784). Significant interactions between virus×DalNeem and virus×NeemAzal for larval mortality were observed (F=7.5; df=4, 25; P=0.0004 and F=7.3; df=4, 25; P= 0.005, respectively). Granulosis virus and DalNeem combined at low rates (10^4 OBs/mL+4 mg/L) caused a significantly greater mortality than each agent applied alone (P< 0.05), resulting in an additive effect (χ^2<3.84, df=1, P< 0.05; Table 2). The combined treatment of 10^4 OBs/mL virus and 8 mg/L DalNeem inflicted significantly greater mortality than the virus itself (P>0.05), but did not differ from the mortality observed in single treatments with DalNeem. Conversely, combined treatments between virus and NeemAzal did not enhance larval mortality, since no statistically significant difference was found when compared to each agent applied individually was observed (P>0.05). As a result, all combinations between NeemAzal and granulosis virus were considered as antagonistic (χ^2> 3.84, df=1, P<0.05), with the exception of an additive interaction between virus and NeemAzal when both were applied at lower concentrations (Table 2).

Both concentrations of DalNeem significantly reduced the recovery of infected larvae when applied together with 10^5 OBs/mL of granulosis virus (F=29.74; df=2, 7; P< 0.0001), but no detrimental effect of DalNeem on infected larvae yield was observed when combined with the low virus concentration (10^4 OBs/mL; F=1.45; df=2, 9; P= 0.2839). On the other hand, NeemAzal did not display any detrimental effect on the percentage of infected larvae recovered from tubers (low virus concentration: F=1.03, df=2, 8, P=0.3985; high virus concentration: F=1.33, df=2, 8, P=0.3166; Table 3).

Generally, larvae that died from combined treatments between virus and neem products had septicemia, especially at higher concentrations of both agents.

Effect of a dry powder formulation of granulosis virus against the potato tuberworm

The average larval mortality from untreated control tubers was 16.7% for the aqueous treatment bioassay and 30% for the formulation bioassay. However, these natural mortalities found in both experiments were not significantly different (t=3.37; df=1, 10; P=0.0963). Aqueous virus suspensions and granulosis virus formulated with dry powder both significantly increased larval mortality in comparison to their respective untreated controls (Wald χ^2=15.91; df=1; P<0.0001 and Wald χ^2=26.03; df=1; P<0.0001, respectively), with mortality ranging from 16% to 100% for aqueous treatments and 58.3% to 100% for dry powder treatments (Fig 1). The corrected mortality for larvae exposed to tubers treated with talc alone (negative control) was 56%, and was not included in the analysis of concentration–response relationship. Moreover, single talc treatment was as much as effective in relation to virus formulations at 5×10^2 and 5×10^4 OBs/g (P>0.05). We only noticed additive effects regarding larval mortality at 5×10^6 and 5×10^8 OBs/g among virus talc treatments. Three weeks after treatment, 50% mortality was observed at 166 OBs/g with virus-based talc formulation and at 5×10^5 OBs/mL with aqueous virus suspension (Table 4). The virus formulated with dry powder was 3,000-fold more effective at causing mortality than the aqueous virus suspension based on the LC_{50} values, since their fiducial limits did not overlap. However, there was a nonsignificant difference in terms of effectiveness between unformulated and formulated granulosis virus based on LC_{90} values, since their fiducial limits did overlap (Table 4). Difference between regression slopes of unformulated and formulated virus

Table 2 Interaction between granulosis virus and neem products on larval mortality of *Phthorimaea operculella*, analyzed by the χ^2 test (df=1).

Combined treatments of GV+AZA (OBs/mL+mg/L)	Corrected mortality (%)[a]		χ^2 calc[b]	Type of interaction[c]
	Observed values (M_{gv+aza})	Expected values (M_e)		
DalNeem				
10^4+4.0	83.7	69.2	3.03	Additive
10^4+8.0	90.8	86.1	0.26	Additive
10^5+4.0	68.7	81.0	1.86	Additive
10^5+8.0	75.5	91.4	2.77	Additive
NeemAzal				
10^4+4.0	45.3	59.1	3.23	Additive
10^4+8.0	29.0	71.1	24.92	Antagonist
10^5+4.0	44.0	83.2	18.47	Antagonist
10^5+8.0	49.0	85.3	15.45	Antagonist

[a] Corrected percent mortality values for GV + neem treatments are presented as observed values (M_{gv+aza}) and the expected values (M_e).

[b] Chi-squared values calculated by the equation: $\chi^2 = (M_{gv+stressor} - M_e)^2 / M_e$, and then compared to the chi-squared table value for df=1 at α= 0.05 (χ^2 table=3.84).

[c] Additive effect: χ^2 calc ≤3.84, and antagonistic effect: χ^2 calc >3.84 and ($M_{gv+aza} - M_e$) <0.

was statistically significant (χ^2=6.05, df=1, P=0.0139), rejecting the hypothesis of parallelism. Also, the pattern of response of increasing concentrations was really different for each virus preparation, mostly likely due to high initial mortality caused by the talc used in the virus formulation. At the maximum virus rates (5×10^8 OBs/mL and 5×10^8 OBs/g), mortalities reached 100% for both viral preparations. In addition, most larvae infected by granulosis virus at concentrations ≥5×10^6 OBs/mL failed to pupate and died.

Discussion

This study evaluated whether azadirachtin or talc powder could be used as stressor agents to enhance virus control against neonates of the potato tuberworm by tuber pretreatment and, consequently, reduce tuber damage using economically feasible pathogen rates. Susceptibility of potato tuberworm larvae varied according to neem products and granulosis virus treatment rates. In fact, larval mortality was directly proportional to concentration of both agents. There was a significant increase in mortality when DalNeem (4 mg/L) and granulosis virus (10^4 OBs/mL) were combined in relation to both agents applied alone at the same rate. Furthermore, no significant difference in mortality was observed between NeemAzal combined with the virus and the virus alone. Most combined treatments between NeemAzal and granulosis virus resulted in antagonistic effect.

Several studies have demonstrated increasing susceptibility to nucleopolyhedrosis virus by additives such as

Table 3 Recovery of virus-infected larvae (in percent, ±SE) of *Phthorimaea operculella* after 3 weeks exposure to single and combined treatments of granulosis virus with DalNeem or NeemAzal (24±0.5°C; 55±5% RH; 12 h photophase).

Virus (OBs/mL)[a]	Infected larvae recovered (%)[b]					
	DalNeem (mg/L)[c]			NeemAzal (mg/L)[c]		
	0	4.0	8.0	0	4.0	8.0
10^4	10.8±4.4 a	7.5±2.1 a	3.3±2.4 a	11.1±3.8 a	5.0±2.2 a	9.2±2.8 a
10^5	54.8±1.0 a	17.5±5.0 b	18.9±2.2 b	24.4±5.9 a	15.8±3.7 a	15.0±2.9 a

[a] Treatments consisted of two concentrations of aqueous virus suspension applied alone or as paired combinations with oil-based neem formulations (mixtures).

[b] Means followed by the same letters within rows are not significantly different by Tukey's HSD test (P>0.05).

[c] Concentrations of neem products were standardized based on milligrams per liter of azadirachtin A.

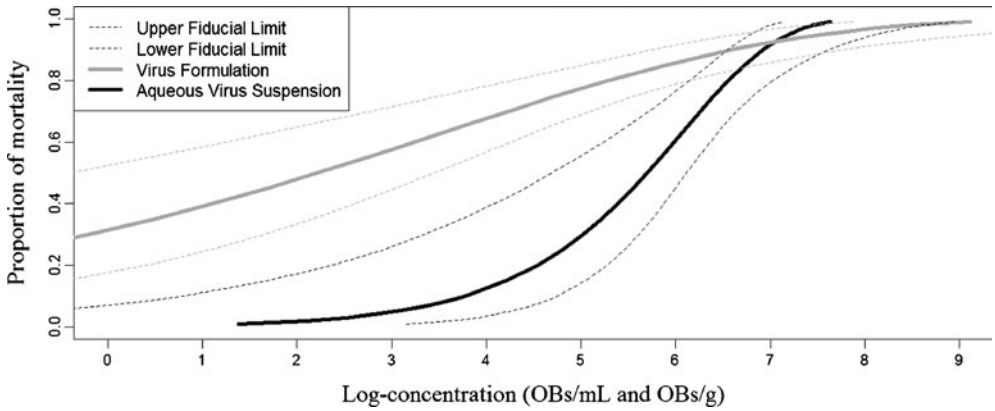

Fig 1 Relationship curves between lethal concentration and larval mortality estimated by Gompertz binomial model for aqueous virus suspension (unformulated) and virus-based talc formulation, 3 weeks after potato tuberworm inoculation on pre-treated seed tubers.

fluorescent brighteners, GV-enhancing protein, and neem extract against lepidopteran hosts at low doses of each agent (Murugan & Jeyabalan 1998, Murugan *et al* 1998, 1999). Results from studies testing neem-based agents against insect pests vary depending on several factors, including neem preparation, concentration of azadirachtin, and insect species and age. Thus, making a direct comparison with the data from this study is difficult.

Both water extract- and oil-based formulations of neem prevent potato tuberworm damage to stored potatoes (Kroschel & Koch 1996, Siddig 1988). Kumar *et al* (2008) observed a decrease in azadirachtin and nucleopolyhedrosis virus required when both agents were combined to control *Helicoverpa armigera*. In the present study, potato tuberworm larvae that fed on tubers treated with virus+DalNeem at low rates exhibited higher mortality levels rather than each treatment alone.

Studies evaluating the interactions of neem and nucleopolyhedrosis viruses at low doses against lepidopteran pests indicate synergistic or additive effects, such as enhancing speed of kill and increasing larval mortality (Cook *et al* 1996, Nathan *et al* 2005, Nathan & Kalaivani 2006). We observed that additive effects were found when combining virus with DalNeem, whereas NeemAzal with virus mostly rendered antagonistic interactions. Therefore, it might be concluded that the outcome of mixing insect virus with azadirachtin to achieve higher insect mortality relies on a range of factors, including the insect host, concentrations of active ingredients, type and composition of product formulation.

A significant reduction in the percentage of infected larvae recovered from the tubers was observed when DalNeem was applied in mixture with the higher rate (10^5) of granulosis virus in comparison with the virus treatment alone. This unexpected result might be explained by the observation that larvae exposed to both agents died of septicemia prematurely due to rapid action of azadirachtin in disrupting the insect gut, which could have affected in somehow the progression of the viral disease. This fact was most often observed while the tuberworm was still inside the tuber. Percentage of infected larvae (i.e., inoculum source) and virus yield within the larval cadaver should be a primary concern when evaluating neem products in combination with entomopathogenic viruses (Cook *et al* 1996). In this study, we found that, NeemAzal did not affect percentage of infected larvae when it was applied in mixture with granulosis virus.

Formulated virus with talc powder was more effective than its unformulated counterpart; 50% mortality of neonate potato tuberworms was obtained at a much lower virus concentration when formulated with talc in comparison with the crude virus treatments (unformulated). The

Table 4 Insecticidal activity of unformulated and formulated granulosis virus to potato tuberworm larvae (24±0.5°C; 55±5% RH; 12 h photophase).

Virus preparation	No. of insects tested	Slope (SE)[a]	χ^2 (*P* value)	LC_{50} (95% FL)[b]	LC_{90} (95% FL)[b]
Aqueous suspension	480	0.98 (0.25) a	43.02 (0.0047)	5.0×10^5 ($0.5–13\times10^5$) a	8.4×10^6 ($3.6–42\times10^6$) a
Dry powder formulation	480	0.27 (0.05) b	32.03 (0.0769)	166.2 (0.4–2,850) b	3.9×10^6 ($0.6–58\times10^6$) a

[a] Parameter estimates followed by different letters indicate significant difference ($P<0.05$).

[b] Values within a column followed by the same letter are not significantly different based on the fiducial limits (95% FL). Lethal concentration values were expressed as OBs per milliliter (active ingredient) of aqueous virus suspension and as OBs per gram of formulated virus, and determined by the binomial model with a complementary log–log link function for dose–response relationship.

smaller slope for formulated virus was due to the high mortality levels at low virus concentration afforded by talc. At the highest rate (5×10^8) of both aqueous suspension and virus formulation, no feeding damage of first instars on the tubers was observed (data not shown), and most likely due to overdose. Arthurs *et al* (2008) also verified 100% potato tuberworm larval mortality with concentrations as low as 5.75×10^8 OBs/kg. Talc was tested as a dust carrier for virus OBs, but we observed that the talc itself strongly affected larval mortality of potato tuberworm due to its fast desiccation effect against newly hatched larvae, as previously described elsewhere (Alcázar *et al* 1992b, Arthurs *et al* 2008). Some dry powder carriers have been used as an alternative to synthetic insecticides and are thought to act by imposing mechanical disruption (e.g., mouth parts injuries on chewing insects such as beetles and caterpillars) and physiological stress by absorbing wax from the insect cuticle resulting in water loss (Ebeling 1971, Korunic 1998). Our results demonstrated that talc mixed with low or medium concentrations of the granulosis virus for dusting tubers provides better effectiveness than aqueous virus suspension treatment against potato tuberworm. The use of granulosis virus with talc is simple and nontoxic to humans, and can be an economically viable option recommended to farmers to protect seed tubers for at least 2 months in rustic potato storage systems (Alcázar *et al* 1992b, Arthurs *et al* 2008).

In conclusion, mixing DalNeem containing azadirachtin with the granulosis virus at low rates provides an effective, cost-efficient method for controlling the potato tuberworm, attaining mortality levels that are greater than either product yielded on their own. Additionally, low rates of virus formulated with talc powder for dusting seed tubers may offer more advantages than aqueous crude virus, since talc has low cost and cause a high mortality by itself.

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