Effect of Temperature on Long-Term Storage of Codling Moth Granulovirus Formulations

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ABSTRACT Codling moth, *Cydia pomonella* (L.), is the major pest of apple (*Malus* spp.) in the western United States and many other regions of the world. The codling moth granulovirus (CpGV) provides a selective and safe means of its control. We assessed the long-term stability and storage potential of two commercial formulations of CpGV, Cyd-X, and Virosoft. All assays were performed with individual *C. pomonella* neonate larvae in 2-ml vials on 1 ml of artificial larval diet that was surface inoculated with 10 μl of the test virus suspension. Baseline quantitative assays for the two formulations revealed that the LC_{50} and LC_{95} values (occlusion bodies per vial) did not differ significantly between the formulations. For year-long studies on Cyd-X stability, the product was stored at −20, 2, 25, and 35°C, and quantitative bioassays were conducted after 0, 3, 6, and 12 mo of storage. Cyd-X retained good larvicidal activity from −20 to 25°C, and it was the least negatively affected at the lowest temperature. Storage of Cyd-X at 35°C was detrimental to its larvicidal activity within 3 mo of storage. For longer term storage studies, Cyd-X and Virosoft formulations were stored at 2, 25, and 35°C, and assayed for larvicidal activity over a 3-yr period. For recently produced product, a 10-μl sample of a 10^{-5} dilution of both formulations resulted in 95–100% mortality in neonate larvae. Larvicidal activity for the Cyd-X formulation remained essentially unaffected for 156 wk when stored at 2 and 25°C, but it began to decline significantly after 20 wk of storage at 35°C. The Virosoft formulation stored at 2°C also remained active throughout the 3-yr study, but it began to decline in larvicidal activity after 144 wk at 25°C and 40 wk at 35°C. The information reported in this study should be useful to growers and commercial suppliers for avoiding decreases in CpGV potency due to improper storage conditions.

KEY WORDS *Cydia pomonella*, temperature, granulovirus, storage, shelf life

Codling moth, *Cydia pomonella* (L.), is the major pest of apple (*Malus* spp.) and a significant pest of pear (*Prunus* spp.) and walnut (*Juglans* spp.) in the western United States and many other regions of the world (Barnes 1991, Beers et al. 1993). The codling moth granulovirus (CpGV) offers an alternative to broad-spectrum insecticides, and it provides a selective and safe means of codling moth control with a negligible preharvest interval (Cross et al. 1999, Lacey and Shapiro-Ilan 2008). CpGV was first commercially produced on a large scale in Europe in the late 1980s (Cross et al. 1999). Although earlier development of the virus in the United States and Canada was promising (Falcon et al. 1968, Jaques et al. 1987), it has only been registered in the United States since 1995 and in Canada since 2000 and in significant use since 2003 (Lacey and Shapiro-Ilan 2008). Three commercial CpGV products (Carpovirusine, Cyd-X, and Virosoft) are now available in the United States. Virosoft is the only product available in Canada. Using fresh product, all three commercial formulations have provided effective control of codling moth in western North America (Arthurs and Lacey 2004, Cossentine and Jensen 2004, Lacey et al. 2004, Arthurs et al. 2005). However, once CpGV products are purchased, the manner in which they are stored is highly variable. Storage of CpGV formulations by some growers under the same conditions as conventional chemical insecticides (i.e., in non–air-conditioned storage sheds), often with temperatures exceeding 40°C, has resulted in curtailed shelf life and treatment failures (L.A.L., unpublished observations). Our studies using quantitative assays were designed to compare the stability and effectiveness of two commercial products after storage in different temperature regimes (2, 25, and 35°C) for up to 3 yr.

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Materials and Methods

Virus Formulations. Two formulations of CpGV, Cyd-X, and Virosoft, were obtained from Certis USA (Columbia, MD) and BioTepp Inc. (Mont-St-Hilaire, QC, Canada), respectively. The products were briefly stored at 2°C until prepared for temperature testing. Label information for Cyd-X and Virosoft indicates that they contain $\approx 3 \times 10^{13}$ and 4.2×10^{13} virus

Table 1. LC_{50} and LC_{95} values for two codling moth granulovirus formulations bioassayed against *C. pomonella* neonates

Formulation	*n*	LC_{50} (95% CI)	LC_{95} (95% CI)	Slope	χ^2	*P*
Cyd-X	802	23.6 (19.6–27.8)	236.4 (175.9–348.2)	1.46	2.0	0.84
Virosoft	416	31.7 (25.2–39.2)	197.7 (146.6–292.8)	1.84	3.6	0.31

The number of occlusion bodies per 10 μl is based on dilutions from label specified virus concentrations. All probits based on dosage $\log_{(10+1)}$ and adjusted for control mortality ($\leq$5.1%).

Bioassays conducted following the methods described in Lacey et al. (2002). The label specified virus concentrations are a min of 3×10^{13} OBs per liter for Cyd-X and 4.2×10^{13} OBs per liter for Virosoft. The surface area of the treated medium in each vial is $\approx$100–110 mm^2.

occlusion bodies (OBs) per liter, respectively. The products used in the baseline and long-term bioassays were conducted with the same samples of Cyd-X and Virosoft. The quantitative year-long bioassays of Cyd-X were conducted with four different bottles of product from the same batch.

Insects and Quantitative Baseline Bioassays. Baseline activity of the two formulations was determined by bioassay of recently produced product by using neonate codling moth larvae and procedures described by Lacey et al. (2002). Codling moth eggs that were ready to hatch were obtained from the colony maintained at the Yakima Agricultural Research Laboratory, and reared on artificial diet using the system of Toba and Howell (1991). Five to seven dilutions of the CpGV formulations were used to produce mortality in neonate larvae ranging from 15 to 95% with at least two data points above and two below 50% mortality. Bioassays were conducted on artificial diet (Southland Products Incorporated, Lake Village, AR) in 2-ml plastic conical autosampler vials (Daigger, Lincolnshire, IL). One milliliter of molten codling moth diet was added to each vial and allowed to cool before application of 10 μl of viral suspension or deionized water for controls. The surface area of the medium ranged between 100 and 110 mm^2. After application of viral suspensions or water, vials were rotated until the surface of the diet was evenly coated and then left to dry before adding a single larva to each vial. A 2-mm-diameter hole in the cap of each vial covered with stainless steel screen (150 mesh) eliminated condensation. The vials were incubated for 7 d at 25 $\pm$ 1.7°C and a photoperiod of 16:8 (L:D) h in a walk-in incubator, and then they were assessed for larval mortality. Bioassays of each concentration of virus and controls were conducted with 30 neonate larvae per replicate test. A minimum of three replicate tests were conducted on separate dates for each formulation.

Year-Long Quantitative Bioassays of Cyd-X. Bioassays were performed with the Cyd-X formulation that had been held at −20, 2, 25, and 35°C for 0, 3, 6, and 12 mo of storage. Assays were conducted as in the baseline studies with five concentrations per replicate test. Four replicate tests were run on separate dates for each storage interval. One-liter samples of the product were stored in their original commercial containers (high-density polyethylene) at the various temperatures with the exception of product stored at −20°C. Four 300-ml aliquots of Cyd-X were stored at −20°C in plastic bottles identical in composition to the 1-liter commercial container. They were thawed one at a time for each assay period to avoid multiple freezing and thawing of a single container. Between individual replicates (performed 2–3 d apart) for each storage period, thawed Cyd-X was stored temporarily at 4°C.

Long-Term Storage Stability of Cyd-X and Virosoft. To test the long-term shelf life of Cyd-X and Virosoft at different temperatures, bioassays were performed using two concentrations of virus formulations and the artificial diet assay method outlined above. For bioassays, we used 1,000-fold (10^{-3}) and 100,000-fold (10^{-5}) dilutions of each product, maintained continuously under three storage temperatures (2, 25, and 35°C). Tests were conducted every 4 wk (four replicates of 30 vials per concentration and control per test) over a 3-yr period for the 25°C storage temperature. Two replicate bioassays were initially conducted at 4-wk intervals for the 2°C regime, but after 88 wk, based on the stability of the virus held at 25°C, assays were performed every 12 wk. For the products stored at 35°C, assays were conducted as for 25°C samples until larvicidal activity declined to <20% mortality, when the study was terminated.

Data Analysis. The quantitative bioassays (i.e., baseline and year-long studies with CpGV formulations) were analyzed based on probit (normal sigmoid) comparisons of the LC_{50} and LC_{95}, respectively, and analysis of covariance (ANCOVA) of the probit slopes (SAS Institute 2001). For the 3-yr storage study, treatments were compared using a generalized linear model assuming a binomial distribution with a logit-link function (PROC GENMOD in SAS, SAS Institute, Cary, NC).

Results

Baseline Quantitative Assays. ANCOVA of the probit slopes revealed no significant difference between Cyd-X and Virosoft, despite the 40% higher concentration (based on label information) of OBs in the Virosoft formulation ($\chi^2 = 3.7$, df = 1, $P = 0.06$). Furthermore, based on the 95% confidence intervals, the calculated LC_{50} and LC_{95} values did not differ between the two CpGV formulations (Table 1). Larvae tended to browse the surface of the medium before making deep entry, and the amount of browsing varied from larva to larva. This fact and the approximate surface area of the medium (100–110 mm^2) provide only an estimate of OB consumption per larvae. Hence, dosage is expressed in OBs per 10 μl.

Table 2. LC_{50} and LC_{95} values for codling moth granulovirus (Cyd-X formulation) stored up to 12 mo at four different temperatures and bioassayed against *C. pomonella* neonates

Storage temp. (°C)	Storage (mo)	*n*	LC_{50} (95% CI)	LC_{95} (95% CI)	Slope	χ^2	*P*
−20	0	718	6.5 (3.6–11.2)	76.2 (34.7–183.5)	1.53	11.7	0.02
	3	709	8.0 (6.2–9.8)	85.8 (64.1–125.5)	1.59	5.0	0.28
	6	708	13.7 (11.4–16.3)	173.9 (126.4–262.2)	1.49	7.5	0.11
	12	716	6.7 (5.2–8.3)	109.5 (78.7–169.3)	1.36	5.1	0.28
2	0	720	3.8 (2.4–5.3)	139.5 (89.6–263.1)	1.05	3.0	0.55
	3	718	9.4 (7.2–11.8)	169.4 (117.0–278.1)	1.31	5.0	0.29
	6	700	12.0 (7.5–18.9)	109.6 (48.6–265.8)	1.71	15.4	0.01
	12	713	12.6 (10.8–14.6)	92.0 (72.2–124.5)	1.91	4.5	0.35
25	0	716	7.1 (5.6–8.7)	74.9 (56.3–108.6)	1.61	8.4	0.08
	3	718	7.8 (5.6–10.1)	206.6 (134.5–373.7)	1.15	6.2	0.18
	6	713	12.8 (10.7–15.1)	145.0 (107.5–212.7)	1.56	6.0	0.20
	12	717	13.1 (11.1–15.2)	110.5 (85.2–153.4)	1.77	5.9	0.20
35	0	719	2.7 (1.6–3.9)	90.9 (60.2–164.1)	1.07	3.0	0.55
	3	720	18.5 (14.3–23.3)	361.9 (235.5–650.4)	1.27	4.5	0.34
	6	718	34.7 (22.3–54.6)	724.0 (227.3–2732.0)	1.25	9.6	0.05
	12	706	Essentially inactive	Essentially inactive		5.0	0.29

The number of OBs per 10 μ l is based on dilutions of Cyd-X with a label specified virus concn of 3×10^{13} OBs per liter. See footnote in Table 1.

Year-Long Quantitative Assessment Storage of Cyd-X. The results of bioassays for 0–12 mo for the −20 to 25°C storage regimes fluctuated somewhat from test to test with LC_{50} values ranging from 3.8 to 7.1 OBs per 10 μl for initial assays (time 0) to 13.7 OBs per 10 μl (6 mo at −20°C) (Table 2). Considering only the initial assays and the 12 mo assays, there was negligible decline in activity for the Cyd-X stored at −20°C; the reason for the increased LC_{50} in the 6-mo sample is not known. However, there were 3.3- and 1.8-fold increases in the LC_{50} values for the 2 and 25°C storage regimes, respectively, over 12 mo; significant increases in these latter confidence intervals at 2 and 25°C occurred consistently after 3 and 6 mo, respectively. Interestingly, such decreases in activity were not observed for the LC_{95} estimates, suggesting that any such declines would be insignificant at operational concentrations used in the field. Storage of Cyd-X at 35°C was markedly detrimental to the larvicidal activity even after just 3 mo of storage. By the end of the 12-mo study, the product held at 35°C was ineffective for control.

Long-Term Storage Studies. Bioassays conducted on Cyd-X and Virosoft stored at different temperatures over 3 yr revealed both formulations retained good activity after storage at 2 and 25°C, even at the lower concentration (10^{-5} dilution) (Figs. 1 and 2). There was a slight decline in Virosoft's activity with the 10^{-5} dilution at 25°C after 144 wk of storage near the end of the study (Fig. 2B). However, significant declines in activity were observed when formulations were stored at 35°C. The logit model over the first 64 wk (subsequently bioassays on the 35°C treatment were terminated) showed highly significant main effect of storage temperature on bioassay activity and an interaction of storage temperature with storage interval ($\chi^2 = 166.0$, df = 2,476, $P < 0.0001$ and $\chi^2 = 200.2$, df = 32,476, $P < 0.0001$, respectively). At 35°C, the activity of Cyd-X declined earlier compared with Virosoft. This earlier decline started after ≈20- versus 40-wk storage at the lowest concentration (Figs. 1B and 2B) of Cyd-X and Virosoft, respectively. This difference is illustrated by a significant two-way (formulation-storage interval) interaction at 35°C ($\chi^2 = 43.1$; df = 16,204; $P < 0.001$). High larvicidal activity persisted at 35°C until 28 and 52 wk for Cyd-X and Virosoft, respectively, for the 10^{-3} dilution (Figs. 1A and 2A).

Discussion

CpGV is one of the most highly virulent granuloviruses (Tanada and Hess 1991). Depending on dosage, it can kill neonate larvae in as few as 3 d (Glen and Clark 1985, Ballard et al. 2000). The LD_{50} has been estimated as low as 1.2–5 OBs per larva (Sheppard and Stairs 1977, Huber 1986) with slightly higher estimates for number of OBs per mm^2 or milliliters of artificial diet (Laing and Jaques 1980, Huber 1981; Lacey et al. 2002, 2005). The data reported here on the LC_{50} of both CpGV products for neonate codling moth larvae is comparable with that reported in the literature for other CpGV preparations. The difference in LC_{50} and LC_{95} values for Cyd-X in the two tables may be due to batch-to-batch variation in the product. For this reason, we did not directly compare these two data sets. Producer specified application rates for Cyd-X and Virosoft are 73–438 ml/ha (2.2×10^{12} to 1.3×10^{13} OBs per ha) and 234 ml/ha (9.8×10^{12} OBs per ha), respectively. The lower concentration (10^{-5} dilution) used in our bioassays is lower than recommended field application rates to avoid overkill (i.e., only produce mortality between 90 and 100%). The 10^{-3} dilution is substantially higher (equivalent to 3–4.2 $\times 10^{13}$ OBs per ha applied in 1,000 liters) than producer recommended application rates for both products, and it was intended to demonstrate residual activity after the optimal shelf life had been surpassed. Because the concentration of virus required to kill neonate codling moth larvae is very low, high mortality rates could be

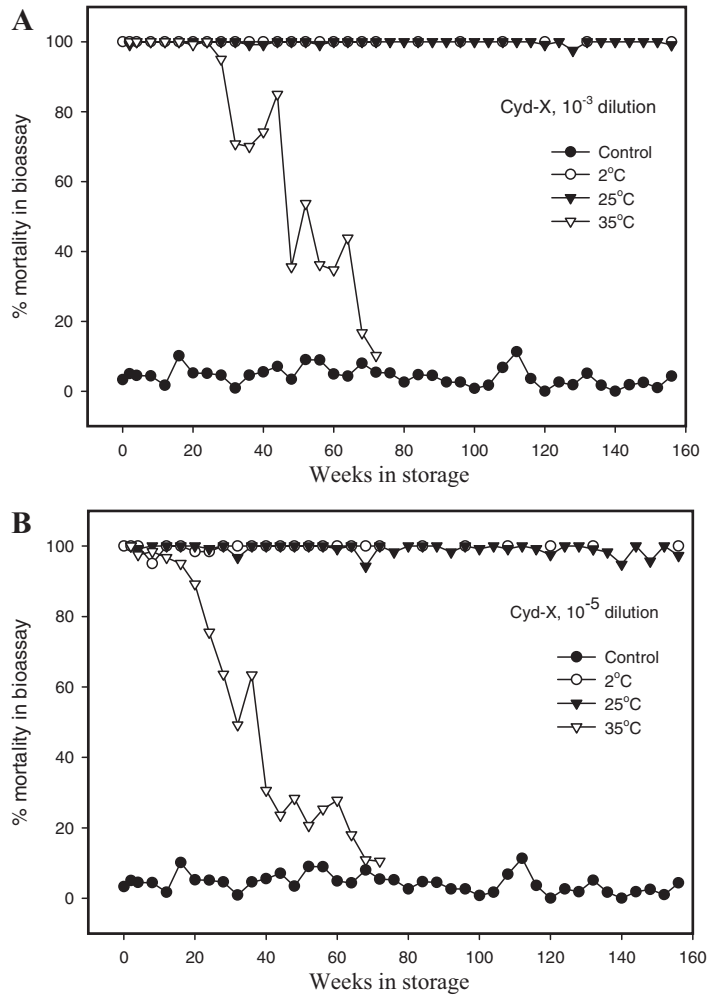

Fig. 1. Larvicidal activity of the Cyd-X formulation of the codling moth granulovirus stored at three temperatures for up to 3 yr. Data show percentage of mortality in bioassays with *C. pomonella* neonate larvae (average of four replicates) by using the 1,000-fold dilution (10^{-3}) (A) and the 100,000-fold dilution (10^{-5}) (B).

still observed after inactivation in storage of a large proportion of the OBs in the 10^{-3} dilution. Conducting bioassays with both high and low dilutions provided a realistic estimate of the shelf life of both formulations. Year-long quantitative studies using four temperatures and a range of concentrations were conducted with only one of the formulations (Cyd-X) due to limitation of resources.

Brassel (1978) stated that CpGV could best be stored when glycerin was added and the preparation kept at −20°C. Geissler (1994) stored the Granupom formulation of CpGV at −18°C for 3 yr and concluded that its activity for the pea moth, *Cydia nigricana* (F.), was similar to a recently produced batch of the virus. Our research indicates that storage of the Cyd-X and Virosoft formulations of CpGV at room temperature or colder will ensure retention of larvicidal activity for >2 yr. However, it is still recommended to purchase fresh product for each season. If excess formulation is available at the end of the growing season, it should be kept as cool as possible until the following season. Keeping product frozen until it is used would be optimal. However, the effects of repeated freezing and thawing are not yet known for CpGV formulations and hence should be avoided.

The exposure of CpGV formulations to 35°C significantly reduced survival of the virus in a relatively short period. However, Fritsch and Huber (1985) demonstrated that exposure of CpGV to temperatures of up to 75°C for 160 min caused no significant loss of activity. The precise mechanism(s) responsible for decline of CpGV activity was beyond the scope of this study. Denaturing of DNA and or the protein coat of the OB at 35° for relatively short-term storage, could be responsible for a decrease in larvicidal activity. Reed et al. (2003) showed that storage of TM Biocontrol-1, a commercially produced nucleopolyhedrovirus (NPV) of *Orgyia pseudotsugata* (McDunnough), for 5–15 yr at −10°C had no adverse effect on the quality of the viral DNA.

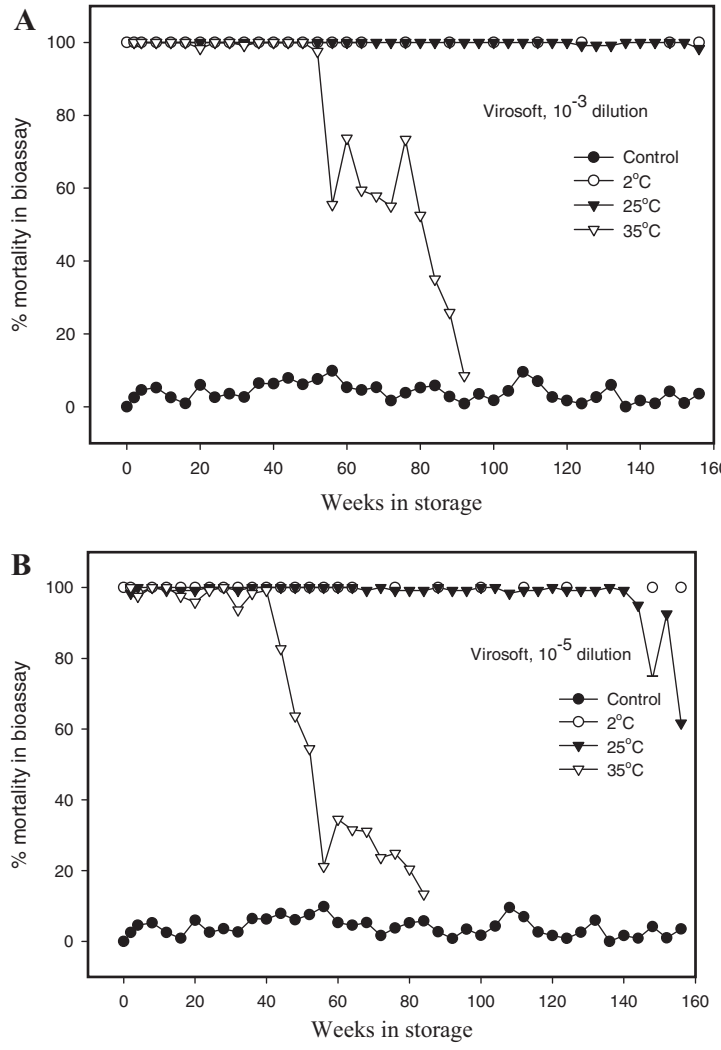

Fig. 2. Larvicidal activity of the Virosoft formulation of the codling moth granulovirus stored at three temperatures for up to 3 yr. Data show percentage of mortality in bioassays with *C. pomonella* neonate larvae (average of four replicates) using the 1,000-fold dilution (10^{-3}) (A) and the 1000,000-fold dilution (10^{-5}).

The effects of higher temperatures also have been studied on other granuloviruses (GVs) and NPVs. David and Gardiner (1967) reported on the exposure *Pieris brassicae* L. GV (PbGV) to 40–70°C. Purified virus was inactivated in as little as 10 min at 70°C. Although PbGV was not entirely inactivated after 20 d at 40°C, its activity was significantly decreased after 10 d at this temperature. Experiments by Behle et al. (2003)) demonstrated that the *Anagrapha falcifera* (Kirby) NPV in lignin-based spray-dried formulations had a shelf-life of up to 3 mo at 30°C and up to 30 mo at 4°C.

Variable storage duration has been reported over the past several decades for entomopathogenic baculoviruses depending on the type of preparation and storage temperatures. Steinhaus (1960) observed that *Bombyx mori* (L.) NPV kept in flame-sealed glass tubes, mostly at refrigerator temperatures, was infectious for *B. mori* larvae after 20 yr of storage. Commercially produced and formulated virus kept in cold storage has the most variability in shelf life. Kaupp and Ebling (1993) noted that storage of Virtuss (an NPV of *O. pseudotsugata*) at 4°C did not prevent loss of activity. They recorded a 46% loss in infectivity after 2 yr of storage and greater losses for product stored for 4–10 yr. Similar losses of virus activity were observed by other studies for this and other NPVs stored at −10 and 4°C (Cunningham 1970, Otvos et al. 2006). Freezing and freeze-drying seemed to be a better method of storing the virus (Cunningham 1970, Geissler et al. 1991, Otvos et al. 2006). Studies of spray-dried and freeze-dried CpGV formulations are warranted for further improvements in shelf life.

The information reported in this study should be useful to growers and commercial suppliers for avoiding decreases in CpGV potency due to improper storage conditions.

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