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New method for testing solar sensitivity of commercial formulations of the granulovirus of codling moth (*Cydia pomonella*, Tortricidae: Lepidoptera)

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Abstract

A method for screening codling moth granulovirus (CpGV) formulation sensitivity to sunlight using specially prepared half apples and a solar simulator is described. The half apple preparation allows an even coverage of virus over the surface of the fruit that would not be possible using whole apples. Leaves and artificial medium were not usable for extended periods of exposure in the solar simulator due to excess drying. Fruit was sprayed with 10^{-3} and 10^{-5} dilutions of three commercial formulations of CpGV (Carpovirusine, Cyd-X, and Virosoft) and infested with codling moth neonates. Half of the sprayed fruit was exposed to 650 W/m^2 for 4 h in an Atlas Suntest CPS solar simulator resulting in an accumulated radiant energy of 9.36×10^6 J/m^2 before they were infested with neonate codling moth larvae. Spraying non-irradiated fruit with the 10^{-3} dilution of Cyd-X and Virosoft resulted in nearly 100% mortality of neonate larvae. Irradiation reduced viral activity by 71–98% at the 10^{-3} dilution and by up to 32% at the 10^{-5} dilution relative to non-irradiated fruit. The procedures utilized enabled good preservation of the fruit throughout the incubation period and minimized invasion of the fruit by plant pathogens and saprophytic organisms. This laboratory method for screening candidate formulations and potential UV protectants could conserve time and resources by eliminating adjuvants with less potential in laboratory tests and field testing only the most promising candidates. It also enables year-round testing.

Keywords: Codling moth; *Cydia pomonella*; UV sensitivity testing; Granulovirus; Bioassay technique

1. Introduction

The *Cydia pomonella* granulovirus (CpGV) was discovered in Mexico over 40 years ago (Tanada, 1964), but despite earlier development in the United States and Canada (Falcon et al., 1968; Jaques, 1990), it has only been registered for use in the USA since 2001 (Lacey et al., 2004b). Significant use of commercial CpGV products did not begin in the US until 2003. One of the major disadvantages of the virus is its sensitivity to ultraviolet radiation (Arthurs and Lacey, 2004; Fritsch and Huber, 1985; Jaques et al., 1987; Keller, 1973; Kienzle et al., 2003; Lacey et al., 2004a), requiring reapplication of the virus at 7–10 days interval when codling moth neonates are present. A variety of studies have been conducted on UV protectants and other adjuvants with the goal of extending or improving the activity of CpGV (Ballard et al., 2000; Charmillot et al., 1998; Keller, 1973; Krieg et al., 1981; Lacey et al., 2004a). Development of a laboratory method to screen candidate formulations and adjuvants could conserve time and resources by eliminating those with less potential in laboratory tests and field testing only the most promising candidates. It would also enable year-round testing. This paper reports a new method for assessing CpGV solar sensitivity using specially prepared apples and a solar simulator that employs wavelengths comparable to natural sunlight.

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2. Materials and methods

2.1. Materials

Black head stage codling moth eggs (ready to hatch) were obtained from the colony maintained at the Yakima Agricultural Research Laboratory (YARL) and reared using the system of Toba and Howell (1991). Fuji apples that had not been sprayed with chemical pesticides were collected from the YARL experimental farm in Moxee, WA, and kept in controlled atmosphere cold storage (1–2 °C) until preparation for testing.

Three formulations of CpGV: Carpovirusine, Cyd-X, and Virosoft were obtained from Sumitomo USA (formulation made by Calliope, Nogueres, France), Certis USA (Columbia, MD, USA), and BioTEPP (Mont-St-Hilaire, Quebec, Canada), respectively. The products were stored at 2 °C until used. The Carpovirusine formulation is currently marketed in the USA by Arysta Life Sciences (San Francisco, CA). Label information for Carpovirusine, Cyd-X, and Virosoft indicates that they contain approximately 10^{13}, 3×10^{13}, and 4×10^{13} virus granules/L, respectively. Producer specified application rates for the three products are 1 L/ha (10^{13} granules/ha), 73–438 mL/ha (2.2×10^{12}–1.3×10^{13} granules/ha), and 234 mL/ha (9.4×10^{12} granules/ha), respectively.

An Atlas Suntest CPS + solar simulator (Atlas Material Testing Technology LLC, Chicago, IL) was used for irradiating virus-treated and control apple halves. The simulator uses a xenon light source and filters to provide irradiance that approximates sunlight. To provide a larger work surface and enable location of apples further from the heat of the light source, the simulator was set on top of a custom-made cabinet ($85.6 \times 60.3 \times 40$ cm) made of 1.9 cm thick plywood and covered with Formica (Fig. 1A). All surfaces on the inside of the cabinet were lined with reflective Mylar (DuPont Teijin Films, Hopewell, VA). The simulator was set over a 33×30 cm opening in the top of the cabinet. A piece of Tefcel plastic (American Durafilm, Holliston, MA) was placed over the opening in the cabinet to reduce heat generated by the light source. McGuire et al. (2000) determined that Tefcel does not reduce energy in the UV wavelengths. The shelf on which the apples were set was 66 cm from the xenon lamp.

2.2. Preparation of apples

The apples were prepared for experiments by first immersing them in a 50 L Sterilite (Sterilite, Townsend, MA) plastic bin partially filled with 21 L of deionized water to which 75.6 mL of germicidal bleach (5.5%

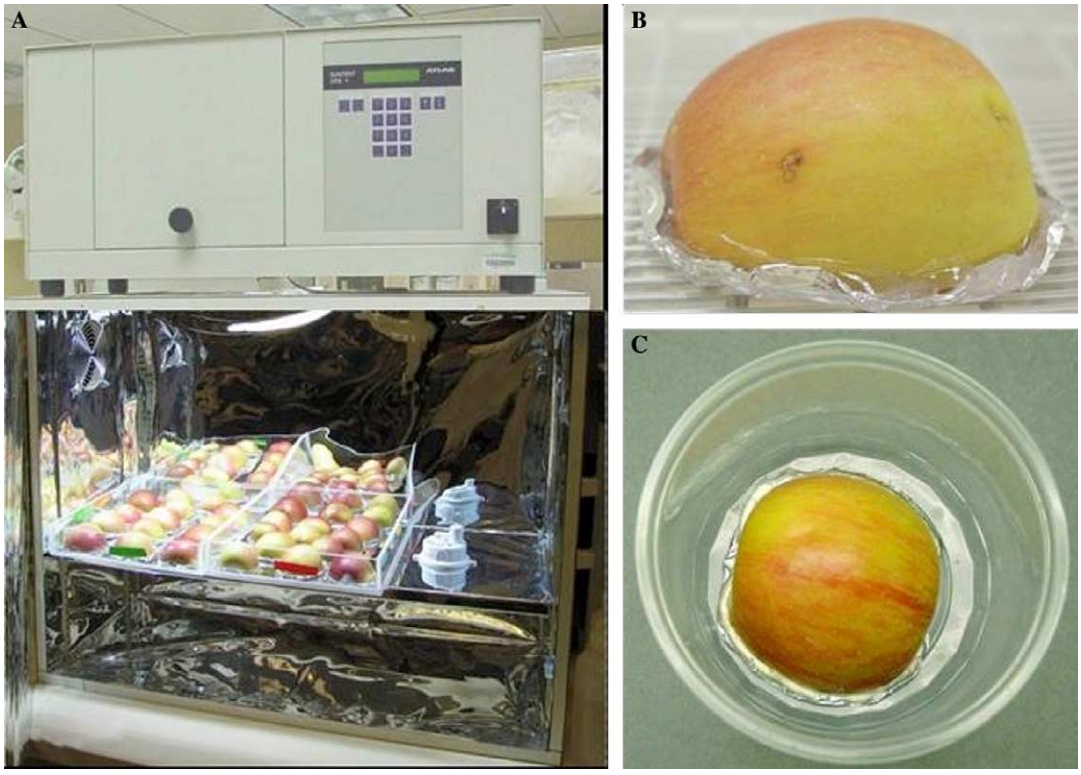

Fig. 1. Solar simulator and half apple system used for determination of solar sensitivity of commercial formulations of codling moth granulovirus. (A) Solar simulator and open reflective cabinet. (B) Detail of half apple; apple has a shallow entry. (C) Half apple in the container used for exposure to neonate larvae; lid not shown.

NaClO) had been added. After 15 min in the bath, they were rinsed with deionized water and air dried under a fan. Each of the apples was cut in half from stem to calyx and the stems were removed. The cut surface of the apple was immersed in deionized water (68 °C) for 1 min, blotted dry and sealed using molten paraffin (68 °C), which was then cooled in a water bath and covered with aluminum foil. The foil was sealed in place with hot glue (Stanley Fastening Systems L.P., E. Greenwich, RI) (Figs. 1B and C—close up of apple half). The prepared half apples were stored up to 72 h at 12 °C until used for experiments.

2.3. Treatment of apples

The three commercial CpGV products were tested at two dilutions (10^{-3} and 10^{-5} dilutions of each product) applied in 35 mL of deionized water to which 0.025% Silwet L77 (Silicone–polyether copolymer, Loveland Industries, Greeley, CO) had been added as a wetting agent. The apple halves were sprayed with CpGV suspensions in a DeVries spray cabinet (DeVries Mfg., Hollandale, MN) using a track-mounted flat fan nozzle (XR TeeJet 8001 VS, Spraying Systems, Wheaton, IL) that provided fine droplets and enabled even coverage. The speed of the track sprayer was adjusted to provide a volume application rate equivalent to 935 L/ha (100 gal/A). Controls apples were sprayed with water and the wetting agent.

Due to constraints on the number of apples that could fit in the solar cabinet, virus formulations were assessed on separate dates. Twenty apple halves were sprayed for both of the virus dilutions and untreated controls, for a total of 60 apple halves per replicate test. After drying, the apples from each of the virus/control treatments were randomly divided into two equal groups. One half (i.e., 10 apple halves per virus treatment) were individually placed in 0.47 L plastic food containers and immediately infested with five neonate codling moth larvae <4 h old. A 6.4 cm diameter hole cut in the lid and covered with polyester mesh provided ventilation. The remaining apple halves were placed on 26.7×33.7 cm Plexiglas trays in the solar simulator cabinet described above and exposed to UV (300–400 nm) and other wavelengths (visible, 400–800 nm and infrared, $\geqslant 800$ nm) at an irradiance setting of 765 W/m^2 for 4 h. The irradiance is measured by a sensor located near the floor of the solar simulator. Since the apple halves were 37 cm below the floor of the simulator, the actual irradiance would be significantly less than that recorded by the sensor in the simulator. The irradiance at the level of the apple halves was measured with a Li-Cor LI-1400 data logger fitted with a pyranometer (Li-Cor; Lincoln, NE) providing a reading of 650 W/m^2. This results in an accumulated radiant energy of 9.36×10^6 J/m^2 over the 4 h of exposure. The door of the cabinet was kept closed during the 4 h exposure period both for maximizing reflected light within the chamber and for shielding laboratory personnel from harmful rays. Temperature was monitored with a Hobo H8 Pro Series data logger (Onset Computer, Pocasset, MA). After irradiation, the apple halves were allowed to cool, placed in plastic containers and infested with larvae as described above.

All samples were incubated at 25 °C, 16:8 L:D in a walk-in incubator for 10 days after which the survival of larvae and fruit damage were determined. The study was repeated four times for each virus formulation.

2.4. Data analysis

Treatment effects were compared using univariate ANOVA (SPSS 12.0.1 for Windows). Significant F-ratio means were further separated with Fisher's LSD for multiple comparisons at $P < 0.05$. Data for fruit damage and larval survival were normalized via $\log_{10}(n+1)$ prior to analysis. For the assessments, each replicate was based on the mean for each test date (i.e., 10 apple halves per treatment) for a sample size of four.

3. Results

Exposure to UV resulted in reduced larval mortality with the Cyd-X and Virosoft formulations applied at two rates and the Carpovirusine formulation at the high rate compared with controls (Fig. 2A). At the 10^{-3} dilution, non-irradiated Cyd-X and Virosoft produced 97 and 99% mortality, respectively, in codling moth neonate larvae relative to controls. The mortality of neonates treated with a 10^{-3} dilution of Carpovirusine was $83 \pm 9\%$ relative to controls. This dilution is equivalent to the field application rate for Carpovirusine, but represents higher than label rates for Virosoft (4.3 times label rate) and Cyd-X (4.6 times median label rate). At the same dilution, larvicidal activity of irradiated Cyd-X, Virosoft, and Carpovirusine was decreased by 98, 95, and 71%, respectively, relative to non-irradiated fruit. Application of the 10^{-5} dilution of the three commercial CpGV products to the half apples resulted in substantially lower mortality (Fig. 2A). Reduction in viral activity at the 10^{-5} dilution after irradiation was significant for Virosoft and Cyd-X (19 and 32% reduction relative to non-irradiated fruit), but not for Carpovirusine. Despite these differences in larval mortality, the numbers of fruit injuries were statistically similar among the different treatments (Fig. 2B). It was noted that infected larvae placed directly on the fruit survived long enough to cause shallow stings <5 mm deep.

ANOVA tests revealed both UV exposure and dosage, but not virus formulation, were significant factors in the number of live larvae recovered from virus-treated fruit (Table 1). Exposure to UV was also involved in

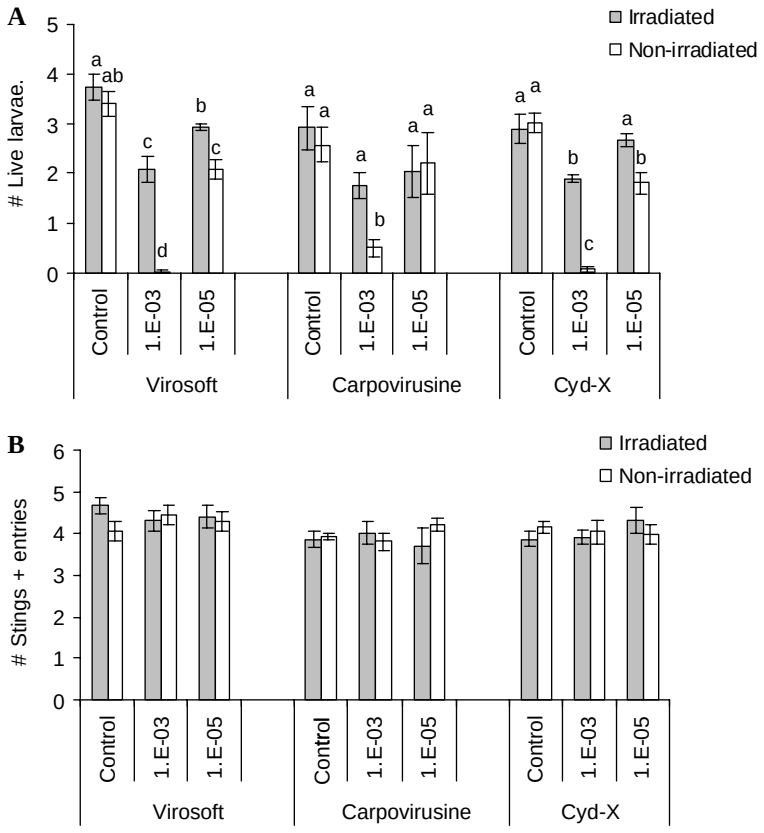

Fig. 2. Number of live larvae recovered (A) and fruit damage (B) on apple halves treated with 3 virus formulations at 2 rates (10^{-3} and 10^{-5} dilutions) and exposed to simulated solar radiation (9.36×10^6J/m^2) together with controls. Data show average ± SEM for four tests. Letters indicate Fishers LSD at $P < 0.05$ (for each virus formulation assessed separately). The 10^{-3} dilution of Virosoft, Carpovirusine and Cyd-X is equivalent to 4×10^9, 10^9, and 3×10^9 granules/m^2, respectively.

Table 1
Univariate ANOVA describing effect of virus (three commercial formulations), dosage (dilution rates of 10^{-3}, 10^{-5}) and UV (exposure to 9.36×10^6J/m^2 and controls) on fruit damage (number of stings + entries), and number of live larvae recovered

Source	Type III SS	*df*	*F*	Significance
Dependent variable: number of live larvae recovered				
Formulation	0.18	2	2.54	0.09
UV	3.12	1	88.09	0.00
Dosage	3.78	1	106.76	0.00
Formulation × UV	0.44	2	6.25	0.01
Formulation × dosage	0.13	2	1.81	0.18
UV × dosage	1.73	1	48.83	0.00
Formulation × UV × dosage	0.01	2	0.10	0.90
Error	1.27	36		
Dependent variable: fruit damage (stings + entries)				
Formulation	0.061	2	2.67	0.08
UV	0.000	1	0.03	0.86
Dosage	0.001	1	0.13	0.72
Formulation × UV	0.007	2	0.31	0.73
Formulation × dosage	0.004	2	0.17	0.84
UV × dosage	0.000	1	0.01	0.91
Formulation × UV × dosage	0.033	2	1.43	0.25
Error	0.410	36		

Apple halves were infested with five neonate larvae and assessed 10 days post-treatment.

two-way interactions with both dosage and formulation. The two-way interactions are explained by the proportionally greater loss of virus viability on irradiated fruit at the 10^{-3} dilution compared with the 10^{-5} dilution, especially for the Virosoft and Cyd-X formulations. By contrast, neither UV-exposure, dosage nor virus formulation significantly influenced the level of fruit damage (Table 1, Fig. 2B). There were no significant interaction terms between these factors. Temperature in the closed cabinet ranged from 22 to 24 °C at the beginning of the exposure periods to 35–40 °C at the end of the 4 h exposure.

4. Discussion

The half apple preparation allows an even coverage of virus and irradiation over the surface of the fruit that would not be possible using whole apples. The procedures utilized in the preparation of the apples enabled good preservation of the fruit throughout the incubation period and successfully prevented invasion of the fruit by plant pathogens and saprophytic organisms. Earlier attempts in our laboratory to use leaf disks, whole leaves, or artificial medium as exposure surfaces in the

solar simulator resulted in excess drying of leaves and medium. Potted apple seedlings are less affected by drying in the simulator, but present multiple distances from the light source and uneven coverage of irradiation. Also, the age and texture of leaves on seedlings that would fit into the cabinet are considerably different from what would be encountered in the orchard at the time of initial spraying in the late spring. The apple halves also provided the surface waxes and fruit volatiles that would be encountered by neonates in the orchard. While the 10^{-5} dilution of the three commercial CpGV products produces close to 100% neonate mortality on artificial codling moth rearing medium (Lacey et al., 2002; Lacey unpublished data), larvicidal activity was substantially reduced at this concentration on the apple halves, ostensibly as a result of interactions by neonates and virus with the surface of the fruit. The non-irradiated 10^{-3} dilution of the three formulations produces substantial mortality in codling moth neonates. The 4 h exposure to simulated sunlight resulted in a substantial, but not total reduction of virus activity at this dilution which will enable side by side comparisons of CpGV with and without adjuvants for the determination of protective effects. Due to the differences in producer-specified granule counts, the data reported here are not intended for comparisons among products, but rather to provide baseline background on the solar sensitivity of each product.

The increase in temperature in the cabinet, as would occur during exposure of treated fruit in the orchard, could possibly play a role in virus degradation. In laboratory tests with CpGV-treated apples, Keller (1973) reported that neonate codling moth mortality was significantly reduced at 34 °C, compared with studies conducted at 15–30 °C, but this may have been due to the effect of temperature on larval feeding rather than on the virus. In our studies, the infestation of apples with neonates took place at room temperature after the apples were removed from the exposure cabinet. Fritsch and Huber (1985) studied the effects of artificial UV radiation and high temperature on the inactivation of CpGV. They reported that exposure to temperatures of up to 75 °C for 160 min caused no significant loss of virus activity. However, at high temperatures, the rate of inactivation by UV was substantially increased, but this effect was significant only above 50 °C. For future tests our exposure cabinet has been modified to include an air intake port (11.4 cm diameter) on one side of the cabinet and an 18 W exhaust fan (Dayton Electric Mfg., Niles, IL) fitted into an 11.4 cm port on the opposite side. A substantial decrease in temperature was obtained by the air circulation with a maximum temperature of 28 °C recorded at the end of a 4 h exposure period.

The Atlas solar simulator has been used to test formulation effects with other baculoviruses and *Bacillus thuringiensis* (McGuire et al., 1997, 2000) with consistent results. McGuire et al. (2000) highlighted the variability that is possible when comparing exposure to natural sunlight at different times of the day or year, or between locations, and suggested that the appropriate measure for effect of light is accumulated radiant energy (J/m^2). After measurement of irradiance in the Suntest CPS solar simulator with a spectroradiometer, they concluded that the solar simulator provided light at a flux level higher than sunlight (i.e., greater intensity of irradiance). This enables faster exposure in the simulator to equivalent amounts of accumulated radiant energy in the orchard. Ignoffo et al. (1997) estimated that 1 h of the UV-producing artificial light used in their experiments was equivalent to 4 h of natural sunlight.

The most damaging wavelengths of light for entomopathogens and other biological systems are in the ultraviolet range (Diffey, 1991; Ignoffo, 1992). Although UV wavelengths account for only 3.5–4.0% and ≈3% of the total energy in sunlight and the solar simulator, respectively (McGuire et al., 2000), the photons have much more energy than longer wavelengths (Ryer, 1997). A variety of additives have been utilized for screening entomopathogens including CpGV from UV radiation, but the results in terms of extending virus activity have been variable (Burges and Jones, 1998). Lignin microencapsulation provided extended protection for other baculoviruses (Behle et al., 2003; Farrar and Ridgeway, 2000) and appears promising as a candidate for CpGV. Lignin formulations and certain UV-protectant adjuvants will be screened in future studies in our laboratory using the half apple preparation and the Suntest CPS solar simulator.

Although accumulated radiant energy can be compared between the solar simulator and natural sunlight, daily changes in light intensity and shading make extrapolations to field conditions difficult. Nevertheless, the method described in this paper will allow repeatable and consistent solar simulation for the purpose of testing UV protectants for CpGV on apple. Ultimately, field testing will provide the definitive information on the effectiveness of formulations and adjuvants for protection of virus from solar degradation in the orchard.

Research on phagostimulants to enhance virus uptake before larvae reach the fruit is also warranted to reduce the number of shallow entries that are observed even when high mortality is attained with CpGV treatment (Arthurs and Lacey, 2004).

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