Nucleopolyhedrovirus infection in obliquebanded leafroller (Lepidoptera: Tortricidae)

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Abstract—A virus isolated from obliquebanded leafroller, *Choristoneura rosaceana* (Harris), larvae collected in an apple, *Malus domestica* Borkh. (Rosaceae), orchard of Saint-Joseph-du-Lac (Quebec, Canada) was studied. Microscopic studies revealed that it was a uninucleocapsid nucleopolyhedrovirus from the family Baculoviridae. Larval mortality was approximately 75% (0% mortality in control group) in larvae infected as third instars immersed in a suspension of 1.7×10^8 occlusion bodies/mL. The average time for larval mortality was 23 ± 3 d after treatment. The majority (95.5%) of infected larvae died as fifth or sixth instars. Infection was observed primarily in fat body cells, and occasionally in the tracheal matrix and epidermis. Mean larval development time of infected larvae surviving to pupae was 20 ± 3 d, significantly greater than the 18 ± 3 d observed in control larvae. Adult emergence was significantly lower in pupae of treated larvae (73.6%) than in the control group (93.5%). Our work constitutes the first baseline study of naturally occurring virus of the obliquebanded leafroller.

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Résumé—Un virus a été isolé de chenilles de la tordeuse à bandes obliques, *Choristoneura rosaceana* (Harris), collectées dans un verger de pommiers, *Malus domestica* Borkh. (Rosaceae), à Saint-Joseph-du-Lac (Québec, Canada). Des observations microscopiques ont révélé qu'il s'agissait d'un virus de la polyhédrose nucléaire, possédant une nucléocapside par enveloppe, de la famille des Baculoviridae. La mortalité larvaire était d'environ 75 % (aucune mortalité dans le groupe témoin) lorsque les chenilles étaient infectées au troisième stade par immersion dans une suspension de $1,7 \times 10^8$ corps d'inclusions polyédriques/mL. La durée de vie moyenne avant la mort de ces individus était de 23 ± 3 j. La majorité (95,5 %) des chenilles malades étaient mortes au cours du cinquième ou sixième stade larvaire. L'infection virale était localisée principalement dans le tissu adipeux et occasionnellement dans la matrice trachéale et le tégument. Pour les individus ayant survécu à l'infection, la durée moyenne du développement larvaire des individus infectés ayant atteint le stade de chrysalide de 20 ± 3 j était significativement allongée par rapport aux 18 ± 3 j des individus témoins. L'émergence des adultes était réduite à 73,6 % pour les individus traités par le virus en comparaison des 93,5 % observés dans le groupe témoin. Notre travail constitue la première étude de base concernant un virus qui survient de façon naturelle chez la tordeuse à bandes obliques.

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Introduction

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is native to and widely distributed in temperate North America. Larvae are polyphagous and are frequently reported in apple, *Malus domestica* Borkh. (Rosaceae), orchards where they damage leaves, flowers, and fruit (Chapman and Lienk 1971). Some populations from North America have developed insecticide resistance (Reissig *et al.* 1986; Carrière *et al.* 1994; Smirle *et al.* 1998; Pree *et al.* 2001) and alternatives to synthetic insecticides are needed.

Little is known about the viruses of *C. rosaceana*. Smirnoff and Burke (1970) reported a uninucleocapsid nucleopolyhedrovirus causing 60% larval and 10–20% pupal mortality in leafrollers randomly collected in 15 forest habitats in Quebec. This virus was found infecting the fat body, hemolymph, epidermis, and tracheal matrix of the larvae. Lucarotti and Morin (1997) isolated a nucleopolyhedrovirus from obliquebanded leafroller larvae collected in New Brunswick (Canada). This viral isolate induced 58% larval mortality at a dose of 1000 occlusion bodies per larva ingested by third and fourth instars. Lucarotti and Morin (1997) also determined that the polyhedron gene of this viral isolate showed high homology to those of a virus isolated from *Choristoneura fumiferana* (Clemens). Although granulovirus, cytoplasmic polyhedrosis, and entomopox viruses have been reported for other species of *Choristoneura* Lederer (Martigoni and Iwai 1986; Zimmermann and Weiser 1991), only nucleopolyhedrovirus have been recorded from *C. rosaceana*.

Diseased obliquebanded leafroller larvae showing symptoms of viral infection were isolated from overwintered individuals collected in a commercial apple orchard at Saint-Joseph-du-Lac (Quebec, Canada) in 1997 (Pronier 2000). In this paper, we report on the identification and development of a virus found in *C. rosaceana* larvae using light and electron (scanning and transmission) microscopy to examine larvae infected in laboratory, and basic information on the pathology induced by this virus.

Materials and methods

Origin of larvae

Obliquebanded leafroller larvae (with no apparent viral symptoms) were collected in spring 1996 in an apple orchard at Saint-Joseph-du-Lac (45°29′N, 74°03′W), Quebec, Canada. Individuals used for the bioassays were reared in the laboratory for 10 generations on a pinto-bean-based artificial diet (Shorey and Hale 1965) at 24°C, 16L:8D photoperiod, and 60–65% RH.

Virus preparation

Larvae with apparent viral disease were collected in this same apple orchard in spring 1997. A viral suspension was obtained by triturating four (randomly selected) of these diseased larvae in 10 mL of microfiltered water and filtering through several layers of muslin. This inoculum was quantified by counting occlusion bodies using a hemocytometer and phase-contrast microscopy. This suspension was adjusted to a concentration of 1.7×10^8 occlusion bodies/mL with sterile distilled water.

Bioassay

Third instar larvae were starved for 24 h and immersed by groups of 10–15 individuals in 10 mL of viral suspension (1.7×10^8 occlusion bodies/mL) ($n_{\text{treated}} = 1210$)

or 10 mL of microfiltered water ($n_{control}$ = 180) for 10 s and transferred to individual plastic cups containing artificial diet. In both treated and control groups, larvae that died within 72 h after treatment were considered to have been injured in the course of manipulation and were discarded from the experiment.

Histological studies

To study the development of the viral disease 20 larvae from the treated and 10 from the control groups were randomly sampled every second day after treatment as long as larvae were available (*i.e.*, 38 d in treated group and 24 d in control group). A total of 350 and 101 larvae for treated and control groups, respectively, were prepared for light or electron microscopy. These larvae were fixed in FAA solution (ethanol: 90 mL, acetic acid: 5 mL, formaldehyde: 5 mL) for 48 h and stored in 70% ethanol.

For light microscopy, the larvae were dehydrated in a graded ethanol series and embedded in paraffin. Serial sections of 15 µm were cut. The Azan staining technique (Gabe 1968) was used to reveal occlusion bodies.

For scanning electron microscopy, serial sections of 5 µm thickness were deparaffinated with acetone, air dried, and coated with gold-palladium. For transmission electron microscopy, larvae were fixed in a solution with 2.5% glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH = 7.4) for at least 48 h and postfixed with 4% osmium tetroxide in a 0.2 M sodium cacodylate buffer for 3 h. After dehydration in a graded acetone series, infiltration was achieved by a 12-h immersion in a 1:1 mixture of 100% acetone and epon-araldite followed by a 1-h immersion in epon-araldite at 37°C. Samples were embedded in epon-araldite for 3 d at 60°C. Sections were cut with an ultramicrotome (Ultracut Reichert-Jung) and mounted on slides or on copper grids. The sections (1–2 µm) mounted on slides were stained for 1 min with 1% azur blue. Sections (80 nm) mounted on copper grids were stained first with 4% aqueous uranyl acetate for 10 min and with lead citrate for 5 min (Glauert 1974).

Mortality and sublethal effects

To determine larval mortality and sublethal effects (*i.e.*, duration of larval development after treatment and percentage of adult emergence), larvae ($n_{treated}$ = 768, $n_{control}$ = 62) were monitored daily for mortality. Dead larvae were triturated in microfiltered water and observed using a phase-contrast microscope to detect occlusion bodies.

Data analysis

The average time for larval development for treated and control was compared using Student's *t* tests (P = 0.05). A chi-squared analysis was used to detect significant (P = 0.05) differences among proportions of adult emergence. All analyses were performed using SYSTAT 8.0 (SPSS Inc 1998).

Results

Histological studies

Only one type of virus was observed during the microscopic examinations in the infected larvae. It infected the nucleus of the cells that became hypertrophied. Occlusion bodies were cuboidal, ranging in size from 0.5 to 2 µm (Fig. 1*a*). The virus was also characterized by having several virions, each composed of a uninucleocapsid per envelope, within an occlusion body (Fig. 1*b*). Occlusion bodies were found in the nuclei

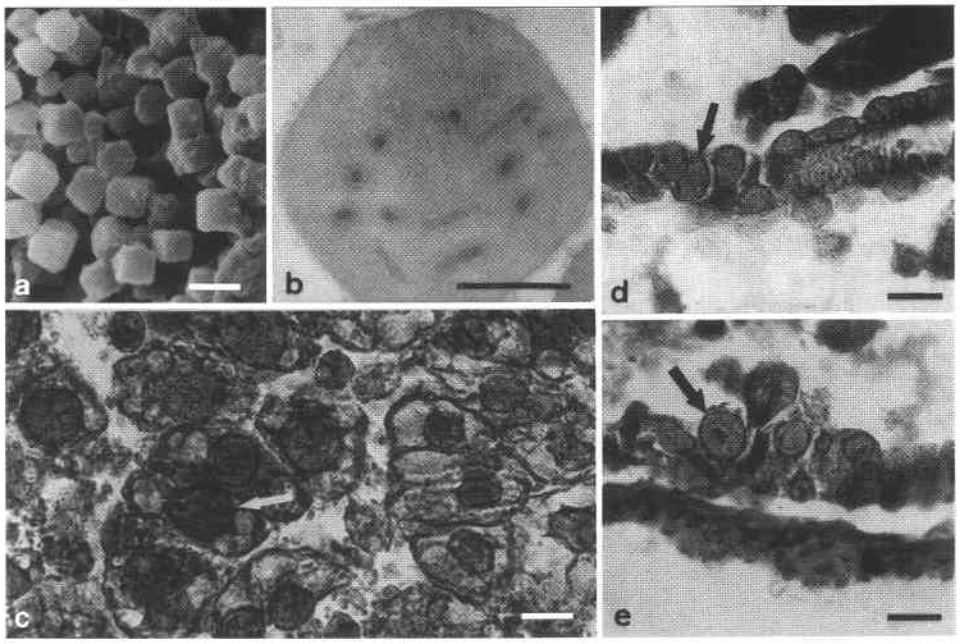

FIGURE 1. Nucleopolyhedrovirus from *Choristoneura rosaceana* larvae. (*a*) Several polyhedra that multiplied in fat body cells (scanning electron micrograph; scale bar = 2 μm). (*b*) Several virions, each composed of a uninucleocapsid per envelope, included in a polyhedral occlusion body (transmission electron micrograph; scale bar = 0.25 μm). Infected cells (arrow) in fat body (*c*), tracheal cells (*d*), and integument (*e*) (light micrograph; scale bar = 25 μm).

of fat body (Fig. 1*c*), tracheal cells (Fig. 1*d*), and epidermis (Fig. 1*e*). They were also found in the cytoplasm of hemocytes, presumably resulting from phagocytosis of occlusion bodies from the hemocoel. They were found free in the hemolymph by lysis of infected cells. The virus was first observed in the fat body 8–10 d after ingestion. In late stages of the disease, all fat body cells were infected and were dissociated. Infections of tracheal cells and integument were restricted to a few cells (Figs. 1*d*, 1*e*) even in late stages of the disease. Infection of these tissues was not observed in all larvae. Epidermis infection was apparent only 16 d after treatment in 12 (n = 19) larvae observed, whereas tracheal cells infection was observed 20 d after treatment in 3 (n = 16) larvae from 20 d after treatment onward.

Mortality and sublethal effects

There was no larval mortality in the control group (n = 62), whereas in the treated group 74.9% (n = 768) larvae died within 38 d after treatment. The majority (*i.e.*, 95.5%) of dead larvae reached the fifth or sixth instars. Symptoms of viral infection, larval sluggishness, abdominal segments slightly inflated and development of white zones visible through the cuticle, were apparent 5–10 d before larval death. Mortality of larvae showing symptoms of viral infection was observed 14–38 d after treatment. The average time for mortality was 23 ± 3 d. No mortality was observed in control larvae beyond 72 h after the beginning of the bioassay. In the treated group, few (4.5%) individuals died as third or fourth instars 6–14 d after treatment. No symptoms of viral

infection were observed for these larvae and no occlusion body was observed by phase-contrast microscopy.

All larvae in the control group pupated within 24 d, whereas pupae were collected up to 32 d after treatment in the treated group. Time for larval development (from the third instar to pupation) was significantly longer in the treated (20 ± 3 d, $n = 193$) than in the control (18 ± 3 d, $n = 62$) group ($t_{253} = 2.016$, $P < 0.05$). The proportion of adult emergence in the treated group, 73.6% ($n = 193$), was significantly lower than in the control group, 93.5% ($n = 62$) ($\chi_1^2 = 11.12$, $P < 0.05$). In the treated group, pupae from which no adult emerged were deformed or their abdominal segments were shortened.

In the treated group, seven larvae were collected on the 38th day after the beginning of the experiment as third or fourth instar and showed no external infection symptoms. No occlusion body was found within these larvae. Fat body tissues were poorly developed in these individuals as in untreated larvae at the same instar.

Discussion

The viral particles associated with the mortality of *C. rosaceana* exhibited the typical morphology of uninucleocapsid nucleopolyhedrovirus from the family Baculoviridae (Tanada and Kaya 1993) and its development in obliquebanded leafroller larvae was similar to nucleopolyhedrovirus found in other Lepidoptera. During a baculovirus infection cycle, infection of midgut epithelium cells is the typical route (Keddie *et al.* 1989). This step of viral infection was not observed in our histological studies, which can be explained by the rapid slough of diseased cells and the replacement of diseased cells by healthy ones (Volkman 1997) that could occur during the first 72 h after our treatment or the passage of viral particles through midgut cells without replication directly to the hemocoel (Blissard 1996). Viral multiplication mainly located in fat body tissues was apparently correlated with larval development. The arrest of larval development disrupting viral multiplication has been reported (Amargier *et al.* 1981). No hypertrophied fat body cells were observed in our larvae still in third instars 38 d after treatment. Nucleopolyhedrovirus inducing diapause has been also reported by Amargier *et al.* (1981) who observed that after diapause larvae developed but died of viral infection, allowing the viral inoculum to remain for the next season in the habitat.

Immersion of third larval instar in a nucleopolyhedrovirus suspension of 1.7×10^8 occlusion bodies/mL caused approximately 75% larval mortality. Most obliquebanded leafrollers died as fifth or sixth instars and after a prolonged larval stage. Observations of food particles in digestive tracts of both control and infected individuals suggested that viral infection does not stop larval feeding; however, quantifying the effects of viral infection on feeding should be done because larvae cause extensive injury to fruit. Nevertheless, prolonged larval stage and limited mobility of diseased larvae (Tanada and Kaya 1993) may increase exposure to other mortality factors (*e.g.*, predators, parasites, or insecticides). Sublethal effects, such as host reproduction, should be researched because they may play an important role in host population dynamics (Sait *et al.* 1994; Duan and Otvos 2001).

In 1998 and 1999, obliquebanded leafroller larvae showing viral infection symptoms (10–20 larvae out of 500) were observed in the same apple orchard in Saint-Joseph-du-Lac where we collected larvae in 1997 (B Rancourt and I Pronier, unpublished data). This suggests that the virus was endemic at a sufficiently low level to be ineffective in reducing the population to economically acceptable levels. In this orchard, where *C. rosaceana* resistance to organophosphates has been reported (Carrière *et al.* 1994; Smirle *et al.* 1998), leafroller populations had reached outbreak levels. Further

studies should investigate how modified fitness of resistant insects to insecticides may influence the pathological effect of this viral disease.

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