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A Field Release of Genetically Engineered Gypsy Moth (*Lymantria dispar* L.) Nuclear Polyhedrosis Virus (LdNPV)

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The gypsy moth (*Lymantria dispar* L.) nuclear polyhedrosis virus was genetically engineered for nonpersistence by removal of the gene coding for polyhedrin production and stabilized using a coocclusion process. A β-galactosidase marker gene was inserted into the genetically engineered virus (LdGEV) so that infected larvae could be tested for its presence using a colorimetric assay. In 1993, LdGEV-infected gypsy moths were released in a forested plot in Massachusetts to test for spread and persistence. A similar forested plot 2 km away served as a control. For 3 years (1993–1995), gypsy moths were established in the two plots in Massachusetts to serve as test and control populations. Each week, larvae were collected from both plots. These field-collected larvae were reared individually, checked for mortality, and then tested for the presence of β-galactosidase. Other gypsy moth larvae were confined on LdGEV-contaminated foliage for 1 week and then treated as the field-collected larvae. The LdGEV was sought in bark, litter, and soil samples collected from each plot. To verify the presence of the LdGEV, polymerase chain reaction, slot blot DNA hybridization, and restriction enzyme analysis were also used on larval samples. Field-collected larvae infected with the engineered virus were recovered in the release plot in 1993, but not in subsequent years; no field-collected larvae from the control plot contained the engineered virus. Larvae confined on LdGEV-contaminated foliage were killed by the virus. No LdGEV was recovered from bark, litter, or soil samples from either of the plots. © 1999 Academic Press

Key Words: **β-galactosidase; field release; genetic engineering; genetically engineered baculovirus; LdNPV; *Lymantria dispar*.**

INTRODUCTION

Over the past decade, there has been increasing interest in the use of formulations of genetically engineered baculoviruses to control insect pests in a variety of agricultural and silvicultural settings (Bishop *et al.*, 1988; Wood and Granados, 1991; Wood *et al.*, 1993, 1994; Cory *et al.*, 1994; Mulock and Faulkner, 1997). As insecticides, viruses possess several advantages over broad-spectrum chemical agents; they have no harmful effects on nonarthropods, such as birds, mammals, and plants, and they are often specific to the pestiferous insect, allowing beneficial insects to survive. To date, no negative health or environmental effects have been documented as a result of viral pesticide applications.

The gypsy moth (*Lymantria dispar*) nuclear polyhedrosis virus (LdNPV) is a baculovirus that, when ingested, kills larval gypsy moths in 9–15 days. A formulation of LdNPV registered as the general-use pesticide GYPCHEK is currently one of the biological control methods being used to suppress gypsy moth populations that threaten environmentally sensitive habitats (Reardon *et al.*, 1996). Although field research has shown that LdNPV is a safe and effective alternative to chemical pesticides, at present the expense of producing the virus is much greater than the cost of producing chemical agents. Recent advances in biological engineering have provided the tools required for genetically altering baculoviruses, such as LdNPV, to produce viruses with quicker kill, higher virulence, or other qualities that would make them more effective as insecticides. This has been accomplished in some baculoviruses through insertion of foreign genes into the viral genome, e.g., toxin-producing sequences from various arthropods (Carbonell *et al.*, 1988; Cory *et al.*, 1996). This strategy has also been considered for use with LdNPV. However, unlike crop pests that inhabit controlled, carefully monitored, and often ephemeral agricultural systems, the gypsy moth is often a pest of large, wooded areas in which the spread of a genetically

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altered virus is more likely to occur and more difficult to detect once introduced. These considerations prompted the design of a preliminary release experiment using LdNPV that was genetically engineered for nonpersistence and tagged with a marker gene for easy tracking in the field (the engineered form of the virus is hereafter referred to as LdGEV).

For the purposes of our experimental release, the β-galactosidase gene (*LacZ*) was inserted into the genome of an LdNPV isolate whose polyhedrin gene had been rendered nonfunctional. β-Galactosidase production as a reporter system has been used to monitor field introductions of genetically engineered pathogens (Cook *et al.*, 1990; Kluepfel *et al.*, 1990, 1991; Mulock *et al.*, 1997). The resulting viral line (poly-minus) does not produce polyhedral occlusion bodies (POBs); its naked virions are highly susceptible to degradation by ultraviolet light and conditions in the insect midgut, the main causes of viral inactivation in nature (Ignoffo *et al.*, 1977; Jacques, 1977). Studies by Hamblin *et al.* (1990) have indicated that the dynamic interactions of poly-minus and wild-type viruses in nature would ensure that poly-minus viruses could not persist in the virus population for extended periods. Addition of the *LacZ* gene to the LdNPV genome and expression of β-galactosidase was thus considered to present no health or environmental dangers; therefore, this method was used to render LdGEV nonpersistent and easily tracked in the field via a colorimetric assay performed on dead larvae. Because it is recognized that β-galactosidase and β-galactosidase-like compounds can be produced by naturally occurring microorganisms that are sometimes associated with gypsy moth larvae (Podgwaite and Campbell, 1972; Podgwaite and Cosenza, 1976), slot blot analysis and polymerase chain reaction (PCR) were also used to confirm the presence of LdGEV in larvae.

The initial release of LdGEV required it to be protected until it had produced infections in host larvae in the field. A genetically engineered strain of LdNPV lacking the polyhedrin gene can be stabilized by the coocclusion process described by Wood *et al.* (1994) and used on other baculoviruses by Miller (1988) and Hamblin *et al.* (1990). Cooccluded LdGEV was produced by coinfecting host cells with both wild-type LdNPV (which contains a functioning polyhedrin gene) and LdGEV (without a functioning polyhedrin gene). When the wild-type virus replicates inside of a host cell, some of the LdGEV particles are occluded by the polyhedral protein along with the wild-type particles. The protection afforded by the polyhedron is thus "borrowed" by LdGEV.

The purpose of our field release was, first, to study the dynamics of the cooccluded LdGEV in a forest ecosystem. Previous work with a baculovirus engineered for nonpersistence in a crop system (Wood *et al.*, 1994) showed that recovery of the engineered form diminished rapidly over a period of 2 years. It was anticipated that LdGEV would be lost from the population even faster because of the potentially higher dilution associated with forest ecosystems. The information gained in this experiment was expected to help determine the feasibility of using the coocclusion process as a method for producing other baculoviruses genetically engineered for enhanced pesticidal properties against forest pests.

Second, we felt that this release would provide useful information regarding the ecology of baculoviruses. It is often difficult to monitor mortality caused by viral pesticides such as GYPCHEK because of the uncertainty in assigning cause of death to either naturally occurring or applied virus. We believed that engineering a recombinant LdNPV expressing β-galactosidase would provide a fast, simple, and inexpensive identification method, making it possible to obtain data concerning the movement of the LdNPV in time and space. Such information would be useful in identifying some of the underlying principles which limit and promote natural LdNPV epizootics.

MATERIALS AND METHODS

Virus production and coocclusion. LdGEV containing a *LacZ* gene from the bacterium *Escherichia coli* was constructed *in vitro* from the wild-type LdNPV isolate G2 (Smith *et al.*, 1988) using methods described in Yu *et al.* (1992). LdGEV, which lacked a functional polyhedrin gene, was cooccluded within polyhedra produced following *in vivo* coinfection of insect cells with the recombinant and wild-type virus (Yu *et al.*, 1992). The cooccluded virus was produced in laboratory-reared gypsy moth larvae (New Jersey standard strain described below) at the Boyce Thompson Institute (Ithaca, NY). Viral DNA restriction endonuclease fragment analyses of the occluded virions was used to estimate the percentages of the LdGEV and G2 genotypes in the inoculum at 21 and 79%, respectively.

Treatment of host larvae and eggs. All gypsy moth larvae and eggs used in our field experiments were of the laboratory-reared New Jersey strain ($F_{44–45}$) maintained by the USDA, APHIS Otis Methods laboratory (Otis ANGB, Cape Cod, MA). Gypsy moths used as eggs were kept at 4°C for 6 months before release to allow for the overwintering period necessary for embryonic development and egg hatch. Eggs that were used for the release of LdGEV were placed in groups of 500 eggs in 200 screen bags. A pipette was used to treat the eggs in each of these bags with 1 ml of a suspension of 2×10^8 occlusion bodies/ml LdNPV and cooccluded LdGEV; eggs were then left under a fume hood to dry for approximately 8 h.

LdGEV release and larval sampling. The LdGEV field release was conducted in a mixed oak and pine forest on the Camp Edwards Military Reservation in Cape Cod, MA. This forest is composed predominantly of black oak (*Quercus velutina*), white oak (*Quercus alba*), and pitch pine (*Pinus rigida*) trees 7–10 m in height. We created two octagonal plots at this site (Fig. 1), a control plot and a virus release plot, each ca. 4 ha in area and 225 m in diameter. The release plot and control plot were separated by ca. 2 km.

On 23 May 1993, screen bags of LdGEV-treated gypsy moth eggs were confined on 20 closely grouped black oak trees within the 25-m-diameter circle in the center of the release plot (Fig. 1A). A total of 200 spun polyester bags (Reemay by Dupont, Deer Park, WI) were attached to the tree limbs to enclose foliage, at a rate of approximately 10 bags per tree (Dwyer and Elkinton, 1993; D'Amico and Elkinton, 1995; D'Amico *et al.*, 1996). Each bag contained 500 LdGEV-treated gypsy moth eggs and 500 untreated eggs. The Reemay mesh bag enclosures confined the movement of the gypsy moth larvae during the first 2 weeks after hatch when they are predisposed to wind-borne dispersal (ballooning) (Mason and McManus, 1981). Bags were removed on 18 June 1993 at the conclusion of the ballooning period and after all confined larvae had either died of virus infection or molted to the second instar. The deployment of mesh bags was a condition for U.S. Environmental Protection Agency approval to conduct the LdGEV release.

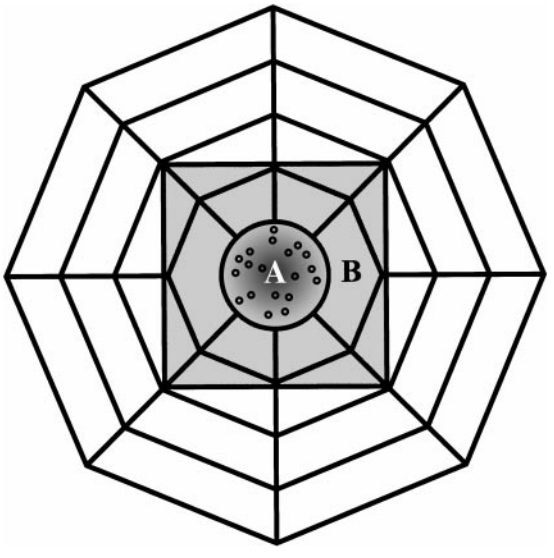

FIG. 1. Release plot consisting of a center circle 25 m in diameter surrounded by four rings 25 m wide divided in the eight compass directions. Both release plot and control plot were 225 m in diameter at their widest point. (A) Eggs contaminated with engineered LdNPV were released on 20 oak trees (○) in the center circle of the release plot (gradient shading). (B) Eggs used to produce the test populations were released within a 100 m^2 (1 ha) square in the center of each plot (gray shading).

TABLE 1

Fraction of Larvae within Ten Initial Virus Release Bags Dying and Testing Positive for the Presence of β-Galactosidase

Tree	Days post-release	Number of larvae sampled	Fraction dead	Fraction dead positive for β-gal
1	5	45	0.86	0.12
2	5	45	0.88	0.25
3	8	50	0.92	0.27
4	8	45	0.65	0.5
5	8	50	0.7	0.21
6	8	40	0.95	0.32
7	19	40	0.72	0.51
8	19	45	0.82	0.51
9	26	46	0.38	0.7
10	26	45	0.5	0.75
Mean ± SE			0.74 ± 0.06	0.41 ± 0.07

Note. Bags were cut down from oak trees immediately outside the release plot on various dates in May and June and ca. 45 larvae from each were reared individually, checked for mortality, and tested using the X-gal assay. This experiment served to test the effectiveness of the LdGEV release method, as measured by the fraction of contaminated eggs hatching into virus-infected larvae, and the fraction of these infected larvae dying from LdGEV.

To test LdGEV for spread, gypsy moth larval infestations were created in the trees surrounding the release and control plots. This was achieved in 1993 by releasing lab-produced eggs within both plots (Gould *et al.*, 1990; Dwyer and Elkinton, 1995) on 10 May. Larger infestations were released in 1994 (on 12 May) and 1995 (on 14 May) to increase the chance of detecting the presence of any persistent LdGEV. Approximately 8–9 million gypsy moth eggs were released in a 1-ha square in the center of each plot in 1993, 15–16 million in 1994, and 9–10 million in 1995. Eggs were placed in screen packets containing ca. 100,000 eggs each, and the packets were attached 1.5 m from the ground on the boles of 55 trees within each 1-ha square (Fig. 1B).

To assess the potential effectiveness of our virus release method, we prepared 10 mesh bags with packets of LdGEV-treated and untreated eggs as described and placed 1 of these bags on a branch of each of 10 black oak trees immediately outside the release plot. The bags were removed over a 3-week period and brought to the lab (Table 1). The 45–50 larvae found alive in these bags were reared individually and assayed for LdGEV as described below.

Larval collections in both plots began on 9 June 1993, before the actual removal of bags and release of infected larvae. This first collection served as a baseline. Collections continued at weekly intervals until pupation. Each week, we attempted to collect 200 larvae from the center of the release plot and 25 from each of the sectors created by dividing 25-m-wide concentric rings into eight coordinate directions to a distance of 112.5 m (Fig. 1), so that we could carefully follow the spread of the

TABLE 2
Number of Larvae Collected from LdGEV Release Plot and Control Plot, by Year and Week of Collection

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
LdGEV release, 1993	760	806	854	846	608	91	20
Control, 1993	99	100	99	99	63	5	7
LdGEV release, 1994	199	200	200	200	200	200	200
Control, 1994	200	200	200	200	200	200	200
LdGEV release, 1995	200	200	200	200	200	—	—
Control, 1995	100	100	100	100	100	—	—

released virus. The 200 larvae were collected at random throughout the control plot. In 1994 and 1995, a lesser number of larvae were collected at random within both plots (Table 2).

Each collected larva was held in an individual container with insect rearing diet (Bell *et al.*, 1981) for 2 weeks, and larvae were checked every other day for mortality. To assay for LdGEV, dead larvae were incubated in a test tube containing 1 ml of a 0.1% (wt/vol) chloro-3-indolyl-β-D-galactopyranoside (X-gal), diethyl formamide in an environment-controlled room for 48 h. The development of a deep blue color indicated cleavage of X-gal by β-galactosidase. Those larvae that produced a blue reaction were confirmed positive for LdGEV by the DNA analyses described below. Tissues and fluids from healthy laboratory-reared *L. dispar* larvae or those infected with the wild-type LdNPV have shown no detectable β-galactosidase activity (Wood, unpublished data).

To show that viable LdGEV was present after the initial release, on 21 June 1993 we selected branches in the release plot that had formerly been confined with LdGEV-treated eggs. We chose one branch on each of 10 randomly selected trees in the center of the release plot and confined 25 healthy third-instar gypsy moths within a mesh bag on each branch. Larvae were allowed to feed on foliage for 1 week, after which bagged branches were cut from the trees and brought to the lab (D'Amico and Elkinton, 1995). Larvae were then reared individually and tested for LdGEV as described above. We also necropsied dead larvae under 400× light microscope to determine whether or not POBs were also present in the cadavers.

Collection of bark, litter, and soil samples. Bark, litter, and soil samples were collected from the LdGEV release plot and the control plot in December of 1993, 1994, and 1995. Bark samples were collected at a height of ca. 1.5 m above ground on the north side of 10 randomly selected trees within a 20-m radius of the center of each plot. Shavings from ca. 20 cm^2 were scraped into a 0.5-L sterile polyethylene Whirl-pak bag (Nasco). Litter and soil samples were collected at 10-m intervals along north–south and east–west transects to a distance of 50 m from the center of each plot. At each sample point ($n = 20$) leaf litter covering ca. 50 cm^2 was removed down to the soil layer and placed in a Whirl-pak bag. Soil immediately below the litter sample was collected to a depth of 15 cm using a 2-cm-diameter stainless steel soil recovery probe (Oakfield Apparatus Co., Oakfield, WI). The top 2.5 cm of each soil core was removed to a whirl-pak bag; the remaining 12.5 cm (bottom) was placed in a separate bag.

Bark, litter, and soil samples taken within a plot were subsampled, pooled, and prepared for bioassay using slight modifications of procedures described by Podgwaite *et al.* (1979). Two grams of each bark sample was placed in 200 ml of sterile, aqueous 0.01 Triton X-100 (Sigma Chemical Co., St. Louis, MO) and blended at high speed for 1 min in a commercial 1 1 Waring blender. The blended bark suspension was poured through cheesecloth into a 250-ml polypropylene centrifuge bottle and held at 4°C for 16 h. The suspension was vigorously shaken for 1 min and spun at 12,000*g* for 30 min. The pellet was held at 4°C until resuspension in 5 ml sterile distilled water immediately before bioassay. Litter subsamples (5.0 g) were similarly pooled and extracted. Soil subsamples (2.5 g) were pooled and suspended in 200 ml of the Triton X-100 solution. Soil suspensions were not blended but were vigorously shaken for 1 min, poured through cheesecloth, and let standing at 4°C for 16 h. Suspensions were again shaken vigorously for 1 min, let standing for 2 min, decanted into a 250-ml centrifuge bottle, and spun at 12,000*g* for 30 min. The pellets were recovered and resuspended as above. Samples were stored at 4°C until bioassayed.

Bark, leaf litter, and soil samples were bioassayed with larvae of the lab-reared strain (New Jersey, $F_{39–41}$) maintained in the Forest Service Rearing Facility, Hamden, CT. One milliliter of pellet suspension from a given pooled subsample was evenly distributed onto the surface (38.5 cm^2) of synthetic diet (Bell *et al.*, 1981) that was dispensed into a 180-ml plastic cup. Ten newly molted second instars were placed in each cup and reared in a growth chamber for 16 days at 29°C and a 16:8 (L:D) photoperiod. Five replicates of 10 larvae were fed uncontaminated diet (controls). Dead larvae occurring over the course of the assay were individually placed in sterile 1-oz plastic cups and held at −20°C prior to analyses for viral genotype.

DNA analyses to confirm viral genotype. Polyhedral occlusion bodies were isolated from larvae testing positive in the β-galactosidase assays described above. Insects were homogenized in the X-gal solution in which they had been tested, and the particulate larval remains were removed. Polyhedra were pelleted by centrifugation at 550*g* for 5 min at room temperature and then resuspended in water and repelleted. This process was repeated three times.

Approximately 1×10^8 POBs were resuspended in 100–200 µl of TE. The POB solution was adjusted to 20 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM EDTA, 0.5% SDS, and 20 µg/ml proteinase K, and incubated at 50°C for 45 min. The solution was adjusted to 1% Sarkosyl and incubated at 50°C for 1 h. The POBs were pelleted by centrifugation, resuspended in 1 ml phosphate-buffered saline, and then resuspended in 250 µl TE. The solution was adjusted to 0.1 M EDTA, 20 µg/ml proteinase K, and the solution was incubated at 37°C for 1 h. $NaCO_3$ (pH 11.7) was then added to a final concentration of 0.125 M, and the solution was incubated at 37°C for 1 h. The solution was extracted with 1 vol of phenol saturated with 0.01 M Tris–0.001 M EDTA, pH 7.5, and 2 vol of chloroform:isoamyl alcohol (24:1). The aqueous phase was dialyzed in TE overnight at 4°C.

For slot blot analyses, DNA samples were prepared and loaded onto a slot blot apparatus according to the method of Brown (1991). LdNPV isolate A21-2 (Bischoff and Slavicek, 1996) was used as a viral standard, and an EGT minus viral strain containing a β-galactosidase gene was used for an LdGEV standard. Viral standard slots contained 1, 5, 25, or 125 ng of DNA. Duplicate slot blots were prepared.

A *LacZ* gene probe was prepared by isolating a KpnI–BamHI 3.5-kb DNA fragment from pCH110 containing the *LacZ* gene. A viral probe was prepared by isolating a BamHI 3.5-kb DNA fragment from pDB109 that covers the LdNPV genomic region from 64.5 to 68.0 kb. The probe fragments were labeled with a nick translation kit (Gibco BRL, Gaithersburg, MD) and [α-32P]dCTP (New England Nuclear, Boston, MA). Hybridizations and washings were carried out under standard high-stringency conditions. Slot blot analyses were performed on samples from the 1993 and 1994 field seasons.

Primers (described below) that would generate a fragment only from the recombinant virus, which contained the *LacZ* gene, were designed for use in the polymerase chain reaction to identify LdGEV. A set of primers was also designed to identify wild-type virus.

Primer A, GTCGATTTCCGCAACTAATC, binds to the region from 92 to 111 bp upstream from the polyhedrin gene translation start site. This primer binds to LdNPV containing wild-type and *LacZ* genes. Primer B, CGCCACGAACTTGTAGCATC, binds to the region from 395 to 414 bp downstream from the polyhedrin gene translation start site. This primer will bind to LdNPV containing wild-type and *LacZ* genes. Primer C, GCAATAATGCCTTTCCATTG, binds to the region from 280 to 299 bp in the xanthine–guanine phosphoribosyl–transferase (*gpt*) gene (Mulligan and Berg, 1981). This primer binds only to LdNPV containing the *LacZ* gene. An amplification product of 525 bp would be obtained using primers A and B with wild-type virus. No amplification product would be obtained using primers A and C with wild-type virus. An amplification product of approximately 3400 bp would be theoretically generated using primers A and B with LdGEV. However, the reaction conditions used were not conducive for the generation of long PCR amplification products. A fragment of 3400 bp was not observed in amplifications of LdGEV. An amplification product of 302 bp would be obtained using primers A and C with LdGEV.

Sample DNA isolated as described above was used for PCR analysis. PCRs in a total volume of 25 µl contained 50 mM $MgCl_2$, 0.05 nM primer A, 0.05 nM primer B or C, 0.2 nM each dNTPs (Promega, Madison, WI), 0.5 units Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN), approximately 3 ng of viral DNA, and one drop of sterile mineral oil. PCR amplifications, carried out in a Perkin–Elmer thermal cycler, were initiated with a denaturation step at 94°C for 3 min. We then ran 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C. DNA amplification products were separated by agarose gel electrophoresis using 1.5% gels and visualized after staining with ethidium bromide. PCR analyses were performed on samples from the 1993 and 1994 field seasons.

For restriction endonuclease analysis, viral DNA was digested with BglII according to the manufacturer's specifications, and the fragments were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide. Restriction endonuclease analyses were performed on samples from the 1993 field season only.

RESULTS AND DISCUSSION

In 1993, larvae from LdGEV-treated eggs released on foliage immediately outside the release plot within mesh bags were found dead of LdGEV, regardless of the date on which they were sampled (Table 1). In the week prior to removal of the bags, 28% (mean) of these larvae were infected with LdGEV, as measured by the β-galactosidase assay.

Of third instars confined within mesh bags on LdGEV release branches, 0.52 ± 0.02 (SE) died from virus, and 0.75 ± 0.01 (SE) of these virus-killed larvae were scored as LdGEV-killed on the basis of a strong blue reaction in X-gal. There was no parasitism of these test larvae or mortality from non-LdNPV causes, because the mesh

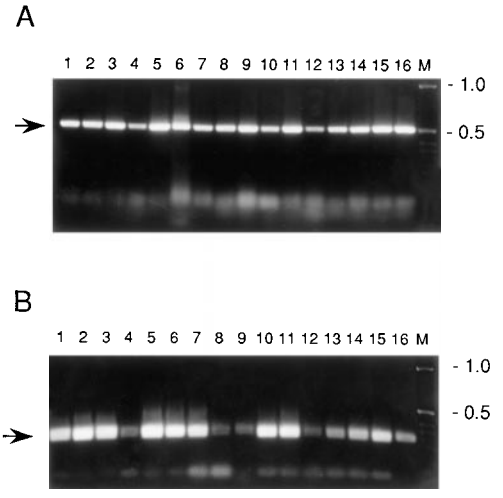

FIG. 2. Amplification products obtained using primers for wild-type virus and the *LacZ* gene containing recombinant virus (LdGEV). (A) Lanes 1–16, products obtained with wild-type virus primers. Lanes 1–15, samples 1–15. Lane 16, wild-type virus control. Lane M contains a molecular weight marker (kb ladder). The 525-bp fragment obtained from wild-type virus is indicated by the arrow. (B) Lanes 1–16, products obtained with *LacZ* gene-specific primers. Lanes 1–15, samples 1–15. Lane 16, LdGEV control. Lane M contains a molecular weight marker (kb ladder). The 302-bp fragment obtained from recombinant virus is indicated by the arrow.

bags successfully prevented adult parasites from access to the larvae. PCR analysis of a subsample of 15 of these larvae ranked as β-galactosidase positive found that all 15 larvae contained both wild-type LdNPV and LdGEV (Fig. 2), as would be expected in larvae dying from the cooccluded LdGEV. From the results of these experiments, we believe that our release methodology succeeded in producing foliage contamination that served as a source of inoculum of LdGEV.

Soon after the field trial had begun in 1993, a much larger than expected percentage of field-collected larvae tested positive for β-galactosidase activity, although the color reaction was less distinct that that observed in the laboratory. These larvae were also collected in the control plot. After dissection, a high proportion of the gypsy moth larvae testing positive were found to be parasitized by the braconid wasp *Cotesia melanoscela*, a common parasite of gypsy moth early instars throughout eastern North America; this was the leading cause of mortality in 1993 (Fig. 3). Female wasps inject an egg into the body cavity of the host, where a single larva develops for ca. 16 days. When we tested pupal *C. melanoscela* for β-galactosidase activity in X-gal, as described for collected gypsy moth larvae, all of 30 individually tested pupae turned blue within 48 h. We also tested 20 gypsy moth larvae that had previously contained a larval *C. melanoscela;* 12 larval preparations turned blue within 48 h. Although β-galactosidase is not produced by organisms associated with dead laboratory-reared gypsy moth larvae (Wood, unpublished data), the range of saprophytic and parasitic fauna in the field is obviously much richer. Such diversity increases the likelihood that some associated organism will produce either β-galactosidase or an enzyme with similar properties. It is highly likely that *C. melanoscela* is such an organism or that *C. melanoscela* contains an organism that produces β-galactosidase. Because parasitized gypsy moth larvae responded as false positives to the X-gal assay, they were not tested using the X-gal protocol after 1993. Instead, polymerase chain reaction, slot blot DNA, and restriction endonuclease analyses were used to verify presence or absence of LdGEV. Despite its ease of use, the *LacZ* marker is inappropriate for use under those field conditions which allow parasites and β-galactosidase-producing microorganisms access to test insects.

We collected 3985 larvae from the release plot in 1993, but of those larvae dying from virus, only 2 from the center of the plot were confirmed positive for LdGEV using PCR analysis. One of these larvae was collected in week 2 (sample L2) and the other in week 4 (sample L4). Agarose gel electrophoresis of DNA amplification products is shown in Fig. 4A. The expected 525-bp fragment was present in the wild-type control lane (lane 1) and in samples L2 and L4 (lanes 5 and 7, respectively). No amplification product was generated using primers A and B with LdGEV (lane 2). The

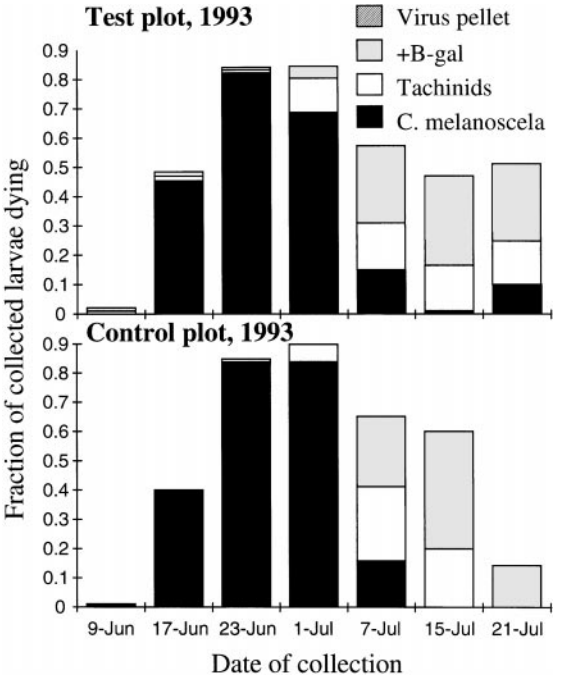

FIG. 3. Mortality of collected larvae by week, 1993. Fraction of collected larvae: parasitized by *C. melanoscela* (Braconidae: Hymenoptera), parasitized by tachinid Diptera, and testing positive for β-galactosidase in the X-gal assay.

expected 302-bp fragment was generated using primers A and C in the presence of LdGEV (lane 3) and in the presence of sample L2 and L4 (lanes 6 and 8, respectively). No amplification product was generated with primers A and C in the presence of the A21-2 virus (lane 4). Lane 9 contains the results of a control amplification performed without template DNA. The relative percentages of LdGEV and wild-type LdNPV in the positive samples were determined by densitometric analysis; sample L2 contained 25.9% LdGEV and sample L4 contained 72.3% LdGEV.

In 1994, we doubled the density of eggs used to create the test population. This resulted in establishment of populations similar in density to those seen in gypsy moth outbreaks, with mortality of larvae falling into categories typical for such a population (Fig. 5). Complete defoliation of oak trees within the plots was observed by June 1994. Because of the high density of larvae present, we felt that we had maximized our chances of detecting persistent virus that had overwintered from 1993. No LdGEV was detected by PCR in the larvae that turned blue in the β-galactosidase assay in 1994, even though larvae parasitized by *C. melanoscela* or tachinid flies were excluded from the assay (Fig. 4B).

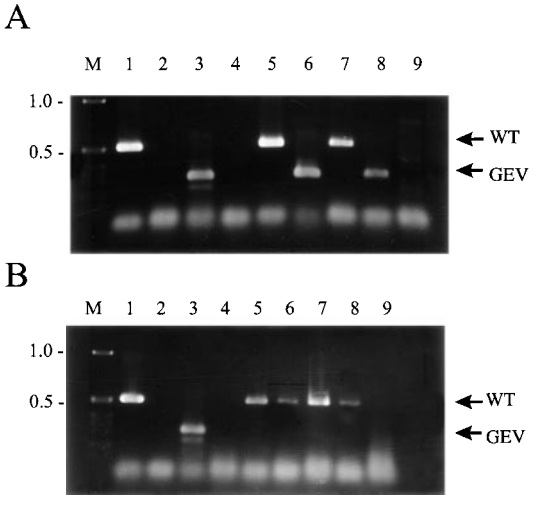

FIG. 4. Amplification products obtained using primers for wild-type virus and the *LacZ* gene containing recombinant virus. (A) Lanes 1, 2, 5, and 7, products obtained with wild-type virus primers. Lanes 3, 4, 6, and 8, products obtained with *LacZ* gene-specific primers. Lanes 1 and 4, wild-type virus. Lanes 2 and 3, *LacZ* gene recombinant virus. Lanes 5 and 6, sample L2. Lanes 7 and 8, sample L4. Lane 9, DNA-minus control. Lane M contains a molecular weight marker (kb ladder). The 525-bp fragment obtained from wild-type virus and the 302-bp fragment generated from the *LacZ* gene containing recombinant virus are indicated by arrows. (B) Lanes 1, 2, 5 and 7, products obtained with wild-type virus primers. Lanes 3, 4, 6, and 8, products obtained with *LacZ* gene-specific primers. Lanes 1 and 4, wild-type virus. Lanes 2 and 3, *LacZ* gene recombinant virus. Lanes 5–8, sample numbers 63, 112, 116, and 121, respectively. Lane 9, DNA-minus control. The 525-bp fragment obtained from wild-type virus and the 302-bp fragment generated from the *LacZ* gene containing recombinant virus are indicated by arrows.

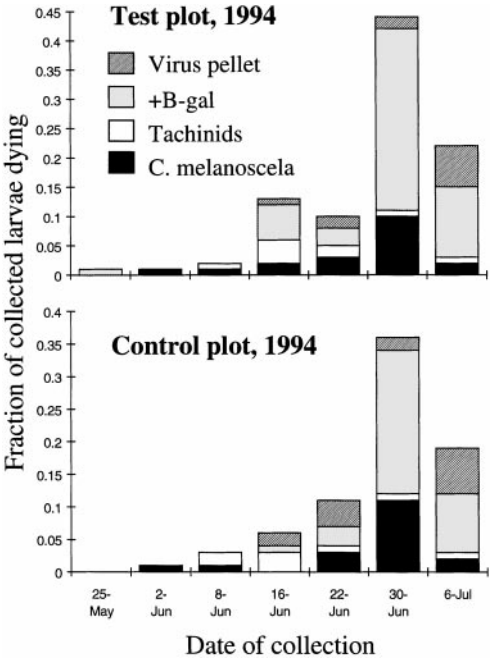

FIG. 5. Mortality of collected larvae by week, 1994. Fraction of collected larvae: parasitized by *C. melanoscela*, parasitized by tachinid Diptera, testing positive for β-galactosidase in the X-gal assay, and containing polyhedral occlusion bodies as determined by light microscopy.

This suggests that another organism associated with dead larvae, perhaps a fungus or bacterium, may also produce a false positive result in the β-galactosidase assay. Whatever the cause of false positives, the density of our test population and our DNA analyses gave us confidence that the virus did not successfully overwinter.

Eggs used to create the test population in 1995 resulted in populations that again reached densities similar to those seen in gypsy moth outbreaks, and levels of parasitism were again relatively low (Fig. 6). Some defoliation of oak trees was observed by June, although not to the same extent as in 1994. No unparasitized larvae tested positive in the β-galactosidase assay in 1995; therefore, no DNA analyses were performed on any field-collected larvae in 1995.

In all 3 years, larvae challenged with extracts of bark, leaf litter, and soil in the plots died of wild-type LdNPV (Fig. 7). According to PCR analyses of dead larvae, none contained the genetically engineered form of the virus. Top soil and bottom soil extracts killed the fewest larvae overall, less than 6% in either plot in any year. Bark extracts killed the greatest number of test insects. The bark of trees is a known refuge for LdNPV (Podgwaite *et al.*, 1979), although typically virus is protected through the winter by an egg mass (Murray and Elkinton, 1989). The presence of LdNPV in samples from 1994 and 1995 presumably resulted from in-

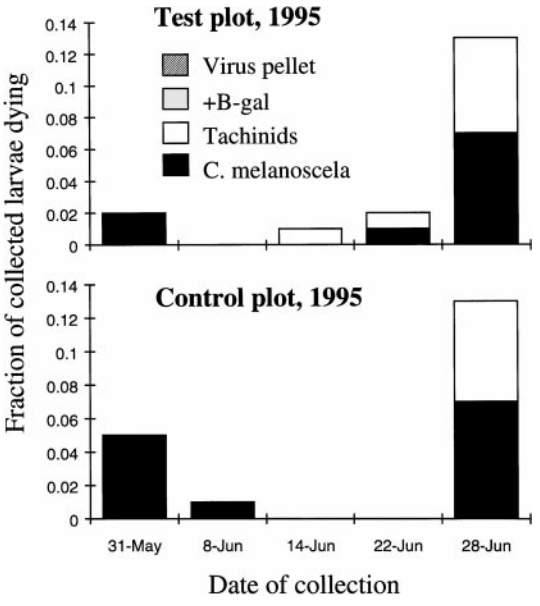

FIG. 6. Mortality of collected larvae by week, 1995. Fraction of collected larvae: parasitized by *C. melanoscela*, parasitized by tachinid Diptera, testing positive for β-galactosidase in the X-gal assay, and containing polyhedral occlusion bodies as determined by light microscopy.

creased mortality from wild-type virus in the denser populations that we created in those years. Because wild-type LdNPV was present in the cooccluded virus and was better able to overwinter, this was expected (Hamblin *et al.*, 1990).

The results presented here detail the first release and recovery of a genetically engineered insect virus in a forested ecosystem in the United States. Because it was the first such release, safety and regulatory issues were paramount. Therefore, we were conservative in our approach, both in the selection of a marker gene and in the methods chosen for release. Clearly, our approach did not allow for uncontrolled spread of LdGEV from the focus of introduction. First-instar gypsy moth larvae are normally borne on the wind, often dispersing several kilometers from an infested area. Our confinement of LdGEV-infected larvae until after the ballooning period, although prudent from the standpoint of safety, severely limited our ability to use the release method to study "natural" LdNPV epizootiology. Further, the β-galactosidase marker system, though safe and easy to use, was problematic. In planning the study, we recognized the risk of false positives that would be caused by β-galactosidase-producing organisms that are closely associated with gypsy moth in its natural habitat. However, the involvement of *C. melanoscela* was quite unexpected and may have limited the usefulness of the β-galactosidase marker for all but the mostly tightly constrained LdGEV release experiments. For those LdGEV releases in which gypsy moth coexists with other β-galactosidase-producing organisms, the X-gal colorimetric assay can only be considered presumptive; LdGEV must be confirmed with the appropriate DNA analytical methods. Hindsight notwithstanding, we have taken a significant step toward the development of genetically engineered viral recombinants for gypsy moth control and have a basis on which to design future studies.

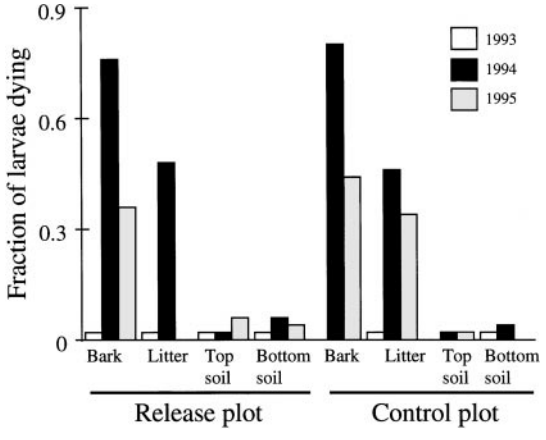

FIG. 7. LdNPV-caused mortality of larvae challenged with extracts from top soil, bottom soil, tree bark, and leaf litter in virus release and control plots, 1993, 1994, and 1995.

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