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# ENHANCEMENT OF BIOLOGICAL CONTROL AGENTS FOR USE AGAINST FOREST INSECT PESTS AND DISEASES THROUGH BIOTECHNOLOGY

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## ABSTRACT

Research and development efforts in our research group are focused on the generation of more efficacious biological control agents through the techniques of biotechnology for use against forest insect pests and diseases. Effective biological controls for the gypsy moth and for tree fungal wilt pathogens are under development. The successful use of Gypchek, a formulation of the Lymantria dispar nuclear polyhedrosis virus (LdNPV), in gypsy moth control programs has generated considerable interest in that agent. As a consequence of its specificity, LdNPV has negligible adverse ecological impacts compared to most gypsy moth control agents. However, LdNPV is not competitive with other control agents in terms of cost and efficacy. We are investigating several parameters of LdNPV replication and polyhedra production in order to enhance viral potency and efficacy thus mitigating the current disadvantages of LdNPV for gypsy moth control, and have identified LdNPV variants that will facilitate these efforts. Tree endophytic bacteria that synthesize antifungal compounds have been identified and an antibiotic compound from one of these bacteria has been characterized. The feasibility of developing tree endophytes as biological control agents for tree vascular fungal pathogens is being investigated.

## INTRODUCTION

Chemical pesticides and fungicides are the preferred control agents for forest insect pests and fungal diseases. In excess of 350 billion pounds of these agents are used annually in the United States to control pests and diseases in forestry, agriculture, and in residential areas. Rachel Carson's Silent Spring, focused widespread attention on the environmental hazards linked to pesticide use. Broad spectrum insecticides and fungicides have adverse impacts not only on their target organisms but also on beneficial insects and fungi, and consequently on the entire ecosystem. In addition, chemical residues may cause health problems among the human population. Interest in biological insect and fungal control agents is growing as a consequence of these concerns regarding chemical pesticide use. A number of bacteria synthesize antifungal compounds, and over 1500 microorganisms or microbial products have been identified that are insecticidal. Generally, natural control agents have little adverse ecological impacts due to their specificity for the target host. Long term environmental hazards and health concerns are not a factor with biological control agents since chemical residues are not present. Unfortunately, biological control agents suffer from several disadvantages in comparison to chemical pesticides, including cost of production, efficacy, and stability. The techniques of biotechnology offer a means of mitigating some of the disadvantages inherent in biological control agents. Efforts in our research work unit focus on the enhancement of an insect virus and a bacteria for control of a defoliating moth and fungal tree pathogens.

## GYPSY MOTH CONTROL THROUGH AN INSECT VIRUS

The Lymantria dispar nuclear polyhedrosis virus (LdNPV), which is pathogenic to Lymantria dispar, the gypsy moth, was selected as a model system for the enhancement of a biological control agent for a forest insect pest. The gypsy moth was imported from Europe into North America near Boston in 1869. Since then the area of gypsy moth infestation has increased to include almost the entire New England area, New York, Delaware, Maryland, New Jersey, Pennsylvania, Virginia, West Virginia, Ohio, and Michigan (1). Several chemical insecticides have been used for gypsy moth control, including DDT which was one of the most effective.

Understanding of the environmental impacts of DDT and other chemical insecticides caused a shift to the use of primarily Dimilin and Bacillus thuringiensis (Bt) for current gypsy moth control efforts. LdNPV has the significant advantage over other control agents of specificity for the gypsy moth. Consequently, LdNPV is the agent of choice for use in environmentally sensitive areas. LdNPV is not used extensively for gypsy moth control primarily as a consequence of high production costs and low efficacy. Efforts are being made to mitigate these problems inherent in LdNPV by generating viral strains that are competitive in terms of cost and efficacy with other gypsy moth control agents.

**LdNPV replication and production for field use.**

Two forms of LdNPV are produced during viral replication, occluded and nonoccluded virus (2). Early after infection nonoccluded virus is produced that leaves the cell and is responsible for secondary infections within the hemocoel of the host insect. A different form of virus is produced late in infection that is occluded into a protein matrix composed primarily of a viral encoded protein termed polyhedrin. These structures, approximately 1 to 3 micrometers in diameter and polyhedral in shape, are termed polyhedral occlusion bodies or polyhedra. Nucleocapsids within polyhedra are protected from most environmental conditions with the exception of ultraviolet light. Virus is applied through sprayers in the field in the form of polyhedra, which are ingested by gypsy moth larvae feeding on tree foliage. Once within the alkaline environment of the insect midgut, polyhedra dissolve, releasing nucleocapsids which infect the insect midgut cells thereby initiating the infection process.

LdNPV is currently produced in gypsy moth larvae at a cost of approximately $30.00 for enough polyhedra to treat an acre of forest. Production is the most expensive component inherent in the use of LdNPV. Limitations exist in increasing the efficiency of production of polyhedra in larvae. Efforts to produce polyhedra in cell culture systems are being made to generate a more economical production methodology. In comparison, production costs of Dimilin and Bt are approximately $3.00 per acre equivalent. LdNPV can be made cost competitive by either increasing viral potency, thereby allowing treatment with fewer polyhedra per acre, or decreasing production costs. We have initiated investigations to gain an understanding of polyhedra production and the molecular basis for viral potency with the goal of enhancing polyhedra production in cell culture and viral potency.

**Enhancement of LdNPV polyhedra production and potency.**

A LdNPV variant has been identified that differs from wild type virus in several characteristics, including polyhedra production and potency (3). This variant, isolate 5-6, was isolated after approximately twenty passages in cell culture (in Lymantria dispar 652Y cells, 4). In comparison to wild type LdNPV (isolate A21), isolate 5-6 produces fewer polyhedra both in cell culture and in vivo (Table 1).

Table 1. Polyhedra production in fourth instar gypsy moth larvae and in 652Y cells.

| Isolate | Polyhedra/larvae | Polyhedra/652Y cell |
|---|---|---|
| 5-6 | $8.6 \times 10^7$ | $4.4 \pm 0.9$ |
| A21 | $2.1 \times 10^9$ | $51.0 \pm 6.0$ |

The relative number of virions present within polyhedra synthesized by these isolates was investigated. Polyhedra were produced in gypsy moth larvae and prepared for examination by electron microscopy. Polyhedra

were sectioned, and the number of virions present in cross sections was quantified by counting and expressed as the number of virions per square micrometer of polyhedra cross section surface area (Table 2). Polyhedra produced by isolate 5-6 were found to be almost devoid of occluded nucleocapsids. The relative potency of LdNPV isolates 5-6 and A21 were examined through bioassay in second instar gypsy moth larvae (Table 3). Isolate 5-6 was found to exhibit very low potency in comparison to isolate A21.

Table 2. Number of virions present within cross sections of polyhedra produced by isolates 5-6 and A21 in fourth instar gypsy moth larvae.

| Isolate | # of Polyhedra Cross Sections Examined | Average Diameter of Polyhedra Cross Sections | Total Number of Virions Counted | Number of Virions Per Square Micrometer |
|---|---|---|---|---|
| 5-6 | 59 | 1.4 um | 14 | 0.13 |
| A21 | 25 | 2.0 um | 941 | 12.2 $\pm$ 5.5 |

Production of few polyhedra, few virions occluded within polyhedra, and extremely low potency are traits exhibited by few polyhedra (FP) viral variants. FP variants have been identified in a number of baculovirus species (4), and have been found to arise during in vitro viral replication as a consequence of insertion of DNA sequences into preferential locations in the viral genome. FP mutations are generated with a high frequency during passage in cell culture, which places limitations on production of polyhedra in cell culture systems. Current investigations on isolate 5-6 are directed to an understanding of the molecular basis that gives rise to the FP phenotype. Once this is understood, a means of increasing the number of virions occluded into polyhedra may be devised which could enhance potency. In addition, if preferential DNA insertion sites exist that give rise to the FP phenotype, site directed mutagenesis could be used to create a viral strain lacking these sites which would preclude the genesis of the FP mutant. This viral strain would enhance cost effective polyhedra production in cell culture systems.

Table 3. Lethal Concentrations of A2-1, 5-6, and LDP 226 for second instar gypsy moth larvae.

| Isolate | $LC_{50}$(95%FL)[a] | $LC_{90}$(95%FL) | Slope[b] | PR[c] |
|---|---|---|---|---|
| A2-1 | 9.90(7.29-13.47) | 29.67(20.80-49.6) | 2.68 $\pm$ 0.35 | 0.94 |
| 5-6 | >7400 | - | - | <0.0013 |
| LDP 226 | 9.31(6.46-13.50) | 48.61(30.37-97.42) | 1.79 $\pm$ 0.23 | - |

a Polyhedra/ml diet X $10^3$, FL= fiducial limits.
b $\pm$ SEM.
c Potency ratio = $LC_{50}$ LDP 226/$LC_{50}$ isolate.

**Enhancement of LdNPV polyhedra production in cell culture.**

Another characteristic of in vitro viral replication under study is the decrease in polyhedra production that occurs with viral passage. Cells in culture infected with nonoccluded virus isolated from hemolymph of virally infected gypsy moth larvae were found to produce the greatest number of polyhedra per infected cell (Table 4). After several passages in cell culture, the number of polyhedra produced per cell dropped approximately 3 fold. This observation has also been noted during the passage of other baculoviruses in cell culture, and appears not to be related to FP viral mutations. The basis for the observed decrease in polyhedra production is under investigation. A recently discovered LdNPV variant, MPV, may aid in the investigation of this characteristic. This isolate was obtained during passage of isolate A21 in cell culture, and was found to maintain a high level of polyhedra production after several passages in cell culture (Table 4). In addition, infection of cells with isolate MPV generates a greater percentage of cells containing polyhedra in comparison to isolate A21. Restriction endonuclease analysis of the genome of isolate MPV is being performed to determine if this isolate was generated by mutation of isolate A21. The economics of polyhedra production in cell culture would be enhanced if a means of maintaining a high level of polyhedra production over repeated passages could be devised.

Table 4. Polyhedra production in 652Y cells.

| Isolate | 1st Passage Production per Cell | Polyhedra Production Per Cell After More than 5 Passages | Percentage of Cells Containing Polyhedra |
|---|---|---|---|
| A21 | 51.0 | | 38.0 |
| A21 | | 14.7 | 34.3 |
| MPV | | 87.9 | 90.0 |

**Identification of LdNPV genotypic variants exhibiting a range of potencies.**

The identification and analysis of viral isolates exhibiting a range of biological activity may lead to a means of enhancing potency through a natural viral characteristic. Several LdNPV isolates were obtained from Gypchek, an LdNPV product currently used for control of the gypsy moth. Viral lines were generated through infection of gypsy moth larvae with amounts of polyhedra that result in from 5 to 10% larval mortality (6). At this mortality level, there is a high probability of generating an infection by a single virion or virion bundle which would generate a population of virus with one or a limited number of genotypes. The degree of genotypic heterogeneity in the LdNPV isolates was assessed by analysis of restriction endonuclease fragment polymorphisms after genomic restriction endonuclease digestion with the enzyme Bgl II. Gypchek was found to be composed of a number of LdNPV genotypes as evidenced by the identification of twenty-two genotypic variants. These variants were grouped into classes on the basis of similarities in the number and length of genomic fragments generated by digestion with the restriction enzyme Bgl II (Table 5).

The biological activity of some of the LdNPV isolates described above were determined through bioassay in gypsy moth larvae. The isolates were found to exhibit a range of potencies in relation to Gypchek, which was used as a standard (Table 6). Several of these isolates were selected for further studies designed to elucidate the basis for observed potency differences.

Table 5. Grouping of Variants According to Similarities in Genomic Bgl II restriction Patterns.

| Class | Isolates | # Of Fragments | # Of Matching Fragments | Genomic Size |
|---|---|---|---|---|
| I | 151 | 20 | 17 | 162.05 |
| | A21 | | | 162.1 |
| II | 201 | 18-20 | 13 | 164.6 |
| | 203 | | | 164.65 |
| | 201-1 | | | 164.2 |
| | 203-1 | | | 163.5 |
| | 203-2 | | | 163.1 |
| | 203-8 | | | 163.15 |
| | 203-10 | | | 164.55 |
| III | 111 | 20 | 17 | 162.0 |
| | 163-3 | | | 161.8 |
| | 141-2 | | | 162.0 |
| IV | 123 | 19-20 | | 160.3 |
| V | 121 | 19 | 16 | 161.6 |
| | 122 | | | 161.5 |
| | 141 | | | 161.7 |
| | 163 | | | 161.8 |
| | B21 | | | 161.7 |
| | B21-2 | | | 161.7 |
| | 131 | | | 161.7 |
| VI | 121-1 | 19 | | 164.5 |
| VII | 162 | 20 | | 163.6 |

Table 6. Relative potencies[1] of viral isolates.

| ISOLATE | $LC_{50}$(LIMITS)[2] | $LC_{90}$(LIMITS)[2] | SLOPE (SEM) | $PR_{50}$[3] | $PR_{90}$[4] |
|---|---|---|---|---|---|
| 203 | 1.5 (1.0- 2.2) | 6.9 (4.4- 14.1) | 1.9 (0.3) | 4.4 | 4.6 |
| A21 | 9.9 (7.3-13.5) | 29.7 (20.8- 49.6) | 2.7 (0.4) | 0.9 | 2.0 |
| 201 | 5.9 (3.9- 8.8) | 43.4 (25.7- 95.8) | 1.5 (0.2) | 1.8 | 1.5 |
| 111 | 8.8 (6.3-12.3) | 35.7 (23.5- 67.0) | 2.1 (0.3) | 1.1 | 1.5 |
| 163 | 3.7 (2.2- 6.0) | 61.3 (32.3- 155.4) | 1.1 (0.1) | 3.0 | 1.1 |
| 121 | 12.4 (8.9-17.3) | 48.5 (32.2- 89.9) | 2.2 (0.3) | 0.8 | 1.1 |
| 226[5] | 8.9 (6.0-13.3) | 48.6 (33.3- 103.8) | 1.8 (0.2) | 1.0 | 1.0 |
| 151 | 7.7 (5.1-11.4) | 52.8 (32.0- 108.2) | 1.5 (0.2) | 1.2 | 0.9 |
| 123 | 15.6(10.6-23.1) | 102.3 (61.7- 212.7) | 1.6 (0.2) | 0.6 | 0.5 |
| 141 | 33.8(23.0-50.0) | 216.1 (130.9- 444.8) | 1.6 (0.2) | 0.3 | 0.2 |
| B21 | 21.8 (9.9-43.4) | 277.1 (117.6-1132.4) | 1.2 (0.1) | 0.3 | 0.1 |
| 131 | 35.3(17.9-69.9) | 648.9 (270.6-2606.3) | 1.0 (0.1) | 0.3 | 0.1 |

1. IN VIVO BIOASSAY IN 2ND INSTAR GYPSY MOTH LARVAE

2. POLYHEDRAL INCLUSION BODIES x $10^3$ / ML DIET

3. RATIO OF $LC_{50}$ STANDARD TO $LC_{50}$ ISOLATE

4. RATIO OF $LC_{90}$ STANDARD TO $LC_{90}$ ISOLATE

5. STANDARD GYPCHEK PREPARATION

**Enhancement of LdNPV efficacy.**

Another disadvantage of LdNPV in comparison to chemical pesticides is that the virus requires from approximately 10 to 14 days to kill its host. During this time period, the virally infected larvae continue to feed, causing defoliation. Alterations to the virus that would either enhance viral killing speed or cause feeding cessation would provide greater protection to tree foliage. The efficacy of the Autographa californica nuclear polyhedrosis has been enhanced through the insertion of neurotoxin genes from the straw itch mite Pyemotes tritici (7) and the scorpion Androctonus australis (8) into the viral genome. In both of these cases, insects infected with the recombinant viruses died in less time compared to insects infected with wild type virus. The

neurotoxin genes from P. tritici and A. australis will be inserted into the LdNPV genome and the efficacy of the recombinant viruses assessed through bioassay.

Through the approaches described in the previous sections, it is anticipated that LdNPV strains will be developed with enhanced control properties that mitigate current disadvantages of LdNPV for gypsy moth control, providing an ecologically sound control agent with commercialization feasibility.

## CONTROL OF FUNGAL VASCULAR WILT TREE PATHOGENS

The fungal vascular wilt pathogens Ophiostoma ulmi and Cryphonectria parasitica have decimated American elm and chestnut trees in North America. Chemical fungicides have proven effective in control of these fungal pathogens when administered through injection. This labor intensive and expensive control strategy is acceptable on a limited basis, but is not feasible for treatment in a forest setting. Treatment of tree fungal pathogens is especially difficult due to the need for delivering the control agent to the vascular system of the tree, thus precluding aerial application methods.

As an alternative to chemical fungicides, the feasibility of using host tree bacterial endophytes is being assessed. Strains of Bacillus and Pseudomonas bacteria that synthesis antifungal compounds have been isolated from tree vascular systems. The structure and activity of an antibiotic of the iturin group has been characterized that is produced by B. subtilis (9,10). This antibiotic is effective against O. ulmi and C. parasitica in in vitro assays. If the bacteria is to provide effective fungal control either the concentration of B. subtilis resident in the xylem of the tree and/or the amount of the antibiotic produced by the bacteria need to be increased. Whether this is possible remains to be determined. Recent studies on control of fungal wilt pathogens by Pseudomonad species suggests that an elicitation of natural tree defense mechanisms was the primary determinant in overcoming fungal infection (11, R. Scheffer, personal communication). These results highlight the complex fungal-host tree interactions that occur upon infections, and suggest the need for obtaining a better understanding of these interactions. Additional research may lead to the development of efficacious and cost effective controls for tree vascular wilt pathogens that can be used in a forest setting.

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