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Popular Methods of Biological Control and the Future of Baculoviruses

Michael Hutchinson

Biological control is a pest management strategy with the goal of reducing pest populations through human collaboration (Bajer et al., 2019). This article presents a broad overview of the field with focus on recent advancements regarding Baculoviruses as biological control agents. The term "biological control" was first used by Harry Scott Smith in 1919, and later was applied by entomologist Paul H. DeBach during research of citrus crops. DeBach was able to control populations of citrus pests through implementation of insect pest predators. This was the first report of someone using an insect species to control an insect pest (DeBach, 2007). A pest is an organism that is either a native or invasive species, causing an array of negative effects on surrounding environments. Negative effects associated with pests may include contaminating food supplies, stunting plant growth, spreading pathogens, and outcompeting native species for resources. Methods of biological control used to combat pests involve the physical removal of organisms and the application of predators, parasitoids, chemical insecticides, microbes including viruses. (Bajer et al., 2019).

The effectiveness of biological control agents greatly depends on the characteristics of targeted organisms. Individual characteristics of targeted organisms may include reproduction rates, location of species, and the ability to adapt to stressors. Specific methods of biocontrol may work efficiently with these characteristics and cause organisms to be more susceptible to containment or eradication. However, individual characteristics can make specific methods less effective and even a poor choice for specific pest populations.

Historically, the first forms of biological control used against pests involved the release of predators and parasitoids. In this process, free-living insect predators are often released into environments to consume large numbers of pests. Predator larvae is also expected to feed on pest populations to further lower their numbers and associated negative effects. Examples of such predators include lady beetles, true bugs, lacewings, and spiders. These predators feed on pests such as aphids or spider mites and can be found in most agricultural and natural habitats (Frodsham et al., 1993).

Predators are favorable biological control agents since they are cost effective and directly contribute to pest mortality. Their release is also important when dealing with invasive species, who lack natural predators and can rapidly reproduce. With high host specificities, many predators target only susceptible pest populations and can sustain such predation for long periods of time. However, other predators are not host-specific and are considered generalists, feeding on both pests and beneficial insects (Frodsham et al., 1993). Another disadvantage is that predators do not provide immediate results like some pesticides do. Lastly, predators need prey to be present to survive, making the complete elimination of a pest unlikely.

Like predators, parasitoids also make great biological control agents. Parasitoids are small insects whose immature stages develop on or within a single insect host. Unlike parasites, parasitoids eventually kill the hosts they are feeding on, making them advantageous biological control agents. Immature stages of parasitoids feed directly on the body fluids and organs of pests until they are ready to pupate (Frodsham et al., 1993). The most beneficial parasitoids used in biological control are wasps, bees, or flies (Frodsham et al., 1993). These organisms are often considered the most important natural enemy for maintaining insect pest populations. The host specificities of most parasitoids allow them to only target one type of host without harming beneficial insects. Another advantage lies in the fact that the life cycles of both prey and parasitoids can greatly coincide, to the point where parasitoids may alter pest life cycles to accommodate their development. Females are also efficient at finding prey by using host-related chemical cues (Frodsham et al., 1993).

Two species of parasitoids have shown to successfully control invasive Winter moth populations (Figure 1). Winter moths are a great concern as their hatching caterpillar larvae directly feed on the leaves of trees they are born on or around. With no natural predators, infested trees can be completely defoliated, which hinders annual tree growth and can even cause direct mortality (Embree, 1965). Both *Cyzebis albicans* and *Agrypon flaveolatum*, lay eggs on the leaves of trees infested by Winter moths. When Winter moth larvae feed on these leaves they become infected. Once infected, the larvae will hatch inside the caterpillar and feed on it from the inside out (Embree, 1965).

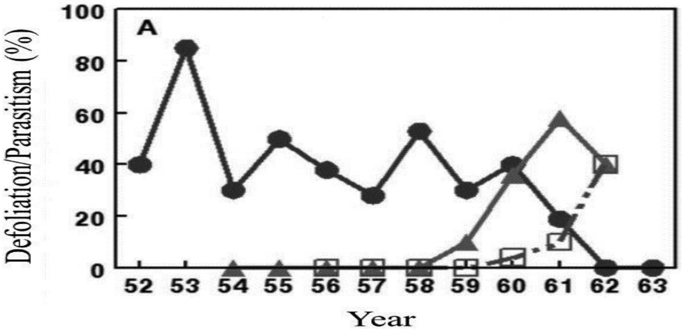

Figure 1. Combatting Winter Moth Infestation via Parasitoids.

Graph of Winter moth populations before and after release of C. *albicans* and A. *flaveolatum*. Both parasitoids were released in the year 1954. Lines with a circle symbol indicate both the population of Winter moths and their rates of defoliation. A line with a triangle symbol indicates the population size of the species C. *albicans.* Lines with a square symbol indicate the population size of the species A. *flaveolatum*. The percentage on the y-axis represents the number of trees that have become defoliated by Winter moth larvae. This figure, Combatting Winter moth Infestation via Parasitoids, was originally published in (Embree, 1965).

In this study, both parasitoid species were released to control Winter moth populations in the year 1956. After 1960, both the population of Winter moths and the percentage of defoliated trees decreased at a rapid rate (Figure 1). By 1962 and 1963, Winter moths were almost driven to extinction while defoliation rates remained as low as ever (Figure 1). While this occurred, the populations of both parasitoid species continued to increase or remain much higher than the population of Winter moths. Despite this scientific evidence showcasing the effectiveness of parasitoids, they too have their fair share of limitations.

Although parasitoids can complete their life cycles and reproduce faster than predators, pests attacked by parasitoids die more slowly. This gives pests a chance to feed or lay eggs before succumbing to an attack. Another limitation with parasitoids involves hyper-parasitism, when parasitoids are parasitized by other parasitoids, greatly reducing their effectiveness (Frodsham et al., 1993). Parasitoids are great biological control agents, but when you combine them with other biological control agents such as predators, their impacts can be greatly enhanced.

A more modern biocontrol method directly combatting the reproductive success of a species are pesticides, specifically insecticides. Insecticides are a chemical form of biocontrol that include ovicides and larvicides, which kill pest eggs and larvae (Bale et al., 2008). There are two main types of insecticides: systemic and contact. Systemic insecticides are incorporated throughout the whole plant. Contact insecticides are toxic to insects when they physically interact with the chemicals involved in the insecticide (Bale et al., 2008). Eventually, insects can develop immunities to these insecticides, which forces the development of new chemicals to combat insecticide resistance. Although, a larger issue stems from insecticide toxicity to humans and surrounding organisms as well as their ability to alter ecosystems. Unfortunately, insecticides remain a popular method of biocontrol that are primarily used in agriculture, particularly for the management of pests that are too difficult to control naturally.

Another biological control agent are bacteria, which is seen as a safer and considerably cheaper alternative of chemical-based pesticides. Bacteria can be found anywhere from the surface of the soil to the floor of the ocean and many species can be reliably grown in laboratory conditions in large amounts (Piggot and Hilbert, 2004). Bacteria from the genus *Bacillus*, is a common biological control agent used against pests. In fact, *Bacillus subtilis* has recently been granted the status of "generally safe" by the USFDA (Piggot and Hilbert, 2004). *Bacillus* is characterized as a gram-positive aerobe, spore-forming soil bacterium. When ingested, *Bacillus* spores bind to the walls of the insect's stomach, triggering starvation (Piggot and Hilbert, 2004). In the case of malaria control, the spores cause fatal cellular alteration when ingested by the larva of Culex and Anopheles mosquitoes (Piggot and Hilbert, 2004). Different strains of *Bacillus* have also been adapted for eradicating larva of Culex, Psorophora, and some Aedes spp mosquitoes. *Bacillus thuringiensis var. tenebrins* has been additionally implemented in the eradication of Colorado Potato Beetle and Elm Leaf Beetle larvae (Piggot and Hilbert, 2004).

Many methods of biocontrol have proven to become ineffective overtime due to target organism adaptability taking place as part of the natural process of evolution. The target organism's adaptability to external factors is a major reason why previous applications of various methods were not effective. The application of only one biocontrol agent at a time can also play a role in these failures, as scientists suggest it is more beneficial to use more than one approach simultaneously (Frodsham et al., 1993). As a result of previously failed attempts of utilizing different forms of biocontrol, the application of viruses as an alternative is in consideration by scientists to control pest populations. Application of viruses is a method of biocontrol that involves the use of modified or existent virus strands being released to infect targeted organisms. Viruses are introduced to targeted organisms through intramuscular injection, aerosol transmissions, or ingestion of contaminated food (Bajer et al., 2019). Findings of research in this field can greatly enhance the potential and future implementation of viruses as biological control agents.

A virus is a type of non-living pathogen that is smaller than a bacterium and is dependent on the living cells of a host. Viruses are also referred to as viral particles or virions. They can enter the bodies of pest organisms through multiple routes, attaching themselves to the outer surfaces of target cells. Next, the viral genome is introduced into the infected cell, effectively forcing it to become a factory for new viruses. In addition to their relevance to medicine and research, viruses are a promising platform for biocontrol efforts. Australia has used viruses as an approach to eradicate the invasive species of *Cyprinus carpio*, the Common carp, and *Oryctolagus cuniculus*, the European wild rabbit (Bajer et al., 2019). The application of viruses will also likely provide safer long-term ecological impacts than commercial chemical insecticides that have become popular in managing lawns and crops. Lastly, future research utilizing recombinant DNA technology may drastically improve the efficiency of viruses as biological control agents. Since virus host interactions are subject to evolution and pest adaptation is inevitable, viral biocontrol must be used in combination with other approaches to ensure lasting results, as well as to be "updated" periodically.

Baculoviruses as a means of Biological Pest Control

Baculoviruses are pathogens that infect and kill insects as well as other arthropods. With high host specificities, these viruses have shown to have no pathogenic effects on humans, plants, mammals, or even non-targeted insects (Hasse et al., 2015). By being exclusively pathogenic to insects, baculoviruses are excellent choices as biological control agents. In fact, they are seen as much safer alternatives to popular synthetic chemical insecticides who have proven to be toxic to non-targeted organisms, including humans. These toxic effects include pollution and increases in cancer, reproductive complications, and neurological disorders (Bale et al., 2008). This has resulted in numerous laws outlawing their use or severely limiting the implication of certain insecticides (Mishra, 1998). Scientists have responded by developing and releasing baculoviruses as biopesticides in pest populations. Baculoviruses in the form of biopesticides have proven to effectively control specific pest populations. Current and future research hopes to improve their efficiency through genetic modification (Afolami and Oladunmoye, 2017).

Baculoviruses belong to the family *Baculoviridae*, the most studied and commonly found group of insect pathogenic viruses (Kamita et al., 2017). These viruses are believed to exist all over the world due to their ability to be spread via wind and rain. In fact, a study in 2011 found that cabbage purchased from five different supermarkets in Washington D.C. were all contaminated with baculoviruses to a large extent (Zang at al., 2006). Baculoviruses are large rod-shaped enveloped viruses containing double-stranded circular DNA genomes housed in nucleocapsids (Ikeda et al., 2015). In the environment, baculoviruses produce protein-based occlusion bodies offering additional protection of the viral genome from detrimental elements such as sunlight and alkaline conditions (Kamita et al., 2017).

The *Baculoviridae* family is currently divided into four genera known as *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* (Ikeda et al., 2015). Collectively, baculoviruses can infect a large range of insects, but targeted insects mainly derive from the orders *Diptera* (flies), *Hymenoptera* (wasps, bees, and ants), and *Lepidoptera* (butterflies and moths). The most commonly studied baculoviruses infect and kill larval stages of caterpillars (Davis, 2019). Primary infection begins when insect larvae ingest food contaminated with occlusion bodies containing occlusion derived viral particles (Ikeda et al., 2015). In biological control, crops are often sprayed with baculovirus biopesticides to complete this process, similar to chemical insecticides (Kamita et al., 2017).

Two forms of virions are produced during the cycle of infection, Budded virions (BV), important for cell to cell spread within the host organism, and occlusion body derived virions (ODV), important for prolonged survival in the environment. Both have identical genetic material as well as the same nucleocapsid and are essential parts of the life cycle of Baculoviruses. Once an occlusion body is ingested by an insect larva, the high alkaline environment of their gut causes the breakdown of the protective protein structure, releasing infectious ODVs. ODVs execute productive infection in midgut epithelial cells, which in turn release BV capable of spreading cell to cell (Clem and Passarelli, 2013). As the infection is spread across the body, more tissues become infected and large number of virions are produced and packaged in occlusion bodies. Within days of first ingestion, infected insects die and liquefy, allowing the dispersal of millions of occlusion bodies to continue the cycle (Clem and Passarelli, 2013).

What makes baculoviruses so sophisticated is they not only infect and kill their hosts, but they do so in ways that maximize their dispersal. For example, baculoviruses are capable of modifying the physiology and behavior of infected larvae. In the case of lepidopteran larvae, known periodically to pause from feeding and go through larval molts, baculoviruses use a viral enzyme to inactivate the major insect molting hormone. This causes infected larvae to grow much larger than normal before succumbing to the virus. The liquefied large larvae can then release upwards of ten million occlusion bodies full of infectious viruses per each milligram of larval tissue (Clem and Passarelli, 2013).

Baculoviruses also use same viral enzyme called UDP-glucosyltransferase (EGT), to induce larval climbing behavior in gypsy moths (Clem and Passarelli, 2013). Gypsy moth larvae typically tend to live in soil, only feeding at night to avoid predation. Infected larvae are compelled to climb to the tops of trees before dying instead, resulting in their liquefied occlusion bodies to rain down on vegetation below (Clem and Passarelli, 2013). By climbing trees, they are also more prone to predation by birds, which would only further enhance the dispersal of baculoviruses. The sophistication of baculovirus dispersal is one of the many benefits to using them to control pest populations.

Every single biological control method has both benefits and shortcomings limiting or extending their implementation. The biggest benefits of baculoviruses center around their high host specificity. Such a specificity allows them to be selected and designed to infect and reduce levels of specific insect pests. More importantly, they have proven to be nonpathogenic to non-targeted organisms, even including beneficial insects like bees and soldier beetles (Davis, 2019). By using safe biopesticides, long term environmental impacts will be improved by reducing risks associated with synthetic chemical insecticides. Another advantage lies in the fact that baculoviruses require an infected host to reproduce. Therefore, baculovirus populations are destined to decline once they have effectively reduced a targeted pest's population.

Not only are there clear benefits to baculoviruses as biological control agents, but also experimental results proving their efficiency. For example, in 2007, a study compared mortality rates of Mottled Willow Moth larvae who were exposed to both a pesticide and a baculovirus (Figure 2).

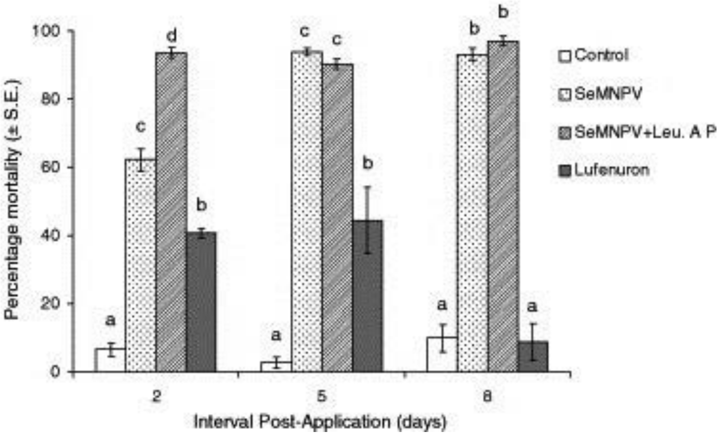

Figure 2. Efficiency of Different Agents of Biological. Mortality of Spodoptera exigua larvae collected from greenhouse pepper crops at 2, 5- and 8-days post-application and reared in the laboratory until death.

The nucleopolyhedrovirus (SeMNPV) was applied alone or mixed with an optical brightener (SeMNPV + Leucophor). A chemical treatment was included for comparison (the insect growth regulator, lufenuron).

Control plants were sprayed with water alone. This figure, Efficiency of Different Agents of Biological Control, was originally published in (Lasa et al., 2007).

In this experiment, control plants were only sprayed with water for comparison's sake. The other three plants were sprayed with either the baculovirus SeMNPV, a chemical insect growth regulator termed Lufenuron, or SeMNPV mixed with optical brightener Leucophor AP, known to increase baculovirus infectivity (Figure 2). Lufenuron is a popular insect pesticide that inhibits the production of chitin in insects. Without chitin, larvae are unable to develop an exoskeleton which causes them to die once hatched or molted via dehydration. Lufenuron has proven to be effective against larval stages of fleas, lepidopterans, and eriophyid mites (Lasa et al., 2007).

Average virus mortality of larvae exposed to control plants varied from 2.7% to 9.9% over the course of eight days (Lasa et al., 2007). This only further proved the natural presence of baculoviruses in surrounding insect environments. Mortality rates of larvae exposed to chemical and virus treatments were much more successful (Figure 1). Larvae exposed to the baculovirus mixed with the optical brightener had the highest mortality rates on days two and eight post-application. Five days post-application, larvae exposed to the baculovirus alone had the highest mortality rates (Figure 1). At the conclusion of this study, both treatments involving baculoviruses resulted in 90% mortality of moth larvae. In contrast, the insect growth regulator Lufenuron, only resulted in less than 45% mortality of larvae when applied at the recommended dose (Lasa et al., 2007). This experiment concluded a baculovirus was far more efficient in controlling larvae than a chemical pesticide, while also potentially shedding light on pests' ability to develop resistance to commercial pesticides.

Despite its many benefits and proven ability to be effective in certain pest populations, baculoviruses are no exception. As much of an advantage the high host specificity of baculoviruses is, this has also proven to be an extreme weakness in the field of agriculture. A common critique of agriculturists is baculoviruses are too expensive of an investment to only control a narrow range of pests (Davis, 2019). If they are going to spend the money, agriculturists would rather purchase a control agent that protects against a variety of insect pests instead.

Other than cost and high host specificity, another critique of baculoviruses deals with their speed of kill. Although producing far fewer negative effects than chemical insecticides, baculoviruses do not provide the immediate results that certain chemical insecticides are capable of. This is an issue in the field of agriculture as it gives infected hosts a small window to continue to feed on crops or harm their production before succumbing to the virus (Davis, 2019). Therefore, the shortcomings of baculoviruses mainly limit their use to high value crops or crops and insects who have become resistant to chemical insecticides.

A new concern regarding baculoviruses has also emerged, one that was not believed to be possible before 2005. During this time, it was first reported that coddling moth larvae in Germany were showing resistance to one of the most efficient baculoviruses, *Cydia pomonella* granulovirus (CpGV) (Gebhardt et al., 2014). Since then, apple plantations in six different countries have also reported this problem. What was once thought to only be a problem regarding chemical insecticides, may now be a developing shortcoming in the field of baculoviruses. Lastly, a final challenge these viruses face is prolonged UV sunlight exposure when occlusion bodies are waiting to be ingested (Afolami and Oladunmoye, 2017). Despite the numerous shortcomings associated with baculoviruses, the work of many scientists suggests these occlusion bodies still represent great potential.

Some of the biggest challenges associated with baculoviruses deal with their speed of kill, host specificity, and potential host development of resistance. Unlike other biological control agents, baculoviruses can be genetically modified to improve these aspects. In fact, scientists have already used genetic engineering to improve the efficiency of these viruses. In 2012, an experiment set out to create a recombinant baculovirus with improved speed of kill. The work of these scientists resulted in a novel recombinant baculovirus, NeuroBactrus, employing a new “three hit” approach which combines the natural killing ability of the virus with the toxicity of two toxins (Shim et al., 2012).

To construct the NeuroBactrus, the *Bacillus thuringiensis* crystal protein gene (termed *cry1-5*) had to be introduced into the *Autographa californica* nucleopolyhedrovirus (AcMNPV) genome (Shim et al., 2012). This was done through fusion of the genetic code of the toxin to the polyhedrin gene, which codes for the major protein found in the occlusion bodies. (Shim et al., 2012). To confirm incorporation of the fusion protein into the produced occlusion body, SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) and immunoblot analyses were performed (Figure 3). These types of analyses allow scientists to detect single proteins (the engineered fusion protein) in rather complex mixtures (protein extract from the entire occlusion body) based on their molecular mass in kilodaltons (kDa) in electrophoresis or their ability to interact with specific antibodies in immunoblot assays.

In figure 3A, a 150-kDa band (labeled with asterisk) absent in wild-type AcMNPV-infected cells was detected in the cell lysate (lane 3) as well as the purified polyhedrin samples (lane 5) from the NeuroBactrus. Further immunoblot analysis confirmed the presence of polyhedrin (Figure 3B, lane 3) and Cry1-5 Bt toxin in the band (Figure 3C, lane 3). In figures 3A and 3B, a 30-kDa band corresponding to the native polyhedrin (labeled with Polh) was also detected in both the infected cell lysate and purified polyhedra. The fusion protein band was later reduced to ~65 kDa after cleavage with trypsin (Figure 3A, lane 7) which corresponds to the size of the activated Cry1-5 Bt toxin, indicating that the active fragment of the Cry1-5 toxin could be released from the fusion protein by proteases (Shim et al., 2012). Therefore, the data suggests the fusion protein of the NeuroBactrus will be active in the gut of host insects (Shim et al., 2012).

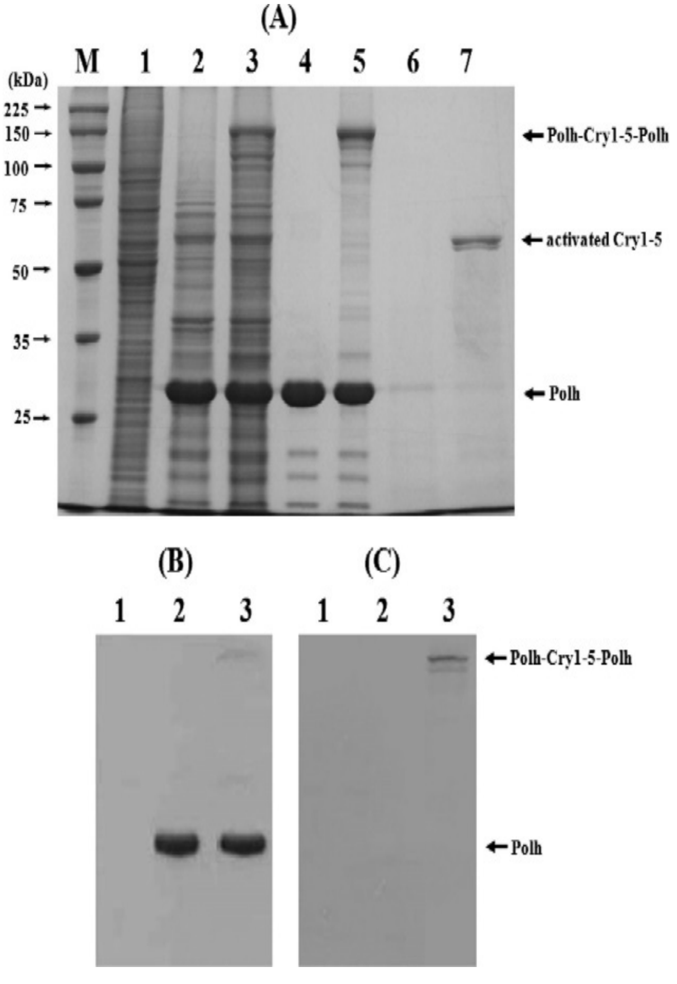

Figure 3. SDS-PAGE and immunoblot analyses of the fusion protein.

(A) Infected Sf9 cells, purified polyhedra, and trypsin-treated polyhedra were electrophoresed in 12%

polyacrylamide gels. (B and C) Immunoblotting was performed against purified polyhedra using anti-polyhedrin (B) or anti-Cry1-5 (C) polyclonal antisera. Lanes: M, protein molecular weight marker; 1, mock-infected Sf9 cells; 2, 4, and 6, wild-type AcMNPV; 3, 5 and 7, NeuroBactrus; 1, 2, and 3, cell lysates; 4 and 5, purified polyhedra; 6 and 7, trypsin treated. This figure, SDS-Page and Immunoblot Analyses of Fusion Protein, was originally published in (Shim et al., 2012).

A similar approach was used to introduce the insect-specific neurotoxin gene, *AaIT*, from *Androctonus australi*, a desert scorpion from North Africa and Middle east (Shim et al., 2012). These experiments portray the great potential baculoviruses hold in the field of biological control. An eco-friendly recombinant baculovirus was amazingly developed and showed signs of faster associated mortality rates. Unlike natural wild types, this NeuroBactrus virus has three modes of action to kill insect hosts. Most pest insects that ingest occlusion bodies will be killed by the first mode of action, which delivers Bt toxins to the midgut of the hosts. Insect larvae that survive *Bacillus thuringiensis* exposure will be controlled by the second mode of action, or insecticidal scorpion neurotoxins that prevent neurotransmissions (Shim et al., 2012). Any infected larvae that survive the action of both toxins should be killed by the Baculovirus infection itself. The described engineered baculovirus eliminates insect larvae with much faster rate due to the action of the two introduced toxins.

In conclusion, baculoviruses are excellent choices as biological control agents. However, like any biological control agent, their shortcomings severely limit their implementation today. Their rates of kill will need to be increased, host specificities will need to branch further out, and the development of host resistance must be prevented at all costs. As we have recently seen, genetic engineering has great potential to resolve the shortcomings of baculoviruses. It is in our best interest to continue to conduct experiments like the one presented in Figure 3. Continued development of these viruses through genetic engineering will likely provide safer long-term ecological impacts. It should also cause public opinion to drift even farther from chemical insecticides, negating the risks associated with such chemicals. If the shortcomings of baculoviruses are not resolved, they will continue to be restricted to high value crops or crops and insects that have become resistant to chemical insecticides.

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Michael Hutchinson graduated from Bridgewater State University in the spring of 2020, earning a Bachelor of Arts degree in Biology. He conducted this research under the mentorship of **Dr. Boriana Marintcheva** (Biology). In addition to pursuing his passion for science, he also was a member of the cross-country, indoor track, and outdoor track teams. He is currently working as an EMT and preparing for a career as a Physician's Assistant.