BIOAg Project Report

Report Type:

Progress

Title:

New Codling Moth Pathogens

Principal Investigator(s) and Cooperator(s):

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Abstract:

Washington organic apple production is at risk from recent codling moth biopesticide resistance development. New tools are needed immediately. Fortunately, during a WTFRC-funded research project, this proposal's PI identified several codling moths collected from commercial orchards that hosted biopathogens. This project's team isolated, cultured, and sequenced three unique fungal pathogens: 1) a *Beauveria bassiana* strain, 2) a *Metarhizium anisopliae* species-specific strain that causes green muscardine disease, and 3) a *Hirsutella*/*Orphiocordyceps* species. We hypothesized that these promising biocontrol agent fungi could be developed into biopesticides and used to manage codling moth in Washington orchards. This project advanced development of biologically intensive, organic, and sustainable practices for Washington pome fruit, stimulated a new research initiative, and advanced game-changing research out into the real world. We began to develop these fungal pathogens into biopesticides that managed codling moth pupating near and in orchard leaf litter and soil.

Project Description:

Nearly 20% of Washington's apple industry (70% of US production, >170,000 acres, valued at >$2 billion annually) is produced organically (NASS, 2023). Codling moth, the primary apple pest in the Pacific Northwest for well over a century (Newcomer and Whitcomb, 1925), has been targeted for control since introduction to North America in the 1750's (Essig, 1931). Larval feeding directly damages fruit, rendering it unfit for sale as a fresh commodity. Poorly managed apple orchards can have substantial losses (Wise and Gut, 2000, 2002) and control with broad-spectrum insecticides has been complicated by the loss of effective compounds through resistance or restrictions (Varela et al., 1993; Knight et al., 1994; Mota-Sanchez et al., 2008).

Recently, insecticide resistance to the main organic control tactic, sprays of granulosis virus, has developed on Washington apple farms (Fan et al., 2023). Control tactics such as pheromone mating disruption (Cardé et al., 1977; Vickers and Rothschild, 1991) are employed with insecticides and biopesticides to manage codling moth on an estimated 243,000 hectares (600,466 acres) of apples, pears, and walnuts worldwide (Gut et al., 2019).

With the development of codling moth resistance to the granulosis virus, organic apple production is at risk in Washington, and new alternatives are needed immediately. Luckily, three prospective fungal pathogens were recently isolated from codling moths collected from Washington farms. This project will work toward developing these pathogens into biopesticides for use in organic apple production. Eventual success will ensure that Washington organic apple production, at least $400 million in value (NASS, 2023), will remain sustainable, and not revert to conventional production. Additionally,

entomopathogens may extend other insecticides' active life by reducing selection pressure (Hatting et al., 2019).

Several species of biological pathogens have been commercialized for the control of a broad range of insect pests. Commercial products containing *Beauveria bassiana* strains target weevils, thrips, spider mites, fall army worm, false codling moth, diamond back moths, whiteflies, stinkbugs, and nematodes (Hatting et al., 2019), while products containing strains of *Metarhizium anisopliae* target locusts, grasshoppers, thrips, weevils, and whiteflies (de Faria and Wraight, 2007; Zimmermann, 2007; Ekesi et al., 2011; Rehner and Kepler, 2017, Hatting et al., 2019). Coombes et al. (2015) developed more virulent strains of *Beauveria* and *Metarhizium* through selections in the laboratory to better manage false codling moth. These studies demonstrate the potential for developing biopesticides from fungal pathogens that target codling moth in Washington apple production.

We are continuing to make progress on our original project objectives: to 1) locate diseased codling moth larvae and adults from Washington apple orchards throughout the state, 2) establish new cultures and sequence prospective pathogens that were isolated from those codling moths, 3) test the infection potential of current and new pathogens on healthy codling moth caterpillars in lab assays and in field studies, and 4) to repeatedly select for infection, pathogenicity, and virulence to increase with each generation.

Outputs:

Overview of Work Completed and in Progress:

Completed Objective 1: 13,423 live and 3,010 dead codling moths were extracted from bands placed in orchards throughout the WA growing regions. Dead codling moths represent potentially pathogen-infected individuals, and may hold many new entomopathogenic fungal strains.

In Progress Objective 2a: Pathogen screening and culturing continues. With 3,010 individuals to screen and culture, this process is more extensive than originally anticipated. We did not expect to obtain this many potentially pathogen-infected individuals when we wrote this project.

Completed Objective 2b: A multiplex tool has been designed and tested to speed up the initial screening process. Testing has demonstrated that it is useful at discerning the different entompathogenic fungi that we found in codling moths (Figure 1).

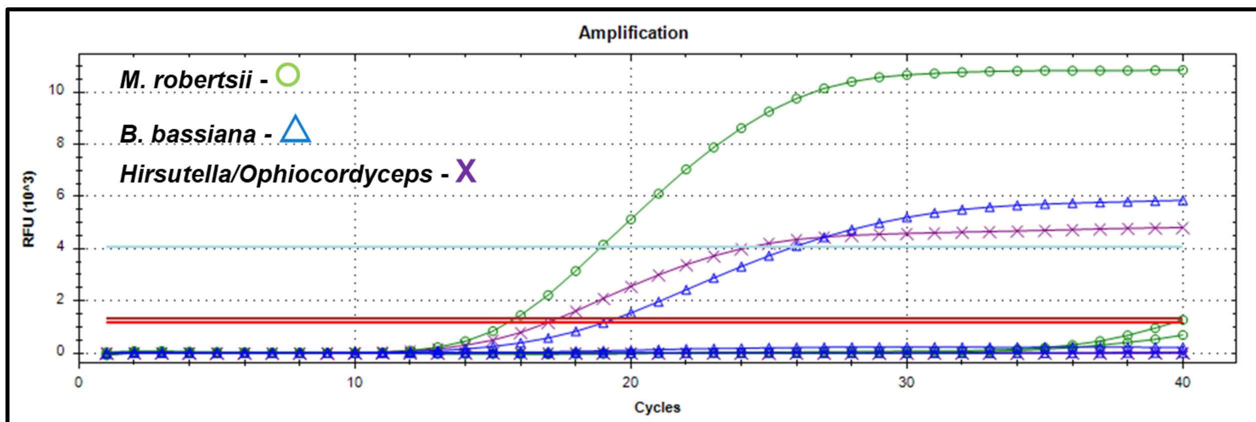

Figure 1. Example of successful amplification of all three targets in the multiplex Real-Time PCR assay. Targets include *Metarhizium robertsii, Beauveria bassiana, and Hirsutella/Ophiocordyceps.* Each probe produced a distinct, non-overlapping fluorescent signal, demonstrating specific and concurrent amplification within the multiplex reaction.

Completed Objective 3: We have completed Koch's postulates and demonstrated that two of the fungi, *Beauveria bassiana* and *Metarhizium robertsii,* are indeed infective to *C. pomonella.*

Completed and in Progress Objective 4: We are currently selecting fungal generation 3 and are observing increases in pathogenicity following genetic selections. In lab assays, *B. bassiana* causes >93% mortality, and *M. robertsii* now causes >96% mortality. Initial assays had significantly lower pathogenicity, demonstrating that our genetic selections are causing rapid evolution in these strains. Although this work will continue into the future, we have completed the objective of performing initial genetic selections.

Methods, Results, and Discussion

Objective 1) Explore – Locate additional diseased codling moth larvae and adults from Washington apple orchards throughout the state

Sample collection

Corrugated cardboard bands (Uline, Pleasant Prairie, WI) were wrapped around trees in commercial apple orchards (i.e., Rock Island, Wenatchee, Sunnyslope, Ephrata, Soap Lake, and Royal City, WA) during August–September 2024 to collect overwintering *C. pomonella* larvae. Bands were retrieved by early October and held in outdoor storage until March 2025, then in cold storage to maintain diapause. Between December 2024 and present, final instar larvae were extracted from bands and separated into live and dead individuals. From this collection, dead larvae displaying visual signs of fungal colonization, i.e., mummification, were selected for pathogen screening. In late September 2025, a subset of specimens were surface-sterilized by sequential rinses in 70% ethanol, deionized H_2O, and 0.5–1% bleach, then air-dried under a sterile vertical laminar flow hood. Dried larvae were placed on 100 x 15 mm Petri dishes (Beckton Dickinson Labware, Franklin Lakes, NJ) containing semi-selective BD Difco™ Sabouraud Dextrose Agar with Yeast (SDAY) medium (Becton, Dickinson Co., Franklin Lakes, NJ) and incubated at 24 °C, 35% RH, 16:8 h light:dark cycle until sporulation occurred (10–15 d).

Objective 1 results

In 2025, we extracted nearly 13,423 live codling moth caterpillars from cardboard bands collected in late 2024. In addition, we collected over 3,000 dead caterpillars, cataloged them, and processed them for storage until screening could begin.

Objective 2) Isolate – Establish new cultures and sequence prospective pathogens that are isolated from those codling moths

Isolation of pure strains

After dead larvae were incubated on SDAY medium, a sterile punch was used to isolate and transfer actively growing mycelia plugs to fresh 100 x 15 mm Petri dishes containing SDAY growing medium. If mycelial growth from single organisms was evident, isolates displaying distinct growth characteristics (e.g., coloration, growth rate, morphological differences) were examined using a compound microscope to characterize spore morphology, shape, and arrangement to provisionally identify them before sequencing and molecular identification confirmed their taxonomic placement. To maintain strains and generate material for future assays, isolates were further propagated as previously described.

Genetic sequencing of initial strains

The DNA from pure fungal isolates obtained from *C. pomonella* larvae was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987, Reyes Corral 2025). The DNA quality and quantity were confirmed by using a NanoDrop™ 2000 spectrophotometer (ThermoFisher, Waltham, MA). The internal transcribed spacer (ITS1, ITS2) region of ribosomal DNA was amplified using universal fungal primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT

TGA TAT GC-3') (Martin and Rygiewicz 2005, Bellemain et al. 2010, Yu et al. 2022). Amplified products were Sanger sequenced (Sanger and Coulson 1975) by Molecular Cloning Laboratories (MCLAB, San Francisco, CA). Sequences were cleaned, aligned, and analyzed in Geneious Prime 2025.0.3 (Dotmatics, Auckland, New Zealand) using Basic Local Alignment Search Tool (BLAST) searches against reference databases to identify matches. Sequences were deposited in GenBank, the NIH genetic sequence database.

Objective 2 results
During 2025, we cultured three groups of pathogens, *B. bassiana*, *M. robertisii*, and *Ophiocordyceps* spp., in the laboratory. We also identified a potential second strain of *B. bassiana* that we now have in culture.

Objective 3) Infect – Test the infection potential of current and new pathogens on healthy codling moth caterpillars in lab assays and in field studies

Reinfection of healthy C. pomonella
To fulfill Koch's postulates and confirm isolated fungal strains' pathogenicity, healthy final-instar *C. pomonella* larvae were exposed individually. For each isolate, spore suspensions were prepared by transferring five mycelium plugs from colonized 100 x 15 mm Petri dishes with SDAY semi-selective medium to 500 mL of sterile 0.1% Tween solution (one plate per 100 mL). The 0.1% Tween solutions were constantly agitated using a magnetic stir bar for spore release. Larvae were immersed in *B. bassiana* (n = 27), *M. robertsii* (n = 27), *Hirsutella*/*Ophiocordyceps* (n = 27), a mixed suspension of all three isolates (RC Mix, n = 29), and 0.1% Tween solution alone (control, n = 27), then transferred to 30 x 10 mm sterile Petri dishes maintained at 24 °C, 50% RH, 16:8 h light:dark. Larval mortality and visible signs of fungal infection were monitored daily for 50 d.

Re-isolation of pure strains
Dead, experimentally infected larvae from each treatment were selected to reisolate entomopathogens. Ten larvae exposed to *B. bassiana*, 6 to *Hirsutella*/*Ophiocordyceps*, 8 to *M. robertsii*, and 10 to RC Mix were processed. Larvae were surface-sterilized and air-dried as previously described, transferred individually to 30 x 15 mm Petri dishes containing SDAY semi-selective medium with penicillin (50 I.U./mL) and streptomycin (50 µg/mL), and incubated under the conditions previously described. After incubation, actively growing mycelium discs were transferred to fresh 30 x 15 mm SDAY + antibiotics plates and incubated under the same conditions for 10 d to obtain pure cultures. Resulting growth was examined microscopically to confirm fungal morphological features, and reisolated strains were used for molecular confirmation.

Genetic sequencing of re-isolated strains
DNA was extracted from 30 pure fungal isolates obtained from larvae used in the reinfection experiment (*B. bassiana* n = 9, *Hirsutella*/*Ophiocordyceps* n = 4, *M. robertsii* n = 8, and RC Mix n = 9) as previously described, and BLAST searches against reference databases to identify matches were conducted. All sequences were deposited in GenBank.

Objective 3 results
Reinfection assays revealed distinct mortality and development patterns across fungal treatments; *B. bassiana* had the highest mortality (Table 1). Visible signs of mycosis and sporulation were evident in all four fungal treatment groups within 10–15 d post-exposure. Overall, *B. bassiana* and the RC mix treatments caused the highest mortality and the greatest reduction in successful adult emergence.

***Table 1**. Mortality of final instar Cydia pomonella larvae that were exposed to three entomopathogenic fungi, a mixture of all three fungi (RC Mix), and a tween control. Rows 2, 3, and 6 correspond to the totals presented in row 1, while rows 4 and 5 collectively represent the values summarized in row 3.*

No.	***Metric***	***Beauveria bassiana***	***Hirsutella/ Ophiocordyceps***	***Metarhizium robertsii***	***RC Mix***	***Tween Control***
1	**No. exposed in assay**	27	27	27	29	27
2	**No. that died pre-pupation**	13	0	3	8	2
3	**No. that pupated**	14	26	24	21	25
4	**No. that died as pupae**	12	5	4	16	0
5	**No. that emerged as adults**	2	21	20	5	20
6	**No. that were unaccounted for**	0	1	0	0	0

From 34 *C. pomonella* specimens that died following fungal exposure, two reisolates were obtained from each individual and examined microscopically. Contamination was rare, occurring in only 3 of 34 specimens. In the *B. bassiana* treatment (n = 10; 9 larvae and 1 pupa), all but one specimen yielded mycelial growth and spore structures consistent with *B. bassiana* originally isolated from field-collected larvae, while a single larva produced *Penicillium* in both re-isolates. Microscopic examination confirmed *Beauveria*-like spore morphology in the remaining samples. In the *Hirsutella*/*Ophiocordyceps* treatment (n = 6; 5 pupae and 1 adult), mycelial growth was slow and visually resembled *B. bassiana*; microscopic features also corresponded to *Beauveria*, with one specimen showing distinct growth suggestive of contamination. Re-isolation of *Hirsutella*/*Ophiocordyceps* was not achieved. In the *M. robertsii* treatment (n = 8; 4 pupae and 4 adults), seven specimens were consistent with *Metarhizium*, while one yielded growth identified as *Aspergillus* (confirmed by spore morphology and Sanger sequencing). In the RC mix treatment (n = 10; 7 larvae and 3 pupae), nine specimens were consistent with *B. bassiana*, while one produced *Penicillium*.

The DNA from 30 pure fungal isolates obtained from the re-infection experiment (*B. bassiana* n = 9, *Hirsutella*/*Ophiocordyceps* n = 4, *M. robertsii* n = 8, and RC mix n = 9) was successfully amplified at the ITS1–ITS2 region and sequenced. The BLAST results revealed consistent identification across most samples. All nine isolates from the *B. bassiana* treatment matched *B. bassiana* (100% pairwise identity, 100% query coverage) (Table 2). Surprisingly, all four isolates from the *Hirsutella*/*Ophiocordyceps* treatment also matched *B. bassiana* with 100% identity and coverage, indicating no recovery of *Hirsutella* by molecular confirmation. Of eight isolates from the *Metarhizium* treatment, seven matched *M. robertsii* (100% identity, 100% coverage), while one isolate was identified as the saprophyte *Aspergillus flavus*, corroborating its distinct morphology. In the RC mix treatment, all nine isolates sequenced matched *B. bassiana* with 100% identity and coverage. These results complete Koch's postulates by demonstrating both pathogenicity and molecular confirmation of the causal agent in the cases of *M. robertsii* and *B. bassiana*.

Objective 4) Select – By repeatedly selecting for infection, pathogenicity and virulence may increase with each generation

Following satisfaction of Koch's postulates, we began selecting genetic lines that are fastest at causing codling moth mortality. By repeatedly infecting and isolating pure pathogen strains from codling moth caterpillars, fungal pathogenicity was increased. The methods used in Obj. 1-3 were repeated, and selected strains were tested for increased pathogenicity. Pathogenicity of each infection generation was recorded to document changes in the virulence of the pathogens. In the future, strains may be selected for other key characteristics, including the ability to be mass-produced, persistence in the field, and field efficacy.

Thus far, we have increased pathogenicity of both *B. bassiana* and *M. robertsii* over 90% in lab assays. These selections show great promise for continuing to develop these pathogens into biopesticides.

Table 2. *Correlation matrix showing genetic similarity among ITS sequences of entomopathogenic fungi isolated from field-collected Cydia pomonella larvae. Each cell represents the percentage of identical bases between sequences, illustrating pairwise similarity across specimens. A two–base pair difference was observed in the consensus sequence of Metarhizium robertsii, attributable to degenerate nucleotides in the original sequence compared to the reinfection sequence (codes M/K).*

Fungi	*B. bassiana*	Bb 7-1 2	*Hirsutella/ Ophiocordyceps*	*M. robertsii*	Ma 2-1 2
B. bassiana		100.0%	79.8%	77.2%	77.4%
Bb 7-1 2	100.0%		79.9%	77.4%	77.6%
Hirsutella/ Ophiocordyceps	79.8%	79.9%		73.8%	73.8%
M. robertsii	77.2%	77.4%	73.8%		99.8%
Ma 2-1 2	77.4%	77.6%	73.8%	99.8%	

Publications, Handouts, Other Text & Web Products:

1) Curtiss, RT; Reyes Corral, C.A.; Leannec-Rialland¸V.; Northfield, T.D. Three Entomopathogenic Fungi Isolated from *Cydia pomonella* (L.) in Washington State. In prep for *Ann. Ent. Soc. Amer.*

Outreach & Education Activities:

1) Yakima Study Codling Moth Circle Dec 2, 2025 - Curtiss
2) Tonasket Codling Moth Study Circle Dec 3, 2025 – Curtiss
3) GS Long Meeting, Yakima, WA Dec 3, 2025 – Northfield
4) WA Hort Show, Wenatchee, WA Dec 8, 2025 – Curtiss
5) WA Hort Show, Wenatchee, WA Dec 9, 2025 – Reyes Corral
6) Orchard Pest and Disease Management annual conference Jan 16, 2026 – Reyes Corral

Impacts:

Short-Term:

Continued development of a potential new management tool for pome fruit farmers, and greater understanding of background infection levels will help farmers prevent losses.

Intermediate-Term:

Farmers will be provided with information on overwintering mortality caused by fungal pathogens in Washington State, and development of a new tool will facilitate more effective organic management.

Long-Term:

Organic production will be stabilized by development of a new tool and will continue to grow in Washington State.

Additional Funding Applied for/Secured:

WSDA Specialty Crop Block Grant - Codling moth pathogens – PI Curtiss – $249,914 2025-2027

Washington Tree Fruit Research Commission - Codling moth pathogens – PI Curtiss –$349,000 2025-2027

Graduate Students Funded:

N/A

Recommendations for Future Research:

New strains of these pathogens will need to be screened for desirable characteristics. In addition, since this project focused on the entomopathogenic fungi, viral or bacterial pathogens were overlooked. Future projects should screen for those taxa and potentially develop them into biological pesticides.

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