# **Project Title:** Understand and mitigate fungicide resistance in *Penicillium* spp.

**Report Type:** Final Project Report

**PI:** Achour Amiri
**Organization:** WSU-TFREC
**Telephone:** 509-293-8752
**Email:** a.amiri@wsu.edu
**Address:** 1100 N Western Ave
**City/State/ZIP:** Wenatchee, WA 98801

**Co-PI (2):** Wayne M. Jurick II
**Organization:** USDA-ARS
**Telephone:** 301-504-6980
**Email:** wayne.jurick@usda.gov
**Address:** Building 002, BARC West
**City/State/ZIP:** Beltsville, MD 20705

**Cooperators:** Prashant Swamy, Jonathan Puglisi, Rice Fruit, PA

**Project Duration:** 3 Years

**Total Project Request for Year 1 Funding:** $97,795
**Total Project Request for Year 2 Funding:** $92,068
**Total Project Request for Year 3 Funding:** $93,730

**Other funding sources: Awarded**
**Amount: $9,643.20**
**Agency Name: State Horticultural Association of Pennsylvania**
**Notes: Awarded to co-PI Jurick II in 2018 entitled "**Evaluating the efficacy of a new postharvest fungicide and developing tools to monitor fungicide resistance in blue mold populations."

**WTFRC Budget:** None

**Budget 1**

**Primary PI:** Achour Amiri
**Organization Name:** Washington State University
**Contract Administrator:** Anastasia (Stacy) Mondy
**Telephone:** 509-335-2587
**Contract Administrator Email Address:** arcgrants@wsu.edu

| Item | 2020 | 2021 | 2022 |
|---|---|---|---|
| **Salaries**[1] | 40,925 | 42,562 | 44,264 |
| **Benefits**[2] | 13,464 | 14,003 | 14,563 |
| **Wages** | | | |
| **Benefits** | | | |
| **Equipment** | | | |
| **Supplies**[3] | 7,000 | 3,000 | 2,400 |
| **Travel**[4] | 885 | 885 | 885 |
| **Miscellaneous** | | | |
| **Plot Fees** | | | |
| **Total** | $62,274 | $60,450 | $62,112 |

[1 & 2] Salaries for a Postdoc at 4872/month for 12 months at 0.7FTE and benefit rate of 32.9%. A 4% annual inflation is included for Year 2 and 3
[3] Supplies for lab work for fungal growth and maintenance, molecular reagent for detection and sequencing
[4] Travel to packinghouses for sampling and collaborative work for 1,500 miles a year at $0.59/mile

**Budget 2:**

**Co-PI (2):** Wayne M. Jurick II
**Organization Name:** USDA-ARS
**Contract Administrator:** Kristy Wallace
**Telephone:** 979-260-9659
**Contract Administrator Email Address:** Kristy.wallace@usda.gov

| Item | 2020 | 2021 | 2022 |
|---|---|---|---|
| **Salaries** | | | |
| **Benefits** | | | |
| **Wages**[1] | $30,118 | $30,118 | $30,118 |
| **Benefits** | | | |
| **Equipment** | | | |
| **Supplies**[2] | $4,900 | $1,000 | $1,000 |
| **Travel**[3] | $500 | $500 | $500 |
| **Plot Fees** | | | |
| **Miscellaneous** | | | |
| **Total** | $35,518 | $31,618 | $31,618 |

[1]Wages will be used to hire a GS-3 level employee to help conduct the research at USDA-ARS.
[2] Supplies for laboratory work including: genomic DNA isolation, library construction and whole genome sequencing, PCR, and media for fungal growth
[3] Travel for sampling packinghouses and collaborative work for 800 miles a year at $0.69/mile

**OBJECTIVES**

1. **Evaluate the pathogenic fitness of resistant populations having different fungicide resistance phenotypes.** Conidial germination assays indicated a fitness penalty to a lesser extent, but the *in vivo* assay demonstrated that the resistant isolates were more aggressive in disease establishment. The objective is being accomplished and projected to be completed in 2022.
2. **Determine the genetic makeup of *Penicillium* species exhibiting various fungicide-resistant phenotypes to postharvest fungicides.** We identified single, double, and triple fungicide resistant *P. expansum* isolates, and single spore cultures were obtained. Each isolate was confirmed to be *P. expansum* by sequencing of three DNA bar code genes. Elucidation of genome sequences and analysis of resistant phenotypes is underway. One genome has been assembled with a fully sensitive phenotype and 3 other *P. expansum* isolates with single and double resistance to TBZ and FLU that have been sequenced. Assembly and annotation of these genomes is in progress.
3. **Assess the efficiency of various approaches to mitigate resistance in *Penicillium* spp.** Due to limited physical resources and access to commercial facilities due to the ongoing pandemic, we could not accomplish annual and two-year fungicide rotation experiments. However, we screened several chemo-sensitizing agents (CSA) to be used in mitigation strategies and identified four CSAs that are being further evaluated.

**Significant findings**

- Fifteen *Penicillium expansum* isolates with resistance to single-, double, or three fungicides showed some fitness penalties in vitro for some parameters but not all.
- *In vivo* trials on Fuji apples showed that fungicide- resistant isolates can outcompete sensitive isolates.
- Three major *Penicillium* species, i.e., *P. solitum*, *P. roqueforti* and P. commune, apart from *P. expansum,* are found to be abundant in the PNW packinghouses. These *Penicillium* species have different sensitivities to the current postharvest fungicides.
- Four chemo-sensitizing agents (CSA) were tested alone or mixed with current postharvest fungicides on detached fruit to control decay caused by resistant isolates. Some efficacy was seen but higher doses of CSA need to be tested in the future.
- Of the 18 isolates from the Mid-Atlantic region, 15 were *P. expansum*, two were *P. solitum* and one isolate was *P. paneum*
- Whole genome sequence data has been obtained for a total of 36 isolates encompassing fully sensitive, single, and double, and triple resistant *P. expansum* isolates. A mutation (E198K) was found to correlate with TBZ resistance in *P. solitum* and was not observed in our samples representing *P. expansum*.
- None of the isolates examined at the genome level contained known mutations in CYP51A1 that correlate with difenoconazole resistance.
- Known mutations in the Mrr1 or MDL1 genes, that correlate with multiple drug resistance phenotypes, were not discovered.
- Eighteen isolates from PNW have an intact patulin gene cluster indicating their potential to produce this harmful toxin. Thirteen out of fifteen *P. expansum* isolates and one *Penicillium paneum* from Mid Atlantic area have an intact patulin cluster.

## Results and discussion

### 1. Fitness evaluations

We have selected 15 *Penicillium expansum* isolates isolated from decayed apples collected from packinghouses in the pacific northwest (PNW). These isolates exhibited sensitivity or single, double, or triple resistance to thiabendazole (TBZ), pyrimethanil (PYR), and fludioxonil (FDL). Isolate fitness was evaluated both *in vitro* and *in vivo*. *In vitro* experiments were carried out to compare the ability of the 15isolates to germinate, grow, and sporulate under different conditions at 35°F and 72°F. As shown in Table 1, most resistant isolates had lower germination than the sensitive isolates om different agar media (IM. PDA, and WA) regardless of the temperature. The mycelial growth of the resistant isolates does not seem to be affected at 72°F and is slightly affected for some isolates at 35°F (Table 1). The ability of resistant isolates to survive under dry conditions (water stress) and oxygen imbalance (oxidative stress) were measured on PDA in vitro and showed that dual and triple resistant may incur fitness penalties compared to sensitive and single-resistant isolates (Table 1) and would not survive under such harsh conditions.

**Table 1.** Change in conidial germination and mycelial growth abilities of single, dual and trip resistant isolates *in vitro* in comparisons with sensitive isolates.

| | | | Change in resistant isolate fitness relative to the sensitive isolates *in vitro* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Germination | | | | | | Mycelial growth | | | | | | | | |
| | | | 72°F | | | 35°F | | | 72°F | | | 35°F | | | Osmotic stress | | Oxidative stress |
| Sensitivit Group | Sensitivity Phenotype | Isolate | IM | PDA | WA | IM | PDA | WA | PDA | AJA | IM | PDA | AJA | IM | 72°F | 35°F | 72°F |
| Single-Resistant | $TBZ^RPYR^SFDL^S$ | *Pe* -1 | 0% | -4% | -22% | -10% | 4% | -15% | 0.2 | 0.4 | 0.2 | -0.5 | -0.1 | -0.2 | 40.98% | -6.25% | 19.05% |
| | | *Pe* -2 | 0% | 0% | -16% | -20% | 9% | -32% | 0.7 | 0.4 | 0.0 | -0.4 | -0.2 | -0.2 | 21.31% | -2.50% | 24.34% |
| | | *Pe* -3 | 0% | 1% | -11% | -20% | -14% | -34% | 0.9 | 0.6 | 0.0 | 0.1 | 0.1 | 0.0 | 32.79% | -27.50% | 37.04% |
| Dual-Resistant | $TBZ^RPYR^RFDL^S$ | *Pe* -4 | 0% | -1% | -5% | -4% | -13% | -23% | 0.1 | 0.1 | 0.3 | -0.5 | 0.0 | 0.0 | 42.62% | 35.00% | -1.59% |
| | | *Pe* -5 | 0% | -1% | -25% | -15% | -13% | -26% | 0.3 | 0.6 | 0.3 | -0.2 | 0.2 | -0.1 | 34.43% | 2.50% | 10.58% |
| | | *Pe* -6 | 0% | 0% | -19% | -16% | -16% | -31% | 0.3 | 0.6 | 0.2 | 0.0 | 0.0 | -0.1 | -77.05% | -10.00% | 17.20% |
| | $TBZ^SPYR^RFDL^R$ | *Pe* -7 | 0% | 0% | -10% | 17% | 42% | -26% | 0.0 | 0.0 | -0.1 | 0.2 | 0.0 | 0.1 | -100.00% | -45.00% | 13.23% |
| | | *Pe* -8 | 0% | -2% | -9% | 7% | 7% | -17% | 0.2 | 0.3 | 0.0 | -0.2 | 0.1 | 0.1 | -65.57% | -57.50% | -40.21% |
| | | *Pe* -9 | 0% | 0% | -19% | 6% | -4% | -36% | 0.1 | 0.3 | 0.0 | 0.2 | 0.2 | 0.0 | 24.59% | 22.50% | -73.28% |
| Triple-Resistant | $TBZ^RPYR^RFDL^R$ | *Pe* -8 | 0% | 0% | -26% | 0% | -5% | -25% | 0.3 | 0.4 | 0.4 | -0.7 | -0.2 | 0.1 | 14.75% | -73.75% | 11.64% |
| | | *Pe* -9 | 0% | -1% | -19% | -10% | 1% | -25% | -0.2 | 0.0 | 0.2 | 0.3 | 0.6 | 0.5 | 8.20% | -80.00% | -56.88% |
| | | *Pe* -10 | 0% | 1% | -17% | -15% | -8% | -25% | 0.4 | 0.6 | 0.2 | 0.6 | 0.6 | 0.3 | -49.18% | -77.50% | -83.86% |

Blue and green colors indicate fitness loss and gain, respectively. Values in each case indicate the change in % (germination) or in cm (for growth) relative to the control.

The virulence of 15 *P. expansum* isolates was assessed *in vivo* on Fuji apples by measuring lesion diameter after 90 days at 35°F. Results show a decreased decay severity in triple and dual-resistant isolates (Figure 1).

Virulence of the same isolates was also tested on Gala, Honeycrisp, Granny Smith, and WA 38. Overall, Gala and Granny Smith were the least susceptible (data not shown).

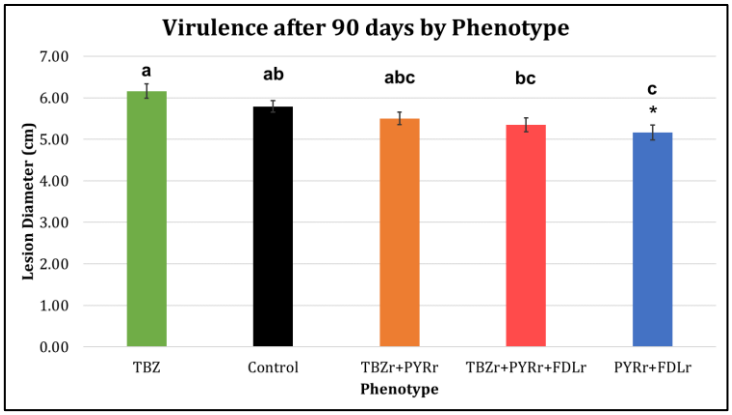


**Figure 1.** Lesion diameters (cm) caused on Fuji apples after 90 days of incubation by sensitive and resistant isolates of *P. expansum*.

**Activity 2.1. The genetic makeup of *Penicillium* isolates**

A total of 644 *Penicillium* isolates that did not exhibit characteristics "*expansum-like"* symptoms when grown on fungal isolation media were characterized using visual distinctions that included the predominant color on PDA, CYA, YES, and MEA after 10 days (colorless, green tint, or orange), the color of the fungal colony (dark green, tan/green, or cream), colony appearance (flat or raised), size of the colony (<2 cm, 2.5-3 cm or >3.5 cm), and the color of the colony on the reverse side of the plate. Based on these criteria, isolates were sequenced using 3 genetic markers for species identification. The DNA sequencing results confirmed that the predominant *Penicillium* species in the PNW was *P. expansum* followed by *P. solitum, P. roqueforti, and P. commune.*

Isolates from these major species were tested for fungicide sensitivity to four postharvest fungicides, i.e., fludioxonil (FDL), pyrimethanil (PYR), thiabendazole (TBZ) and difenoconazole (DIF). The results indicated that a large percentage of major "non-expansum" species developed a high level of tolerance to FDL, TBZ, and PYR but not to DIG.

**Activity 2.2. Elucidate whole genome sequences of *Penicillium* isolates with different fungicide resistance phenotypes**.

We have identified isolates with varying levels of resistance to postharvest fungicides (Tables 2). These isolates were obtained from commercial packinghouses in WA, OR, PA and MD from infected fruit and cull piles. Single spore isolates were obtained, and glycerol stocks were preserved for each isolate. High quality genomic DNA was isolated for each isolate and quantified using gel and spectrophotometric methods. Intact DNA was then used to make libraries for NGS Illumina HiSeq 150bp paired end reads. Twenty-nine isolates have their genomes sequenced, assembled and annotated. Common mutations in B-tub locus have been identified and correlate 100% with resistance phenotypes. We have observed no mutations in the CY51A1 genomes of these 29 isolates, so they should be controlled by postharvest fungicides containing difenoconazole labeled for pome fruit (e.g. Academy). No mutations in common genes (MDL1, Mrr1) were detected as well. Most of these isolates have intact patulin gene cluster and, therefore, are expected to be active producers of patulin.

**Table 2.** Isolates *P. expansum* obtained from commercial storage in the Mid-Atlantic (MD, PA, WV) and Pacific Northwest (WA, OR) regions for their fungicide phenotypes and whole genome sequence analysis.

*: silent mutation, not associated with fungicide resistance

| Region | Isolate # | Phenotype | Total Number of Raw Sequences | GC% Total Reads | Mutation in B-tubulin |
|---|---|---|---|---|---|
| **Mid-Atlantic** | ARS1 | $TBZ^{R}PYR^{S}FDL^{S}$ | 15,864,382 | 47.4 | Yes (E198V, L240F) |
| | ARS2 | $TBZ^{R}PYR^{S}FDL^{S}$ | 15,779,790 | 47.3 | Yes (E198V, L240F) |
| | ARS3 | $TBZ^{S}PYR^{S}FDL^{R}$ | 16,053,534 | 46.7 | No |
| | ARS6 | $TBZ^{S}PYR^{R}FDL^{S}$ | 16,070,506 | 47.1 | No |
| | ARS11 | $TBZ^{R}PYR^{R}FDL^{R}$ | 16,187,888 | 46.8 | Yes (E198A) |
| | ARS15 | $TBZ^{S}PYR^{S}FDL^{S}$ | 17,755,220 | 47.2 | No |
| | ARS16 | $TBZ^{S}PYR^{S}FDL^{S}$ | 16,460,740 | 46.6 | No |
| **PNW** | 219 | $\mathbf{TBZ^{R}}PYR^{S}FDL^{S}$ | 16,053,974 | 47.0 | Yes (E198V, L240F) |
| | 184 | $\mathbf{TBZ^{R}}PYR^{S}FDL^{S}$ | 16,178,330 | 46.9 | Yes (E198V, L240F) |
| | 23 | $\mathbf{TBZ^{R}}PYR^{S}FDL^{S}$ | 16,280,196 | 47.1 | Yes (E198V, L240F) |
| | 2570 | $TBZ^{S}PYR^{S}\mathbf{FDL^{R}}$ | 16,042,060 | 47.2 | No |
| | 2558 | $TBZ^{S}PYR^{S}\mathbf{FDL^{R}}$ | 16,062,764 | 47.7 | No |
| | 2555 | $TBZ^{S}PYR^{S}\mathbf{FDL^{R}}$ | 16,101,260 | 47.2 | No |
| | 2483 | $\mathbf{TBZ^{R}PYR^{R}}FDL^{S}$ | 16,052,898 | 47.1 | Yes (E198V, L240F) |
| | 2311 | $\mathbf{TBZ^{R}PYR^{R}}FDL^{S}$ | 16,301,890 | 47.5 | Yes (E198V, L240F) |
| | 8 | $\mathbf{TBZ^{R}PYR^{R}}FDL^{S}$ | 16,306,660 | 47.0 | Yes (E198V, L240F) |
| | 2501 | $TBZ^{S}\mathbf{PYR^{R}FDL^{R}}$ | 16,037,532 | 47.0 | No |
| | 153 | $TBZ^{S}\mathbf{PYR^{R}FDL^{R}}$ | 15,117,556 | 47.6 | No (G235G, silent) |
| | 2517 | $TBZ^{S}\mathbf{PYR^{R}FDL^{R}}$ | 16,045,202 | 47.6 | No |
| | 164-5-48 | $\mathbf{TBZ^{R}}PYR^{S}\mathbf{FDL^{R}}$ | 16,029,930 | 47.4 | Yes (E198K) |
| | 164-4-39 | $\mathbf{TBZ^{R}}PYR^{S}\mathbf{FDL^{R}}$ | 16,152,548 | 47.1 | Yes (E198K) |
| | 162-5-42 | $\mathbf{TBZ^{R}}PYR^{S}\mathbf{FDL^{R}}$ | 16,000,486 | 47.4 | Yes (E198K, L240F) |
| | 3045 | $\mathbf{TBZ^{R}PYR^{R}FDL^{R}}$ | 16,184,410 | 47.2 | Yes (F167Y), G235G* |
| | 2754 | $\mathbf{TBZ^{R}PYR^{R}FDL^{R}}$ | 15,118,376 | 46.8 | Yes (F167Y), G235G* |
| | 1020 | $\mathbf{TBZ^{R}PYR^{R}FDL^{R}}$ | 16,135,502 | 47.1 | Yes (E198V, L240F) |
| | 1267 | $TBZ^{S}PYR^{S}FDL^{S}$ | 16,203,278 | 47.3 | No |
| | 40 | $TBZ^{S}PYR^{S}FDL^{S}$ | 16,039,432 | 46.9 | No |
| | 3339 | $TBZ^{S}PYR^{S}FDL^{S}$ | 16,024,584 | 47.1 | No |

**Activity 3.2. Chemo-sensitizing approaches to mitigate fungicide resistance in *Penicillium* spp.**

Of the eight chemo-sensitizing agents **(**CSA) tested initially *in vitro*, the four most effective ones, i.e., cinnamaldehyde, carvacrol, octyl gallate, and thymol, were tested solo or in tank-mix with FDL, PYR, or TBZ on detached Fuji apples inoculated with spore suspensions of four P. expansum isolates with different sensitivity phenotypes to FDL, PYR, and TBZ. Cinnamaldehyde, carvacrol, octyl gallate, and thymol applied solo at 100, 500, 100, and 500 ppm, respectively, showed little efficacy against the 3 isolates after 4 months of storage at 35°F except for oxyl-galate which showed some reduction of the triple-resistant isolate Pe1020 (Table 3). Oxyl-galate and cinnamaldehyde significantly reduced blue mold incidence of the TBZ-resistant isolate P23, whereas all CSAs tank-mixed with pyrimethanil reduced blue mold incidence of the triple-resistant Pe1020. Fludioxonil alone or in tank-mixes was fully effective. This trial indicates some potential for the CSAs to reduce incidence of resistant populations but additional tests including different doses and additional *Penicillium* and *Botrytis* isolates will be needed.

**Table 3.** Blue mold incidence on Fuji apples treated with different fungicides and chemo-sensitizing agents and inoculated with different *Penicillium expansum* isolates after 4 months of storage at 35°F.

| | Isolate | | | |
|---|---|---|---|---|
| | Pe1267 | Pe23 | Pe08 | Pe1020 |
| Treatment\ Phenotype | TBZ PYR FDL | TBZ* PYR FDL | TBZ PYR* FDL | TBZ* PYR* FDL* |
| **Control** | **100.0 a** | **100 a** | **100 a** | **100** a |
| TBZ | 16.7 bc | 91.7 ab | 91.7 a | 91.7 a |
| PYR | 0.0 c | 0 c | 100 a | 16.7 cd |
| FDL | 0.0 c | 0 c | 0 b | 0 d |
| Thymol | 100.0 a | 91.6 ab | 100 a | 100 a |
| Carvacrol | 83.3 a | 100 a | 83.3 a | 100 a |
| Octyl gallate | 83.3 a | 91.7 ab | 100 a | 66.7 ab |
| Cinnamaldehyde | 100.0 a | 91.7 ab | 100 a | 100 a |
| TBZ+ Thymol | 33.3 b | 100 a | 100 a | 100 a |
| TBZ+ Carvacrol | 0.0 c | 75 ab | 91.7 a | 83.3 ab |
| TBZ+ Octyl gallate | 0.0 c | 16.7 c | 83.3 a | 66.7 ab |
| TBZ+ Cinnamaldehyde | 0.0 c | 66.7 b | 100 a | 83.3 ab |
| PYR + Thymol | 0.0 c | 0 c | 100 a | 0 d |
| PYR + Carvacrol | 0.0 c | 0 c | 100 a | 0 d |
| PYR + Octyl gallate | 0.0 c | 0 c | 100 a | 0 d |
| PYR + Cinnamaldehyde | 0.0 c | 0 c | 91.7 a | 50 bc |
| FDL + Thymol | 0.0 c | 0 c | 0 b | 0 d |
| FDL + Carvacrol | 0.0 c | 0 c | 0 b | 0 d |
| FDL + Octyl gallate | 0.0 c | 0 c | 0 b | 0 d |
| FDL + Cinnamaldehyde | 0.0 c | 0 c | 0 b | 0 d |

Asterisks next to each fungicide indicate that the isolate is resistant to it. Values within the same column followed by different letters are significantly different.

**Executive summary**

**Project Title:** Understand and mitigate fungicide resistance in *Penicillium* spp.

**Key words:** Blue mold, new species, non-expansum, genome, chemo-sensitizers.

**Abstract:**

Blue mold of apples is a major threat to apples in storage. In this three years project, we conducted a risk assessment study to assess whether populations of the blue mold fungus *Penicillium expansum* that acquired resistance to one, two, or three postharvest fungicides could cause a greater or lower risk to the packers. We have shown that the resistant populations may endure some fitness penalty which does not seem to prevent them from being as virulent as the sensitive populations on detached fruit. This warrants the implementation of adequate resistant mitigation approaches to reduce risks of control failure. We also investigated whether other *Penicillium* species other than P. expansum can cause a greater risk for fruit packers. We have identified 13 different *Penicillium* species, of which three species are widespread, that can cause blue mold on apples. Most isolates from the 13 *Penicillium* species showed high *in vitro* tolerance to the three fungicides most commonly applied to fruit at harvest. Preliminary detached fruit data indicate that these *Penicillium* species are less virulent than *P. expansum* after two months in storage on apples treated with thiabendazole, pyrimethanil or fludioxonil. Whether their virulence increases after 9 to 12 months is being studied. We used whole genome sequencing to obtain full sequences of 36 *P. expansum* isolates from the west and east coasts and their genomes are being annotated. The knowledge will serve to develop molecular tools for detection of resistant populations in the future. In an effort to help mitigate resistant populations of *P. expansum* to thiabendazole, pyrimethanil and fludioxonil, we have tested eight chemo-sensitizing agents (CSAs) *in vitro* of which four were tested on apple fruit. Results indicate that tank-mixing the three fungicides to which resistance is observed with some of the CSAs could potentially enhance their control although additional studies are warranted to optime dosage of the CSAs.