**FINAL PROJECT REPORT**
**WTFRC Project Number: CP-17-100**

**Project Title:** Rapid lab and field detection of two major apple quarantine pathogens


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**Cooperators:** Stemilt Growers, Borton Fruit

**Total Project Request:** **Year 1:** $35,211 **Year 2:** $15,429 **Year 3:** $3,800

**Other funding sources** None

**WTFRC Collaborative Expenses:** None

**Budget 1 (Achour Amiri)**
**Organization Name: WSU** **Contract Administrator:** Katy Roberts/Shelli Tompkins
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| Item | 2017 | 2018 | 2019 | 2020 |
|---|---|---|---|---|
| **Salaries**[1] | 12,635 | 9,371 | 0 | 0 |
| **Benefits** | 4,449 | 3,358 | 0 | 0 |
| **Wages** | 0 | 0 | 0 | 0 |
| **Benefits** | 0 | 0 | 0 | 0 |
| **Equipment**[2] | 13,735 | 0 | 0 | 0 |
| **Supplies**[3] | 4,000 | 2,000 | 3,000 | 0 |
| **Travel**[4] | 392 | 700 | 800 | 0 |
| **Miscellaneous** | 0 | 0 | 0 | 0 |
| **Plot Fees** | 0 | 0 | 0 | 0 |
| **Total** | 35,211 | 15,429 | 3,800 | 0 |

**Footnotes:**
[1] Salaries are Rachel Bomberger (30% FTE in 2017 and 10% FTE in 2017) and for 6 months 20% FTE for Research Intern (Amiri lab) for 2018.
[2] Equipment will include costs for portable Genie II instrument to be used in and outside lab environments.
[3] Supplies include reagents for LAMP assay optimization and field use.
[4] Travel to Wenatchee for Rachel Bomberger and for Amiri lab to field and packinghouse samplings and testing.

**OBJECTIVES**:

1. Laboratory development and optimization of the LAMP assay to:

   -Identify and distinguish the different species causing in the bull's eye rot complex, including *Neofabraea perennans*, *N. malicorticis*, *N. alba* and *N. kienholzii*.
   ***Deviation from the original objective:*** The initial objective was to develop primers for all the four *Neofabraea* species, however, for the sake of time and funds, we focused on the *N. perennans*, which is the most important and frequent species causing bull's eye rot in Central Washington. Future work, beyond this project, will focus on developing the LAMP assay for the three other species and the findings will be provided to the industry once available.

   - Identify the causal agent of speck rot (*Phacidiopycnis washingtonensis*).

2. Evaluate the sensitivity and reliability of the LAMP assay for early detection of Neofabraea and *Phacidiopycnis washingtonensis* on artificially inoculated (Sunrise) and naturally infected (commercial orchards) fruit before and after harvest.

**SIGNIFICANT FINDINGS:**

**Objective 1.**

- Six primer sets were developed for the detection of *Neofabraea perennans*.
- One primer was specific to *N. perennans* only and did not react to other species.
- The LAMP assay was very sensitive as it detected as low as 1 pg of fungal DNA.
- A Gennie II portable instrument (Figure 2) which is battery-powered has been acquired and was optimized to use without DNA extraction.
- One set of primers was developed for *Phacidiopycnis washingtonensis.*

**Objective 2**.

- The LAMP assay was successfully used to detect *N. perennans* on Golden Delicious apples inoculated with *N. perennans* at the Sunrise orchard and in storage for up to 90 days.
- The LAMP assay was able to detect as low as 1,000 spores/ml of *N. perennans* on Golden Delicious fruit.
- Commercial fruit were sampled 90, 60, 30 and 0 days preharvest in 2018 from one conventional and one organic commercial orchard (cv. Pinata) in Quincy. Fruit, from the same orchards, were sampled after 30 and 90 days of storage in RA at 34°F.
- The LAMP assay used the portable device Genie II detected *Neofabraea* inoculum on fruit from both commercial orchards at low (10%) frequency 90 days preharvest.
- The frequency of samples positive to *N. perennans* increased though the growing and storage season to reach about 65% after 90 days of storage.

LAMP identification using purified DNA of on fruit will be assessed using the portable device Genie II instrument (Figure 1). The LAMP portable device battery-powered heat consisting of a thermocycler necessary for the LAMP assay and a screen that show real-time amplification of positive DNA if present in the sample. At each detection a positive sample consisting of DNA of N. perennans will be used as a check-up.


**Figure 1.** Portable Genie II instrument, battery-enabled aquired by Pathology lab at WSU-TFREC to be used LAMP detection of pathogens without prior DNA extraction.

## RESULTS AND DISCUSSION

### Objective 1: Primer design and LAMP assay optimization for *N. perennans*

<u>*Specificity of LAMP primers to detect N. perennans only*</u>: Initial reactions using template DNA at 100 ng/µl provided negative reactions for all fungal species tested except for *N. perennans* and one *N. kienholzii* isolate (Nk-4480) (Figure 2-left). Although the primer designed to detected *N. perennans* only continued to amplify *N. kienholzii*-4480 in further reactions, the amplification was 6 times lower and significantly delayed (15 min) after that of *N. perennans* (Figure 2, right).

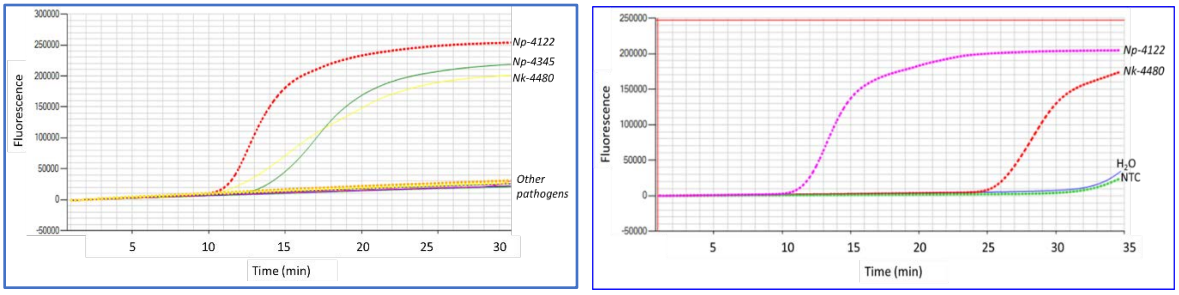



**Figure 2.** Initial specificity LAMP assessment using the portable LAMP Genie® II instrument to amplify *N. perennans* and other pathogens (left) and in comparison, with *N. kienholzii* (Nk.4480) (right). All reactions were run at 65°C and a DNA concentration of 1 ng/µl for each pathogen. Np and Nk indicate *N. perennans* and *N. kienholzii*, respectively. $H_2O$ and NTC are negative controls without DNA.

<u>*Sensitivity of LAMP to detect different DNA concentrations of N. perennans*</u>: A 30 min reaction in a traditional thermocycler followed by gel electrophoresis analysis showed that DNA concentration as low as 0.1 ng/µl of DNA could be detected (Fig. 2A). LAMP reactions conducted in the portable Genie® II instrument using a fluorescent dye was able to detect *N. perennans* DNA concentrations as low as 0.01 ng/µl within 17 min and 0.001 ng/µl after 45 min (Fig. 2B). When the DNA concentrations were higher than 0.1 ng/µl, amplifications occurred between 9 and 15 min after the reaction start.

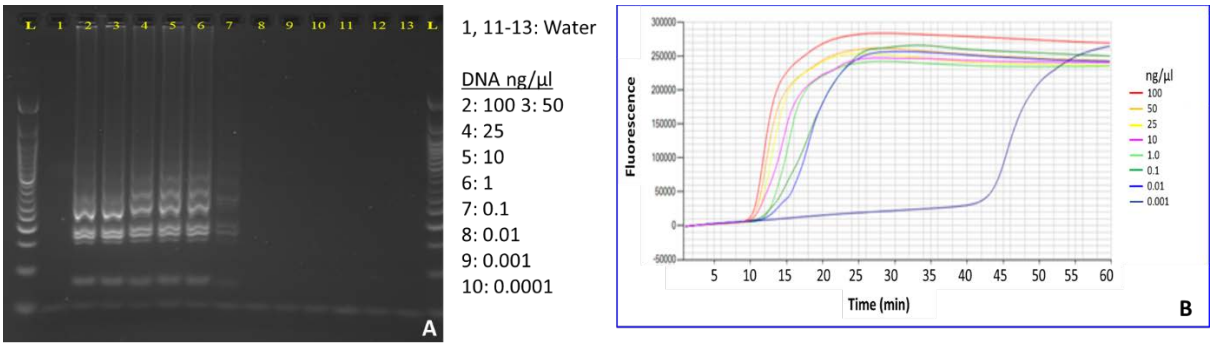



**Figure 3.** (**A**) Image of 1% agarose gel demonstrating the sensitivity of LAMP assay using the primer set 22 at 65°C to detect different DNA concentrations of *N. perennans*. (**B**) LAMP sensitivity in Genie® II portable instrument at 65°C for detection of *N. perennans* at different DNA concentrations between 100 and 0.001 ng/µl. Negative control reactions are not shown as they were included with reactions with DNA concentrations from 1.0 to 0.0001 ng/µl.

**Objective 2. Detection of *Neofabraea perennans* on fruit using LAMP**

<u>*Detection of Neofabraea perennans in artificially-infected fruit.*</u> Apple trees of Golden Delicious, a highly susceptible cultivar to *Neofabraea* spp., were inoculated with spore suspensions of *N. perennans* at concentrations of 0, 100, 1000, and 10,000 spore/ml. Only 5 to 20% of samples were positive to *N. perennans* in non-inoculated fruit or those inoculated with a spore suspension at 100 spores/ml regardless of the inoculation or sampling time (Figure 4). On apples inoculated at 1,000 spores/ml, LAMP detected *N. perennans* in samples collected 0 DPI as well as those sampled during storage, whereas *N. perennans* was detected at all sampling times on almost all fruit inoculated with a spore suspension at 10,000 spores/ml (Figure 4).

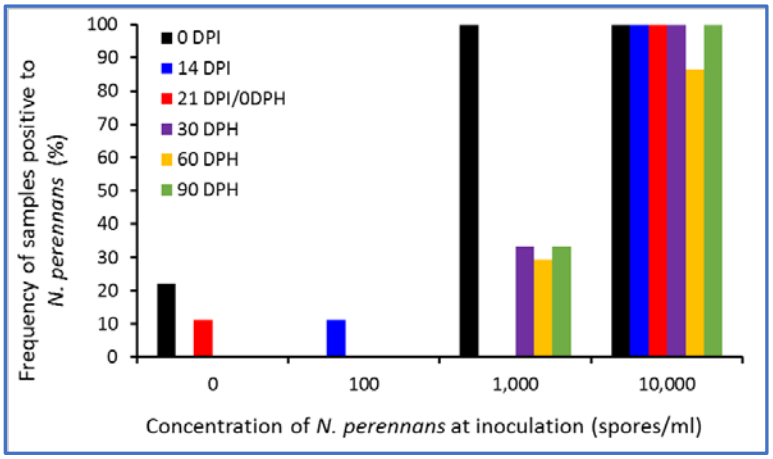
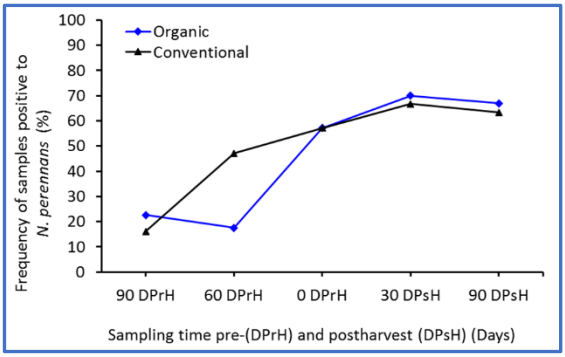



**Figure 4.** Mean frequency (%) of Golden Delicious samples positive to *N. perennans* detected by LAMP in Genie® II from fruit inoculated with different spore concentrations of the pathogen. DPI and DPH indicate day post-inoculation and day postharvest, respectively.

*Detection of Neofabraea perennans on fruit from commercial orchards: naturally-infected fruit*. The portable LAMP assay was used to detect *N. perennans* in asymptomatic Piñata apples from commercial conventional and organic orchards in Quincy. Natural infections of *N. perennans* were detected in samples collected 90 days preharvest in both organic and conventional orchards and the frequency of fruit carrying *N. perennans* increased during the growing season and reached the maximum values of 66 and 70% in conventional and organic orchards, respectively, 30 days postharvest and remained steady for up to 90 days postharvest (Figure 5, on right).





## ACKNOWLEDGMENTS

We thank the WTRC for funding this crucial project. We also thank the participating growers and packers for allowing us to use access their fields and rooms to conduct the work planned.


## OTHER OUTCOMES

### Manuscripts and Abstracts

1. Enicks D.A., Bomberger R.A., Amiri A. 2020. Development of a portable lamp assay for detection of *Neofabraea perennans* in commercial apple fruit. *Plant Disease* **104**:2346-2353.

2. Enicks D.A., Amiri A. 2019. LAMP detection of *Neofabraea perennans*, the causal agent of Bull's eye rot of pome fruit. *Phytopathology* **109**:11-S3.9.
3. WSU-developed field test could detect fruit-rotting diseases months before harvest. *CAHNRS News*. Dec. 2020
4. An early peek at pathogens. *Good Fruit Grower*. Oct. 2020

### Talks

1. Amiri A., Enicks D. Development of a LAMP portable assay to detect two quarantined pathogens of apple fruit. *Annual Phytopathological Society-Pacific Division meeting, Fort Collins, CO*, June, 2019.
2. Enicks D., Amiri A. Development of a LAMP portable assay to detect two quarantined pathogens of apple fruit. *WA Tree Fruit Association Annual meeting*, Yakima, December 4th, 2018.

## EXECUTIVE SUMMARY

**Project title:** Rapid lab and field detection of two major apple quarantine pathogens

**Key words:** Bull's eye rot, speck rot, early field detection, DNA, quarantine

**Abstrac**t: Most pathogens that cause decay in storage start fruit infections in the field during the growing season. Decay symptoms only develop after several months in cold storage and early detections of latent (dormant) infections is critical for decay management pre and postharvest and for fruit shipping in case of quarantine pathogens such as Neofabraea and Phacidiopycnis which cause bull's eye rot and speck rot, respectively. Herein, we developed a sensitive DNA-based assay to detect these two pathogens before decay symptoms become visible. This assay can provide an accurate diagnostic in less than 30 min and can be used by stakeholders at their facilities given that they have a received a minimum training. We were able to detect the bull's eye rot pathogen and the speck rot pathogen 3 and 2 months before harvest, respectively, in commercial apples which will be very useful to understand the epidemiology of the diseases and make timely sprays to reduce infections well before fruit are harvested and stored. We were also able detect the pathogens at harvest and follow their dynamics for up to 3 months in storage. We hope this will help packers decide about the best storage conditions and time based on infection levels to avoid consequential fruit loss in storage. We also hope this assay will ease restriction on fruit shipping about detecting quarantine pathogens as this assay can be used by packer of phytosanitary staff at ports of entry of exports to make final decision and avoid extra costs for shipped in case fruit are highly infected with latent pathogens. We plan to organize a workshop funded by the WTFRC in 2021 to provide a training to interested stakeholders and will continue top provide trainings in the future to interested growers or packers. We plan to extend this assay to detect the other major pathogens to improve overall decay management. Ultimately, we will develop a video to help interested stakeholder carry-out the assay independently or with minimum supervision.