# 2025 Apple Horticulture and Postharvest Research Review

Freshly Harvested WA64 in Quincy, Washington

Photo Source: David Gonzalez

## January 30, 2025

## Hybrid Format
## Wenatchee, WA

# **Project/Proposal Title:** Measuring storage reserves to assess severity of biennial bearing


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**Cooperators**:


**Project Duration:** 1 Year

**Total Project Request for Year 1 Funding:** $ 58,927
**Other related/associated funding sources:** None

**Budget 1**
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| Item | 2023 |
|---|---|
| **Salaries**[1] | $29,315 |
| **Benefits**[2] | $13,062 |
| **Wages**[3] | $6,500 |
| **Benefits**[4] | $650 |

| | |
|---|---|
| **Equipment** | $0 |
| **Supplies[5]** | $8,900 |
| **Travel[6]** | $500 |
| **Miscellaneous[7]** | $0 |
| **Plot Fees** | $0 |
| **Total** | $58,927 |

**Footnotes:**

**[1] Salary is requested for 16.7% of a post-doc and 50% of a research intern (technician).**

**[2] Benefits are calculated at 39.9% for the post-doc and 46.5% for the research intern (technician).**

**[3] Wages are for covering summer salary for the graduate assistant**

**[4] Benefits are calculated at 10% for summer graduate students**

**[5] Supplies are for field and lab consumables to conduct applied experiments for objective 1 and 2.**

**[6] Travel funds are requested for frequent travel to the Sunrise research orchard for PIs and personnel.**

## OBJECTIVES

1. To establish baseline levels of carbohydrate concentrations in storage organs of apple trees that are in different biennial bearing cycles.

2. To establish cost-effective and industry-adoptable methods for measuring non-structural carbohydrates in apple trees.

## SIGNIFICANT FINDINGS:

Based on data from 2022 and 2023 dormant sampling, carbohydrate levels were higher in high cropping trees across different plant tissues for 'Honeycrisp', while 'Gala' showed little to no differences at low and high crop loads. Nitrogen levels had a more inconsistent response, showing greater variation from year to year.

- 2022 had better relationships between crop load and carbohydrate and nitrogen content than 2023, likely because of leaves freezing on the trees and poor translocation of reserves to stored tissues.
- Crop load affects end-of-year carbohydrate stored in woody tissues but with an unexpected positive relationship in some cases. We are continuing to explore these relationships further.
- Nitrogen can be related to crop load but appeared to be dependent on year (2022 vs. 2023)
- There are simple methods of assessing soluble sugars in woody tissues but not starch. We will continue to evaluate simpler methods of assessment during the winter of 2025 like visual iodine staining of different tree parts with low and high crop loads from the previous year.
- We are testing agronomic practices that improve the stability of return bloom in Honeycrisp apple that are based on this data

## RESULTS AND DISCUSSION:

In 2022, carbohydrate levels of 'Honeycrisp' apple trees were significantly higher in high-cropped trees for all tissue types that were sampled. Stored carbohydrates accounted for 9-12% of total dry weight of different woody tissues. These values align with those reported for other perennial horticultural species (Fernandez et al. 2018). There were no differences in stored carbohydrates in tissues in 2023 (Figure 2) compared to 2022 (Figure 1). 'Honeycrisp' apple trees in 2023 showed no significant difference in carbohydrate levels among different bud types, although slightly higher carbohydrate levels in high-cropping trees were observed (Figure 2). Carbohydrate levels of 'Gala' apple trees in 2023 did not show significant differences at different crop loads (Figure 3). However, spurs and terminal shoots had lower carbohydrate levels in high cropping trees.

Nitrogen levels of 'Honeycrisp' apple trees in 2022 displayed an inverse relationship to carbohydrate levels. Nitrogen levels were significantly lower for different bud types in high cropping trees (Figure 4). Nitrogen content ranged from 0.8 to 1.2% of total dry weight for dormant above-ground woody tissues. There were no differences in stored nitrogen content in 2023 compared to 2022. Gala had slightly lower nitrogen content in stored tissues compared to Honeycrisp (Figures 5 and 6). Nitrogen levels of 'Honeycrisp' apple trees in 2023 were not

significantly higher for different bud types, although non-significant differences were observed (Figures 4-6). Higher nitrogen levels were observed among different bud types of high cropping 'Honeycrisp' trees. Nitrogen levels of 'Gala' apple trees with high crops were lower in spurs, apical buds, and bourse shoots (Figure 6). Conversely, terminal shoots had higher nitrogen levels in high-cropping trees.

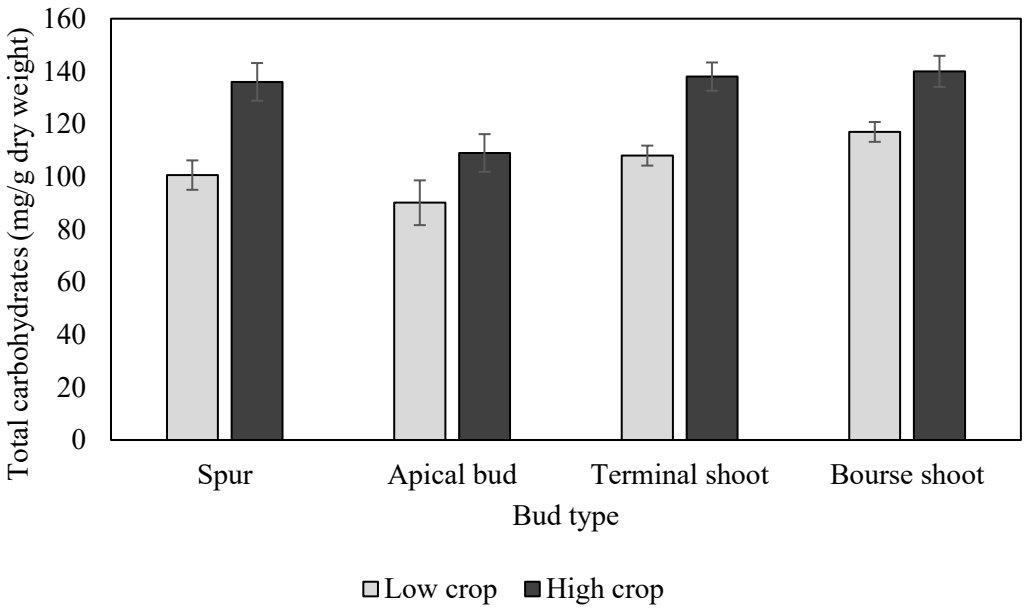


Figure 1. Total soluble carbohydrate (TSC) content (soluble sugars and starch combined) of spurs, apical buds, terminal shoots, and bourse shoots for 'Honeycrisp' apple trees in 2022.

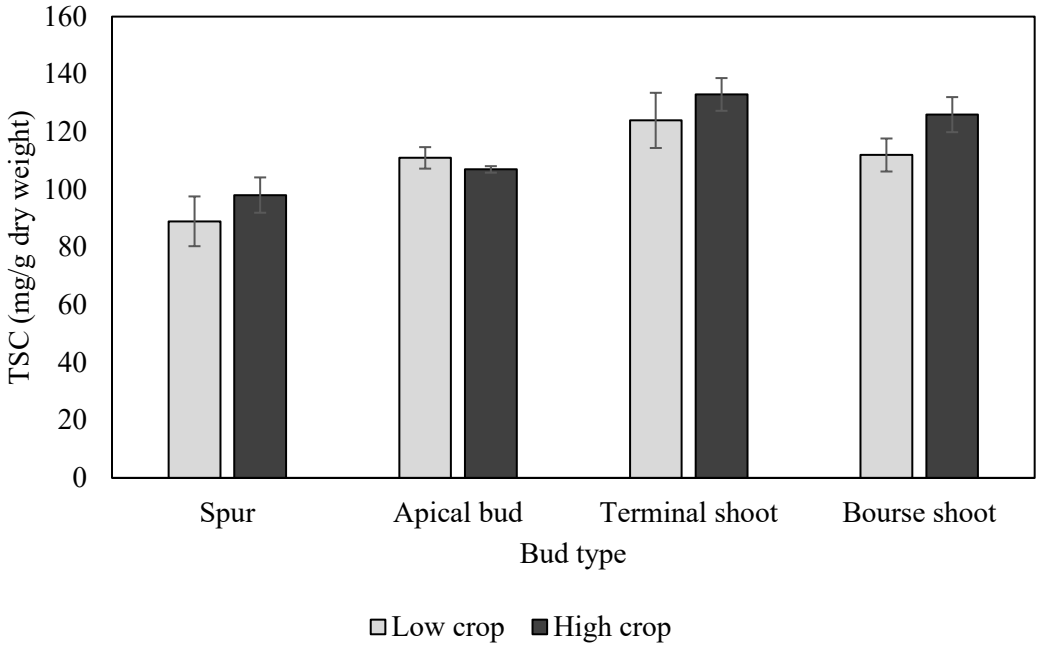

Figure 2. Total soluble carbohydrate (TSC) content (soluble sugars and starch combined) of spurs, apical buds, terminal shoots, and bourse shoots for ‘Honeycrisp’ apple trees in 2023.

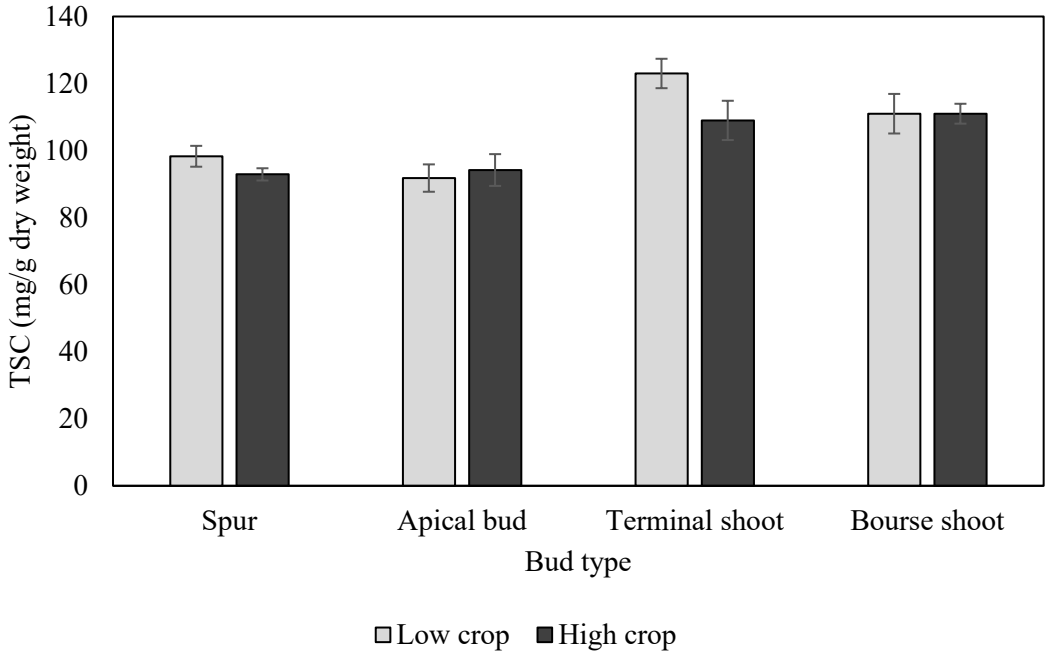


Figure 3. Total soluble carbohydrate (TSC) content (soluble sugars and starch combined) of spurs, apical buds, terminal shoots, and bourse shoots for ‘Gala’ apple trees in 2023.

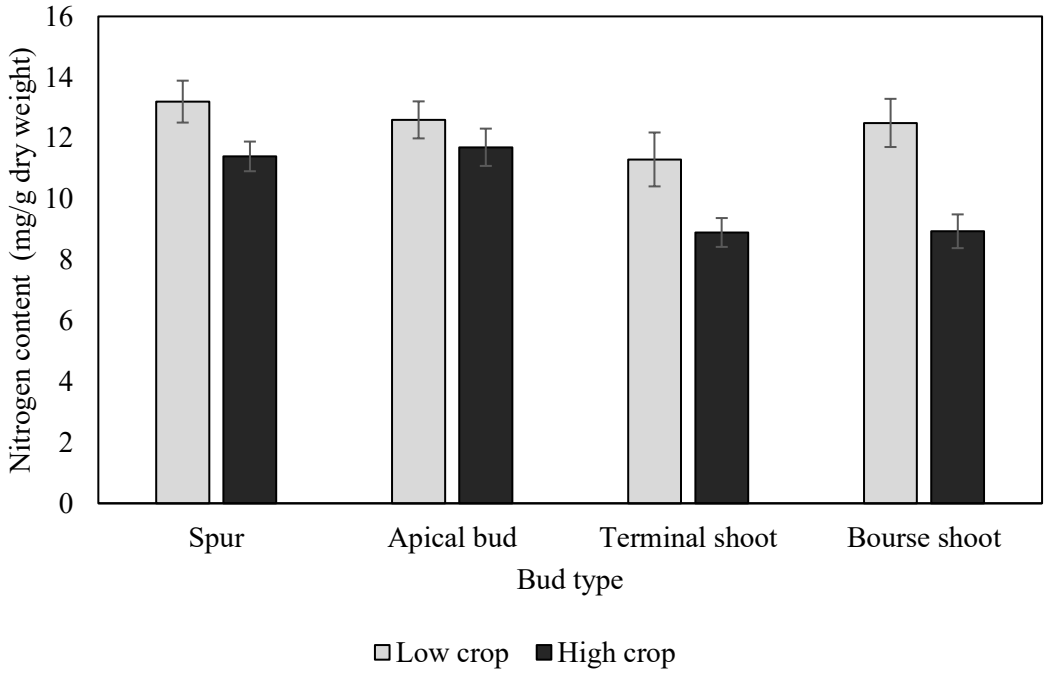

Figure 4. Nitrogen levels of ‘Honeycrisp’ apple trees in 2022 by bud type (spur, apical bud, terminal shoot, bourse shoot).

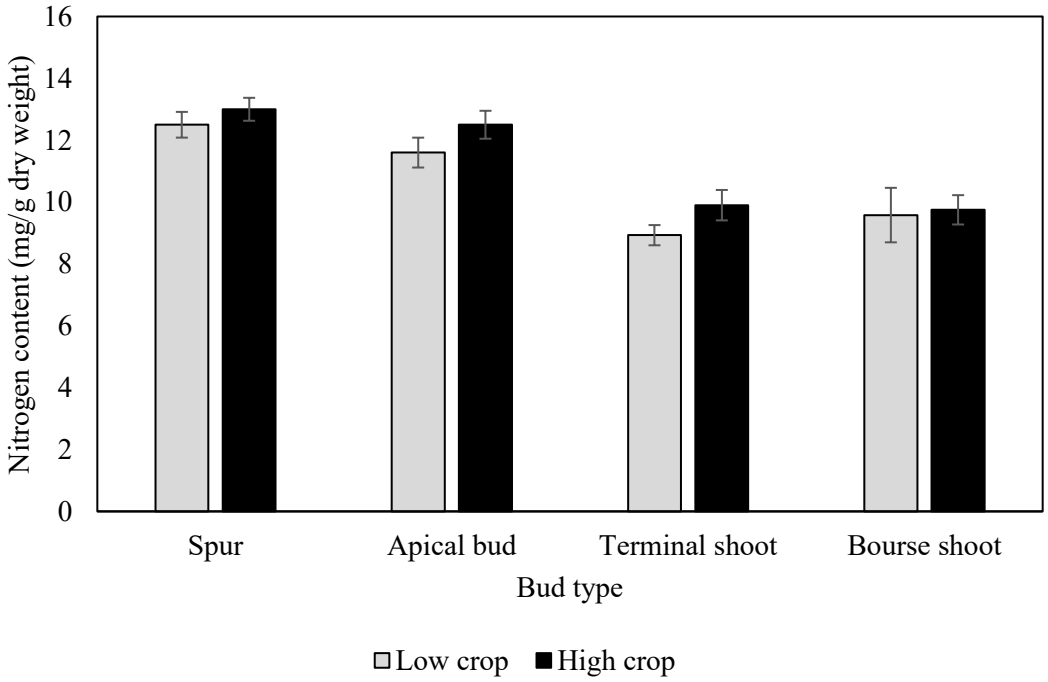


Figure 5. Nitrogen content of ‘Honeycrisp’ apple trees in 2023 by bud type (spur, apical bud, terminal shoot, bourse shoot).

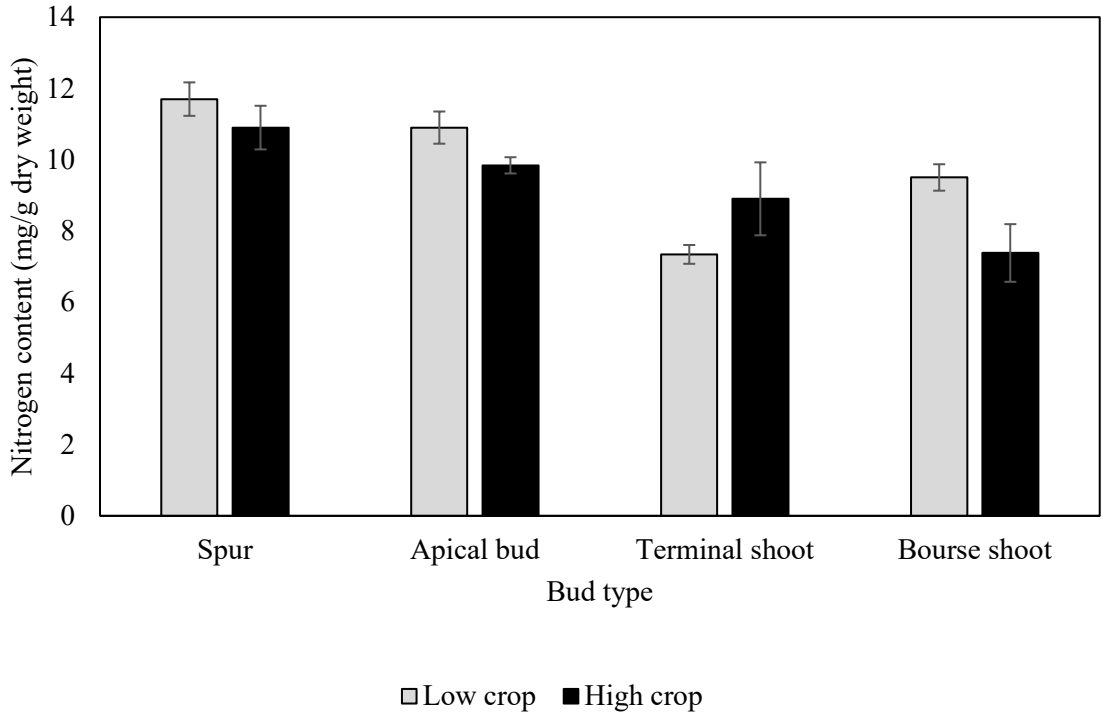

Figure 6. Nitrogen levels of ‘Gala’ apple trees in 2023 by bud type (spur, apical bud, terminal shoot, bourse shoot).

Crop load was positively related to stored carbohydrate levels in plant tissues at the beginning of dormancy. In 2022, effects were significant across different plant tissues. This relationship was less clear in 2023, although trees with higher crop loads still had higher stored carbohydrate levels than lower crop loads. Effects were inconsistent from year-to-year, highlighting the complex factors affecting stored carbohydrate content in apple trees. This is contrary to previous publications, where low-cropped trees were found to have higher carbohydrate levels in plant tissues. Higher starch concentrations have also been reported in flower buds when crop loads were low, supporting a negative correlation with tree crop load (Goldschmidt and Golomb, 1982).

Nitrogen was also measured from the dormant plant tissues that were collected in 2022 and 2023. Trees with low crop loads had significantly higher nitrogen concentrations in 2022. In 2023, the effect was not significant for any type of plant tissue. In general, nitrogen levels varied less with different crop loads when compared to carbohydrate levels. Year-to-year variation, however, was apparent in nitrogen content.

Carbohydrate and nitrogen levels changed significantly from 2022 to 2023, possibly due to environmental factors. In 2022, a quick sudden freeze in fall caused leaves to remain on trees throughout winter without undergoing regular senescence. Although it is well understood that leaf remobilization of carbohydrates and mineral nutrients contributes to stored reserves in woody tissue for the following year, the impact of this early freeze in this specific scenario is unknown. Without undergoing senescence, the leaves may have not been able to transport carbohydrates and nitrogen into the reserve pools of spurs and branches, increasing variation detected in 2022. However, this is a theory, and testing it is quite complicated. More long-term results are needed to identify trends and controlled experiments are needed. Most of the stored carbohydrates in woody tissue is already present prior to leaf senescence and it is possible that the final remobilization in healthy trees contributes a smaller portion of total stored carbohydrates than previously estimated.

*In-season leaf/spur sampling:*

Leaves were also collected throughout the 2023 growing season to study carbohydrate level fluctuations throughout the season. This was an addition to the project to better understand seasonal fluctuations in non-structural carbohydrate content prior to harvest. Leaves were collected starting on May 26 and continued weekly until November 6. Soluble sugars and starch content were measured on leaves collected throughout the season.

Soluble sugar content for low cropping trees was higher for the first four weeks of the season (Figure 7). This is the period where cell division is occurring, which is a significant resource demand, especially when crop load is high. Since the fruit are likely undergoing the peak rate of cell expansion in June and July, high cropping trees may be more affected by greater carbohydrate demands by fruit followed by an increase in leaf soluble sugar content later in the summer. A dramatic drop in sugar concentration occurred on August 18, which could be attributed to soluble sugar remobilization into fruit during the final stages of fruit development. This could be related to fruit ripening and maturation (harvest occurred on September 5 and 6). Soluble sugar content increased following harvest for trees with either low or high crop loads. Once fruits are harvested, carbohydrate demand would drastically decrease. Overall, sugar concentrations increase under both crop loads following harvest due to the lack of sink demand posed by fruits that were once on the trees.

Sugar concentration of ‘Gala’ leaves showed little differentiation throughout the season between high cropping and low cropping trees (Figure 8). Higher sugar levels in the beginning of the season are concurrent with photosynthetic activity and are subsequently used to fuel shoot growth, root growth, and fruit growth. A notable decrease in sugars from June 2 to June 9 could be attributed to the period where cell division is speeding up and fruits are placing greater demands on the overall soluble sugar supply in the tree. Another notable drop in sugars occurs on August 18 immediately following harvest. After harvest, sugars accumulate in leaves and increase, regardless of crop load. This lack of differentiation is explained by the lack of fruit on the trees after harvest, reducing the influence of crop load on sugar accumulation in leaves.

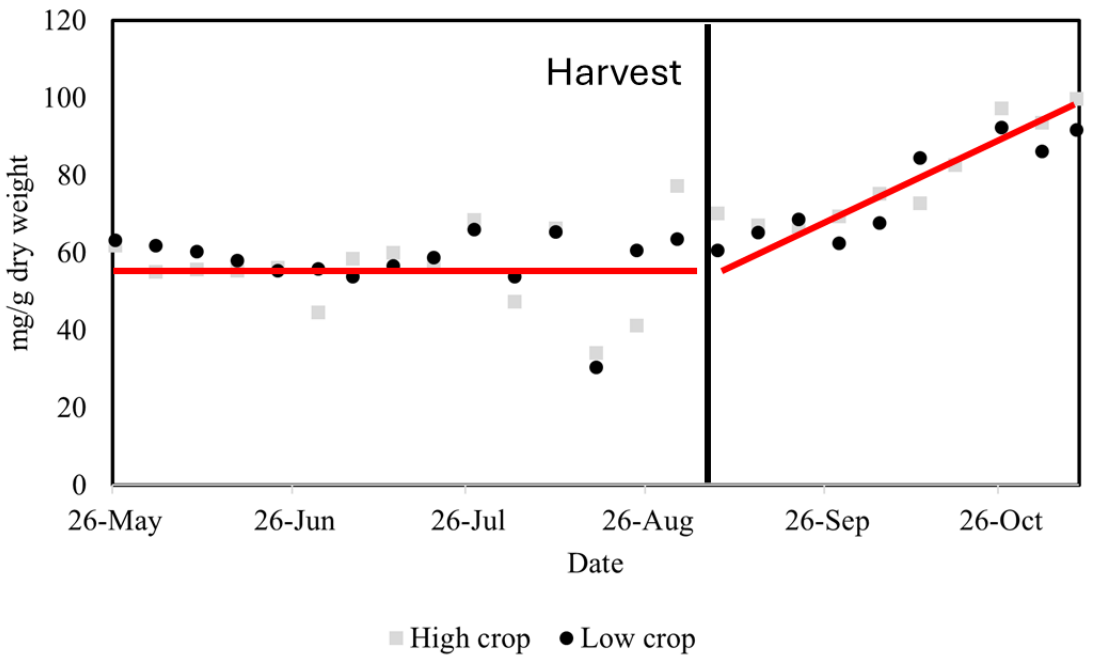


Figure 7. Weekly soluble sugar content of ‘Honeycrisp’ leaves in 2023 at low (black circles) and high crop loads (grey squares). Red lines are not statistical and are simply for illustrative purposes.

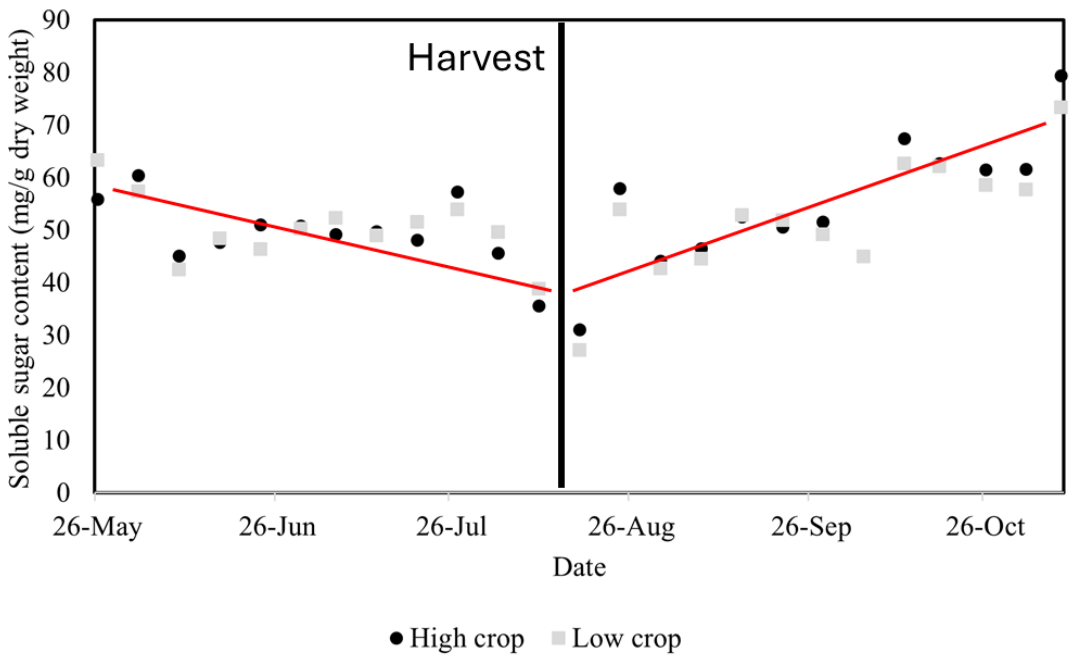


Figure 8. Weekly soluble sugar content of 'Gala' leaves in 2023 at low (black circles) and high crop loads (grey squares). Red lines are not statistical and are simply for illustrative purposes.

In 'Honeycrisp', starch concentrations were generally higher (although similar at certain points) for trees with low crop loads (Figure 9). These follow observations by Snyder-Leiby and Wang (2008) where starch grains accumulated in Honeycrisp leaves when crop loads were low. Trees with low crop loads had lower carbohydrate demand, which may contribute to higher starch content, as observed on June 30 and July 6. There was variability in starch content among sampling dates that could be caused by variability in environmental conditions (warm nights, cloudy/smokey days, or cold days with low photosynthetic activity). After harvest, starch levels appear to begin to accumulate rapidly. Low cropping 'Gala' trees had notably higher starch concentrations throughout the season. While not all samples have been analyzed for starch, those analyzed so far show consistently lower starch levels in high cropping trees. Comparing 'Gala' to 'Honeycrisp', 'Gala' shows much greater differentiation between high and low crop loads. This highlights a consistent relationship between crop load and starch concentration in 'Gala' which is less biennial. A stronger response occurred from June 23 to June 30 in high cropping trees, possibly due to fruits having greater demand and putting greater strain on leaf starch levels when crop levels are higher. Significant reductions in starch concentrations were observed on June 30 and August 3. After harvest, starch levels appear to accumulate at a slower pace, although data from high cropping trees shows a rapid increase on Sept 14.

Following up on this research, we conducted experiments where we looked at the impact of girdling and crop load on carbohydrate and hormone movement between branches as the rest of the tree. This research was conducted with support from the WSU Tree Fruit Endowment Funds to Lee Kalcsits and results will be published in addition to this report.

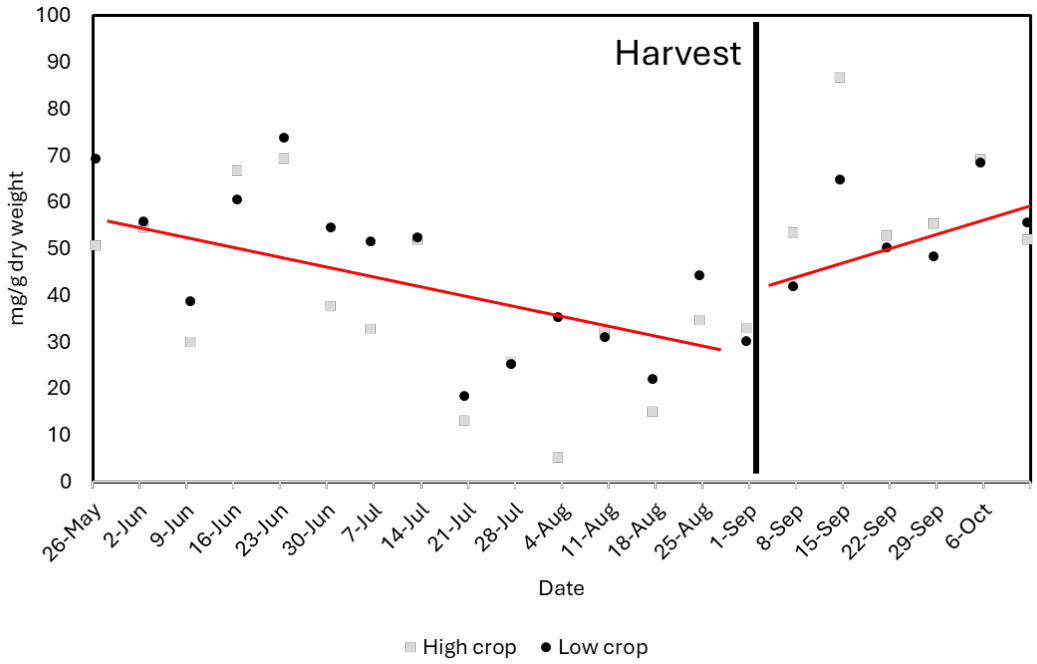


Figure 9. Leaf starch content of 'Honeycrisp' in 2023 at low (black circles) and high (grey squares) crop loads. Red lines are not statistical and are simply for illustrative purposes.

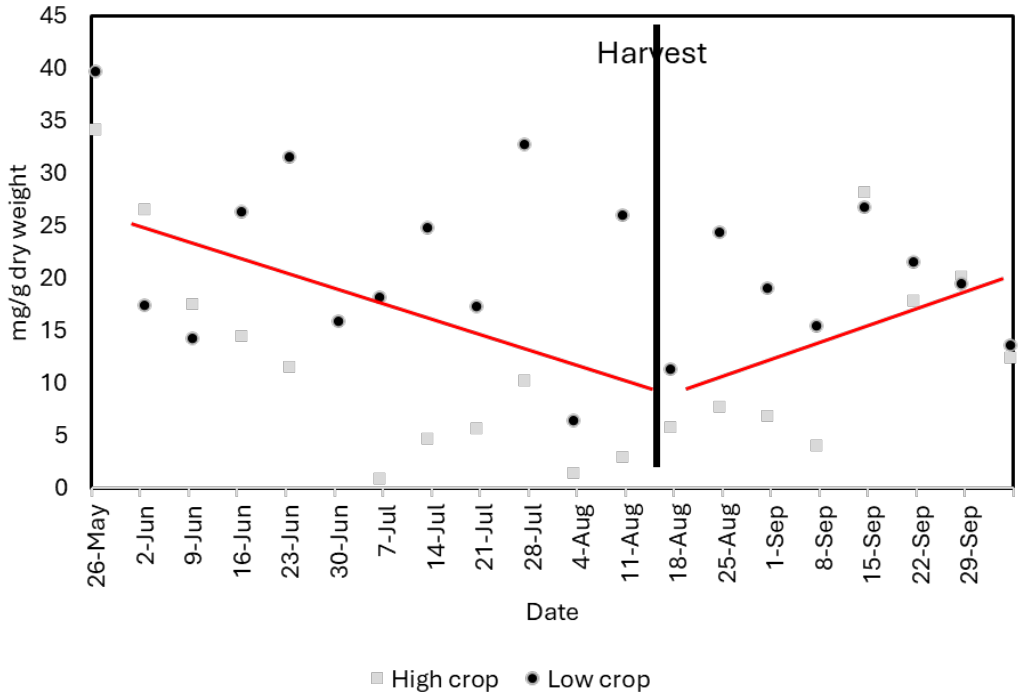


Figure 10. Leaf starch content of 'Gala' in 2023 at low (black circles) and high (grey squares) crop loads. Red lines are not statistical and are simply for illustrative purposes.

## ADDITIONAL ITEMS

*Timing of carbohydrate analysis procedure:*

Spurs, bourse shoots, terminal shoots (1-year-old wood), and apical buds were collected for sugar and starch measurement. Samples were microwaved for 180 seconds at 800W to deactivate NSC-modifying enzymes (Quentin et al., 2015) and dried in a freeze-dryer (Labconco, Kansas City, MO). Samples were then homogenized with a Powergen High Throughput Homogenizer (Fisher Scientific, Waltham, MA). Protocols from Landhäusser et al. (2018) were followed for non-structural carbohydrate extraction. Samples were placed in 80% hot ethanol (EtOH) at 90°C for 10 minutes. The supernatant was extracted for soluble sugar quantification, while the remaining starch pellet was washed twice before being left to dry. Soluble sugars were determined and analyzed using an anthrone-sulfuric acid assay (Leyva et al., 2008), then read in a multi-detection microplate reader (Bio-Tek Instruments, Winooski, VT). Starch was quantified using a glucose hexokinase-6-phosphate (GHK) enzymatic assay (Landhäusser et al., 2018), then read in a multi-detection microplate reader (Bio-Tek Instruments, Winooski, VT).

The method of measuring starch and sugar concentrations can be time-consuming, although practicing the procedure allows for more efficient processing. There are 5-6 main components of the process: (1) microwaving, freeze-drying, and grinding samples; (2) weighing samples into tubes; (3) sugar extraction and starch wash; (4) starch extraction for absorbance reading; (5) absorbance reading of starch; and (6) absorbance reading of sugar. Here is the breakdown:

(1) 2.12 minutes per sample (freeze-drying takes 1-2 days, but is passive process)
(2) 1.7 minutes per sample (efficient speed after running many samples)
(3) 4.3 minutes per sample
(4) 2.3 minutes per sample
(5) 5.63 minutes per sample (plenty of passive time built into the process while microplate is shaking on device)
(6) 1.88 minutes per sample

In total, the average time is from start to finish is 17.93 minutes per sample. This process is most efficient with a 96-well micro-centrifuge tube holder. Running less than 96 samples becomes less efficient because of steps where samples can be run together, such as the hot water bath. Utilization of an electronic pipette dispenser also allows for quick dispensing of materials into sample tubes.

**References**

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E.E. Goldschmidt, A. Golomb. 1982. The carbohydrate balance of alternate-bearing citrus trees and the significance of reserves for flowering and fruiting. J. Am. Soc. Hort. Sci. 107; 206-208. 10.21273/JASHS.107.2.206

Landhäusser SM, Chow PS, Dickman LT, Furze ME, Kuhlman I, Schmid S, Wiesenbauer J, Wild B, Gleixner G, Hartmann H, Hoch G, McDowell NG, Richardson AD, Richter A, Adams HD. 2018. Standardized protocols and procedures can precisely and accurately quantify non-structural carbohydrates. Tree Physiol. 38(12):1764-1778. https://doi.org/10.1093/treephys/tpy118

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Quentin AG, Pinkard EA, Ryan MG, Tissue DT, Baggett LS, Adams HD, Maillard P, Marchand J, Landhäusser SM, Lacointe A, Gibon Y, Anderegg WRL, Asao S, Atkin OK, Bonhomme M, Claye C, Chow PS, Clément-Vidal A, Davies NW, Dickman LT, Dumbur R, Ellsworth DS, Falk K, Galiano L, Grünzweig JM, Hartmann H, Hoch G, Hood S, Jones JE, Koike T, Kuhlmann I, Lloret F, Maestro M, Mansfield SD, Martínez-Vilalta J, Maucourt M, McDowell NG, Moing A, Muller B, Nebauer SG, Niinemets Ü, Palacio S, Piper F, Raveh E, Richter A, Rolland G, Rosas T, Joanis BS, Sala A, Smith RA, Sterck F, Stinziano JR, Tobias M, Unda F, Watanabe M, Way DA, Weerasinghe LK, Wild B, Wiley E, Woodruff DR. 2015. Non-structural carbohydrates in woody plants compared among laboratories. Tree Physiol. 35(11):1146–1165. https://doi.org/10.1093/treephys/tpv073

Snyder-Leiby, T. E., & Wang, S. (2008). Role of crop load in chloroplast ultra-structure and zonal chlorosis, a physiological disorder in ‘Honeycrisp’apple trees. *HortScience*, *43*(6), 1819-1822.

# EXECUTIVE SUMMARY

*Project Title:* Measuring storage reserves to assess the severity of biennial bearing

*Keywords:* carbohydrates, nitrogen, starch concentration, crop load

*Abstract:* Biennial bearing is not a novel concept for fruit trees, likely accompanying them since before their domestication. Biennial bearing, or alternate bearing, is characterized by a heavy crop one year, the “on” year, accompanied by little to no crop the following year, the “off” year. While environmental factors such as spring frosts and drought can initiate a biennial bearing cycle in fruit trees, genetics play an important role, with certain cultivars having a greater likelihood of bearing biennially. In apples, ‘Honeycrisp’ is prone to biennial bearing, even when environmental factors remain constant and in good supply. Regenerative buds can make up more than 90 percent of buds in an “on” year, while the “off” year maybe 20 percent or less of the buds being regenerative. Nonstructural carbohydrates fulfill distinct roles within plants, influencing flower bud initiation and fruit bud formation. Moreover, nitrogen reserves play a key role in fueling spring growth and flowering. We sought to quantify nonstructural carbohydrate and nitrogen levels of various plant tissues in both “on” and “off” trees of ‘Honeycrisp’ and ‘Gala’. Crop load from each tree was used to allow for greater distinction between “on” and “off” trees. Carbohydrate levels increased linearly with crop load, although results showed greater separation in 2022 than 2023. We theorize that abnormal events in 2022, when leaf senescence did not occur going into winter, could have disrupted carbohydrate and nitrogen translocation patterns in the trees, causing data to become more significant. Crop load was more significant in affecting carbohydrate and nitrogen levels of ‘Honeycrisp’ than ‘Gala’. Weekly leaf sampling indicated a clear impact of crop load and fruit development on sugar and starch concentrations of trees throughout the growing season. Carbohydrate and nitrogen levels of trees impact biennial bearing, although the strength and influence of this relationship are currently unclear. Follow up studies on the interactions between storage reserves and hormone movement during floral bud initiation will help support observations in this report.

**Project/Proposal Title:** Measuring the impact of leaf removal on spur and tree health

**Report Type:** Final Project Report

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**Cooperators**: Orlando Howe, Thiago Campbell, Lissett Gonzalez, McDougall and Sons, Gebbers Farms, Zirkle Fruit

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $ 60,344
**Total Project Request for Year 2 Funding:** $ 66,377
**Total Project Request for Year 3 Funding:** $ 52,580
**Other related/associated funding sources:** None

**Budget 1**
**Primary PI: Lee Kalcsits**
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**Contract Administrator: Anastasia Mondy**
**Telephone: 916-897-1960**
**Contract administrator email address:** Anastasia.Mondy@wsu.edu

| Item | 2022 | 2023 | 2024 |
|---|---|---|---|
| **Salaries**[1] | $40,777 | $43,826 | $31,460 |
| **Benefits**[2] | $6,637 | $7,393 | $10,895 |
| **Wages**[3] | $5,187 | $5,394 | $0 |
| **Benefits**[4] | $518 | $539 | $0 |
| **Equipment** | $0 | $0 | $0 |
| **Supplies**[5] | $3,000 | $5,000 | $5,500 |
| **Travel**[6] | $4,225 | $4,225 | $4,225 |
| **Miscellaneous**[7] | $0 | $0 | $500 |
| **Plot Fees** | $0 | $0 | $0 |
| **Total** | $60,344 | $66,377 | $52,580 |

**Footnotes:**

**[1] Salary is requested for a 25% post-doc in years 1 and 2 and then 50% in year 3 as well as a graduate assistant in year 1 and 2 to complete the applied physiology experiments.**

**[2] Benefits are calculated at 34.6% for the post-doc and 12.6% for the graduate assistant.**

**[3] Wages are for covering summer salary for the graduate assistant**

**[4] Benefits are calculated at 10% for summer graduate students**

**[5] Supplies are for field and lab consumables to conduct applied experiments for objective 1 and 2 and then Extension material for objective 3.**

**[6] Travel funds are requested for frequent travel to the Sunrise research orchard for PIs and personnel and to commercial orchards to conduct deleafing trials.**

**[7] Funding is requested for a small personal service contract for a videographer to capture some of the applied experiments being conducted for this project.**

## Objectives*

*1,2, and 4 are original objectives. #3 is an additional objective

1. Quantify improvements in leaf color and changes to sunburn incidence from leaf removal for an early and late-season bicolor apple cultivar.

2. Determine whether differences in leaf removal severity and timing before harvest impacts energy and nutrient storage and subsequent spur health the following season or an early and late-season bicolor apple cultivar.

3. **(ADDITIONAL OBJECTIVE) Determine whether there are differences in red color or sunburn risk from full tree deleafing compared to lower canopy deleafing (commercial practice) and determine additive effects of reflective fabric with deleafing.**

4. Develop practical operating guidelines and economic cost-benefit thresholds for leaf removal based on commercial trials in WA.

Significant Findings

- In 2022, color development was poor for earlier cultivars but was much improved in 2023 for both cultivars. Leaf removal did not affect red color for WA 38 in a good coloring year like 2023 but was effective in a poor coloring year like 2022.
- Leaf removal greater than 50% reduced return bloom, yields but did not affect vegetative vigor.
- Leaf removal significantly enhanced color development but also increased sunburn damage for Honeycrisp but not for WA 38. Benefits were observed as low as 25% leaf removal. Unsurprisingly, above 75% leaf removal increased sunburn damage in unprotected fruit.
- Leaf removal had limited benefit for a high coloring cultivar like WA 38, but also had limited sunburn risk. In a poor coloring year like 2022, leaf removal would likely have benefits for bicolored cultivars with high color requirements.
- Carbohydrate content in storage tissues were relatively unaffected by deleafing treatments.
- The timing of leaf removal had little impact on red color development. Deleafing can be done in as little as 7 days before harvest with improved color still observed.
- The speed of operation varied by commercial orchard depending on the brand of equipment, age of orchard, vigor, etc.
- An 8’ window from the ground was the targeted location for leaf removal for commercial deleafing
- Growers can be less conservative with the use of deleafing machines and risks for sunburn are only high if forecasted temperatures are high and reflective material is used.

## Results and Discussion

### Severity Experiment:

Goal: To assess how differing leaf removal severities affects sunburn, red color development, return bloom, and following season vegetative vigor for Honeycrisp (hard to color) and WA 38 (easy to color) apples.

Treatments: 0%, 25%, 50%, 75%, and 100% leaf removal.

Conclusion: When leaf removal % is lower than 50%, there is limited risk of sunburn and strong benefits for red color development for Honeycrisp. There were no carryover effects on return yield nor vegetative vigor unless leaf removal % exceeded 75%. Growers should be mindful of possible cumulative effects on tree health and adjust deleafing severity each year based on block vigor and light exposure.

Results and Discussion:

Sunburn incidence and severity

Differences in sunburn development were observed between 'Honeycrisp' and 'WA 38' apples. 'Honeycrisp' is particularly susceptible to sunburn due to its low surface temperature threshold (46-49°C) for sunburn browning, compounded by earlier maturation during periods of high solar radiation (Schrader et al., 2008). In contrast, 'WA 38' apples exhibit extensive red skin coverage, which may mask visible browning and reduce apparent sunburn incidence (Gomez & Kalcsits, 2020). In this study, these findings highlight the relationship between leaf removal severity and sunburn incidence, suggesting that increased defoliation intensifies sunburn severity, particularly for both 'Honeycrisp' and 'WA 38' apples.

Sunburn was affected by defoliation treatments in both years (Tables 1 and 2). The proportion of fruit with no sunburn (SB 0) decreased as the amount of removed leaves increased for both cultivars. When no leaves were removed, an average of 78.2% of fruit had no sunburn but only 16.3% of fruit had no sunburn when 100% of leaves were removed prior to harvest in 2022. Similar patterns were observed in 2023, but a lower proportion of fruit had no sunburn for the control (54.2%) and 100% leaf removal (12.3%). The proportion of mild sunburn (SB 1) was the lowest when there were no leaves removed and highest when greater than 25% of leaves were removed in 2022 ($P = 0.027$). However, there were no significant differences among treatments in the proportion of fruit with SB1 classifications of sunburn ($P = 0.31$). SB2, which signifies a medium severity of sunburn, increased with leaf removal for both years. However, SB2 was greater than the control starting when more than 75% of leaves were removed in 2022 ($P = 0.0012$) and only when 100% of leaves were removed in 2023 ($P = 0.0096$). Only 6.1% of the fruit was classified as SB2 in 2022 when no leaves were removed, and 26.9% of the fruit was classified as SB2 when all the leaves were removed. Similarly, SB3 was also highest when all leaves were removed compared to the control ($P = 0.0011$). The proportion of fruit with SB3 sunburn classification was 5.9% and 3.3% for the control in 2022 and 2023, respectively. The proportion of fruit with SB3 was only higher than the control when all the leaves were removed and was 25.7% and 31.5% in 2022 and 2023, respectively. Even the most severe sunburn classification (SB4) increased in both 2022 and 2023. The proportion of fruit with SB4 was 0.5% and 0% for 2022 and 2023, respectively. Similar to SB3, only full leaf removal had greater proportions of fruit with SB4 where 5.8% and 7.2% of fruit were classified as SB4 in 2022 and 2023, respectively.

‘WA 38’ had less severe sunburn symptoms compared to ‘Honeycrisp’. The proportion of fruit with no sunburn symptoms was 98.3% and 90.3% for the control in 2022 and 2023, respectively. When all leaves were removed, the proportion of fruit with no sunburn was significantly lower (88.7%) compared to the control ($P<0.05$) in 2022. However, there were no significant differences in the proportion of fruit with no sunburn among treatments in 2023 ($P = 0.347$). There were significant differences among treatments for the proportion of fruit classified as SB1 in 2022 ($P = 0.0075$) but not 2023 ($P = 0.141$). The proportions of fruit classified as SB1 were 1.6% and 7.1% for trees with no leaves removed in 2022 and 2023, respectively. When all leaves were removed, 8.4% of fruit was classified as SB1 and was significantly greater than the control. The proportions of fruit affected by SB2 remained very low across all treatments for both years, never exceeding 4.8% and were not significantly different among treatments ($P = 0.614$ and 0.629 in 2022 and 2023, respectively). There were no SB3 or SB4 classifications observations across both years for ‘WA 38’.

Red color

Red color coverage was much higher in 2023 than in 2022. Control trees had an average of 11% red color coverage in 2022, whereas in 2023, the mean red color coverage for the control was 52.4% (Tables 3 and 4). As leaf removal increased, there was a significant decrease in the proportion of ‘Honeycrisp’ fruit with 0-20% red color coverage in both 2022 ($P=0.013$) and 2023 ($P=0.039$) (Table 3). This pattern was accompanied by a corresponding increase in the proportion of fruit with high red color coverage (80-100%) as defoliation severity increased ($P = 0.012$ in 2022, $P= 0.016$ in 2023). For 2022 and 2023, the proportion of fruit meeting premium color standards with >33% red color coverage increased as leaf removal increased, as shown in Figure 1.

For ‘WA 38’, there were significant increases in mean color coverage when deleafing was greater than 50% in 2022 (Table 4). The mean red color coverage was just over 70% for the control (71.5%) and just under 90% when 100% of the leaves were removed (88.6%) (4). There were no differences between the partially defoliated and the control in 2022 ($P>0.05$ for all comparisons) (Table 4). In 2023, mean color coverage was nearly 100% for every treatment, including the control, with mean red color coverage ranging from 97.4% to 98.1%, and there were no significant differences among the treatments ($P>0.05$ for all comparisons) (Table 4). The proportion of fruit with >50% red color coverage increased in 2022 as leaf removal increased, going from approximately 80% of fruit meeting that standard to more than 95% of fruit meet that standard when 100% of the leaves were removed (Figure 2). In contrast, there were no significant changes in the proportion of fruit with >50% red color coverage in 2023.

Vegetative vigor and return yields

Leaf removal treatment affected yield in the following year for both ‘Honeycrisp’ and ‘WA 38’. When 100% of leaves were removed, there was a significantly lower yield in 2023 and 2024 compared to the control and 25% leaf removal treatments (Figure 3 and Table 5). For 'Honeycrisp,' yield decreased by 91% under the 100% leaf removal treatment, while for 'WA 38,' the reduction was 71%. 'WA 38' yields were only significantly different between the control and 100% leaf removal treatments, indicating lower sensitivity to intermediate defoliation levels compared to 'Honeycrisp.' In 2023, vegetative shoot growth in ‘Honeycrisp’ varied significantly across defoliation treatments from 8.77 to 16.6 cm, with the lowest growth recorded when 100% of the leaves were removed ($P = 0.005$), while 2022 had no significance ($P = 0.104$) and growth ranged from 9.8 to 15.5 cm (Table 6). There were no significant differences found in vegetative shoot growth for ‘WA 38’ under the same treatments in both 2022 and 2023 ($P = 0.56$ and $P = 0.649$). The growth for ‘WA 38’ remained consistent across treatments (Table 6).

The impact of leaf removal on vegetative growth was minimal, suggesting that moderate leaf removal might not adversely affect tree vigor over the duration of the experiment. While the short-term effects of leaf removal were not observed in these experiments, it is important to consider that prolonged or extreme defoliation could lead to further depletion of carbon reserves, potentially reducing vegetative growth in subsequent seasons. The current study focused on a limited time frame, and future research would be valuable to explore the long-term consequences of extreme defoliation on tree health and productivity, particularly regarding carbon storage and allocation over time. However, high rates of leaf removal reduced yields in the following season for both cultivars, indicating that extreme defoliation may deplete storage reserves in the tree. Di Lorenzo et al. (2013) reported the photosynthetic response to leaf removal is particularly notable when the source-sink ratio is constrained, leading plants to promote the growth of apical meristems to offset reduced leaf area. Zhou and Wang (2021) highlight that defoliation intensity can impede tree growth by restricting the availability of carbon sources and sinks, which affects carbon storage within the tree. This dynamic was evident in our study, where extreme defoliation (100%) in 'Honeycrisp' apples led to a significant decrease in vegetative shoot extension, indicating a carbon allocation disruption.

Defoliation rates can have differential responses in different plant species. Quentin et al. (2011) reported that moderate defoliation, typically between 50% and 66%, can have varied impacts on the total above-ground biomass of different tree species, ranging from significant reductions to negligible changes. In line with this, our findings show no significant changes in vegetative growth across varying severities of defoliation. Bledsoe et al. (1988) reported that in *Vitis vinifera* (Sauvignon blanc) grape vineyards, neither the timing nor the extent of leaf removal significantly affected yield or its components, prompting further research into its long-term effects on yield stability and fruit quality. However, this finding likely does not universally apply across all fruit types, as evidenced by our study where significant yield reductions were observed in 'Honeycrisp' apples subjected to high levels of defoliation. This contrast highlights the necessity of species- and cultivar-specific research into horticultural practices, as techniques beneficial in one type of fruit or cultivar may be less effective or could even be detrimental in another.

The results of this study suggest that moderate leaf removal (25-50%) can effectively increase red color development in 'Honeycrisp' and ‘WA 38’ apples without severely impacting sunburn incidence or future yields. For 'Honeycrisp,' which is more prone to sunburn, a more conservative approach may be necessary to avoid significant quality losses due to sunburn, particularly during years or in locations where the risk of sunburn is high. On the other hand, ‘WA 38’ can tolerate higher leaf removal rates with less risk of sunburn, making it a suitable candidate for more aggressive defoliation strategies to improve red color. Alternatively, in some years, defoliation may not be necessary since red color development will be sufficient for all levels of light exposure in the canopy depending on the training of the tree and fruit exposure levels. Strategies that increase the amount of light penetrating the canopy, such as reflective ground covers, manual pruning techniques, and training systems tailored to optimize canopy architecture, are frequently used by growers to enhance the red coloration of bicolor cultivars. This study provides valuable insights for apple growers aiming to optimize fruit quality through the addition of leaf removal practices.

Future research should focus on the long-term effects of repeated leaf removal on tree health and productivity to develop sustainable management practices that balance immediate quality improvements with long-term orchard health. While leaf removal enhances apple coloration, its application must be carefully managed to minimize risks such as sunburn and ensure the long-term health of the tree. Additionally, cost-benefit analyses are needed to assess the economic impact of leaf removal on farm profitability. This study emphasizes the need for balanced defoliation practices to prevent financial losses and provides valuable insights for developing horticultural strategies that enhance color while managing sunburn risks and protecting future yields.

Table 1. Mean proportions of fruit affected by sunburn (SB0-SB4) for 'Honeycrisp' apples with 0, 25, 50, 75, or 100% of leaves removed in 2022 and 2023. Lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

| Year | Leaf Removal % | Proportion of Fruit under Sunburn Classification (% Fruit) | | | | |
|---|---|---|---|---|---|---|
| | | SB 0 | SB 1 | SB 2 | SB 3 | SB 4 |
| 2022 | 0% | 78.2 ± 4.4 a | 9.3 ± 2.0 a | 6.1 ± 1.3 a | 5.9 ± 2.1 a | 0.5 ± 0.5 a |
| | 25% | 72.0 ± 9.6 ab | 11.3 ± 3.7 ab | 10.7 ± 2.9 ab | 5.5 ± 3.2 a | 0.5 ± 0.5 a |
| | 50% | 63.7 ± 4.3 ab | 19.6 ± 4.6 b | 12.1 ± 1.8 ab | 4.2 ± 0.1 a | 0.4 ± 0.4 a |
| | 75% | 41.5 ± 4.8 bc | 22.7 ± 4.9 b | 23.6 ± 3.5 bc | 11.1 ± 2.1 a | 1.0 ± 0.7 a |
| | 100% | 16.3 ± 13.2 c | 25.3 ± 2.9 b | 26.9 ± 5.6 c | 25.7 ± 6.1 b | 5.8 ± 1.6 b |
| | p-value | **<0.0001** | **0.0269** | **0.0012** | **0.0011** | **0.0005** |
| 2023 | 0% | 54.2 ± 2.4 a | 28.3 ± 1.5 a | 14.2 ± 2.6 a | 3.3 ± 0.1 a | 0 ± 0 a |
| | 25% | 60.8 ± 3.3 a | 25.8 ± 4.9 a | 12.5 ± 1.9 a | 0.1 ± 0.1 a | 0 ± 0 a |
| | 50% | 55.2 ± 6.4 a | 27.3 ± 5.2 a | 14.6 ± 4.0 a | 2.5 ± 1.2 a | 0.4 ± 0.4 a |
| | 75% | 32.5 ± 4.2 b | 31.8 ± 5.3 a | 24.0 ± 3.1 ab | 11.3 ± 4.9 ab | 0.4 ± 0.4 a |
| | 100% | 12.3 ± 5.1 c | 18.2 ± 4.0 a | 30.3 ± 5.5 b | 31.5 ± 11.8 b | 7.2 ± 4.1 a |
| | p-value | **<0.0001** | **0.306** | **0.0096** | **0.0067** | **0.0465** |

Table 2. Mean proportions of fruit affected by sunburn (SB0-SB4) for 'WA 38' apples with 0, 25, 50, 75, or 100% of leaves removed in 2022 and 2023. Lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

| Year | Leaf Removal % | Proportion of Fruit under Sunburn Classification (% Fruit) | | | | |
|---|---|---|---|---|---|---|
| | | SB 0 | SB 1 | SB 2 | SB 3 | SB 4 |
| 2022 | 0% | 98.3 ± 0.4 a | 1.6 ± 0.4 a | 0 ± 0 a | 0 ± 0 a | 0 ± 0 a |
| | 25% | 97.1 ± 1.4 a | 2.9 ± 1.4 a | 0 ± 0 a | 0 ± 0 a | 0 ± 0 a |
| | 50% | 95.4 ± 1.7 a | 4.2 ± 1.5 ab | 0.4 ± 0.4 a | 0 ± 0 a | 0 ± 0 a |
| | 75% | 93.7 ± 1.1 ab | 5.8 ± 1.2 ab | 0.4 ± 0.4 a | 0 ± 0 a | 0 ± 0 a |
| | 100% | 88.7 ± 0.5 b | 8.4 ± 1.2 b | 1.2 ± 1.2 a | 0 ± 0 a | 0 ± 0 a |
| | p-value | **0.0004** | **0.0075** | **0.614** | **-** | **-** |
| 2023 | 0% | 90.3 ± 2.5 a | 7.1 ± 1.9 a | 2.1 ± 0.9 a | 0.4 ± 0.4 a | 0 ± 0 a |
| | 25% | 89.6 ± 2.7 a | 7.4 ± 0.9 a | 2.5 ± 1.5 a | 0.4 ± 0.4 a | 0 ± 0 a |
| | 50% | 87.9 ± 4.1 a | 7.1 ± 2.1 a | 3.5 ± 1.7 a | 0.8 ± 0.8 a | 0 ± 0 a |
| | 75% | 84.4 ± 2.3 a | 12.2 ± 1.6 a | 2.6 ± 0.8 a | 0.8 ± 0.8 a | 0 ± 0 a |
| | 100% | 83.1 ± 2.7 a | 11.7 ± 2.2 a | 4.8 ± 1.3 a | 0.4 ± 0.4 a | 0 ± 0 a |
| | p-value | **0.347** | **0.141** | **0.629** | **0.972** | **-** |

Table 3. Mean proportions of fruit in red color coverage classes (0-20, 20-40, 40-60, 60-80, and 80-100% coverage) for 'Honeycrisp' apples with 0, 25, 50, 75, or 100% of leaves removed in 2022 and

2023. Lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

| Year | Leaf Removal % | Percentage of fruit belonging to each red color class (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0-20% | 20-40% | 40-60% | 60-80% | 80-100% |
| 2022 | 0% | 80.3 ± 4.3 a | 11.4 ± 3.1 a | 4.0 ± 2.0 a | 2.5 ± 1.2 a | 1.8 ± 0.1 a |
| | 25% | 62.9 ± 9.8 ab | 16.4 ± 3.5 a | 11.4 ± 4.2 ab | 6.1 ± 2.7 ab | 3.2 ± 1.1 ab |
| | 50% | 64.2 ± 12.4 ab | 15.1 ± 5.3 a | 9.6 ± 3.8 ab | 8.0 ± 3.6 ab | 3.2 ± 1.2 b |
| | 75% | 39.9 ± 1.0 ab | 22.1 ± 3.6 a | 18.7 ± 3.5 ab | 13.4 ± 2.7 ab | 5.9 ± 0.1 b |
| | 100% | 26.3 ± 13.8 b | 15.9 ± 2.7 a | 16.0 ± 2.5 b | 22.5 ± 1.8 b | 21.7 ± 8.5 b |
| | p-value | **0.013** | **0.389** | **0.042** | **.0368** | **0.012** |
| 2023 | 0 % | 22.4 ± 8.5 a | 15.8 ± 3.0 a | 13.8 ± 3.0 a | 20.7 ± 1.5 a | 27.2 ± 1.1 a |
| | 25% | 24.5 ± 7.7 a | 17.0 ± 2.1 a | 18.2 ± 3.0 a | 22.4 ± 1.8 a | 17.9 ± 7.0 ab |
| | 50% | 7.1 ± 2.5 a | 13.0 ± 5.0 a | 16.8 ± 4.1 a | 27.6 ± 4.2 a | 35.5 ± 1.0 ab |
| | 75% | 8.8 ± 3.8 a | 11.9 ± 2.1 a | 13.9 ± 3.6 a | 25.6 ± 2.2 a | 39.8 ± 7.7 b |
| | 100% | 2.5 ± 1.7 a | 5.8 ± 2.5 a | 8.3 ± 3.0 a | 17.7 ± 3.1 a | 65.7 ± 9.4 b |
| | p-value | **0.039** | **0.137** | **0.321** | **0.120** | **0.016** |

Table 4. Mean proportions of fruit in red color coverage classes (0-20, 20-40, 40-60, 60-80, and 80-100% coverage) for ‘WA 38’ apples with 0, 25, 50, 75, or 100% of leaves removed in 2022 and 2023. Lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

| Year | Leaf Removal % | Percentage of fruit belonging to each red color class (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0-20% | 20-40 | 0-20% | 60-80 | 0-20% |
| 2022 | 0% | 2.3 ± 0.1 a | 8.3 ± 3.8 a | 17.5 ± 6.2 a | 23.3 ± 2.41 a | 47.9 ± 11.6 a |
| | 25% | 4.7 ± 2.2 a | 8.0 ± 2.0 a | 19.7 ± 4.4 a | 27.3 ± 3.4 a | 40.3 ± 10.5 a |
| | 50% | 3.8 ± 2.4 a | 5.9 ± 3.6 a | 8.8 ± 3.6 a | 17.6 ± 6.1 a | 63.4 ± 13.4 a |
| | 75% | 0 ± 0 a | 2.6 ± 0.9 a | 12.5 ± 6.4 a | 17.6 ± 3.8 a | 67.3 ± 7.17 a |
| | 100% | 0 ± 0 a | 0 ± 0 a | 3.2 ± 1.4 a | 11 ± 0.6 a | 85.8 ± 1.87 a |
| | p-value | **0.195** | **0.220** | **0.159** | **0.077** | **0.059** |
| 2023 | 0 % | 0.8 ± 0.5 a | 0 ± 0 a | 0 ± 0 a | 0.4 ± 0.4 a | 98.8 ± 1.14 a |
| | 25% | 0.4 ± 0.4 a | 0 ± 0 a | 0 ± 0 a | 1.75 ± 1.8 a | 97.8 ± 2.6 a |
| | 50% | 1.4 ± 0.6 a | 0 ± 0 a | 0.4 ± 0.4 a | 0 ± 0 a | 98.2 ± 1.1 a |
| | 75% | 0.4 ± 0.4 a | 0 ± 0 a | 0 ± 0 a | 1.25 ± 1.25 a | 98.3 ± 1.8 a |
| | 100% | 0.9 ± 0.6 a | 0 ± 0 a | 0 ± 0 a | 0.5 ± 0.5 a | 98.6 ± 2.2 a |
| | p-value | **0.639** | **-** | **0.431** | **0.265** | **0.941** |

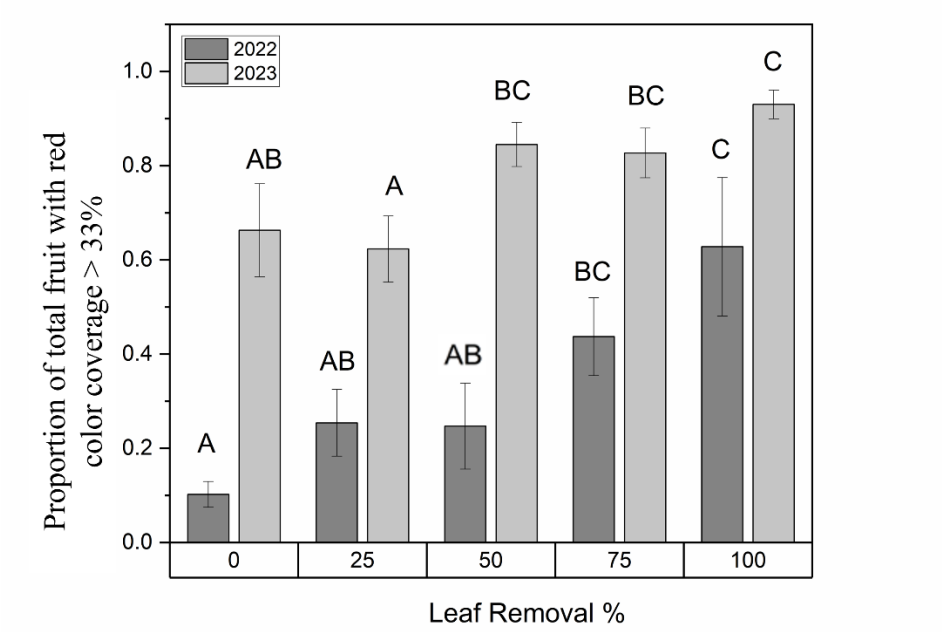


Figure 1. The proportion of 'Honeycrisp' apples with more than 33% red color coverage for five defoliation severities (0, 25, 50, 75, and 100% leaf removal). Error bars denote the standard error of the mean (N=5), and lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

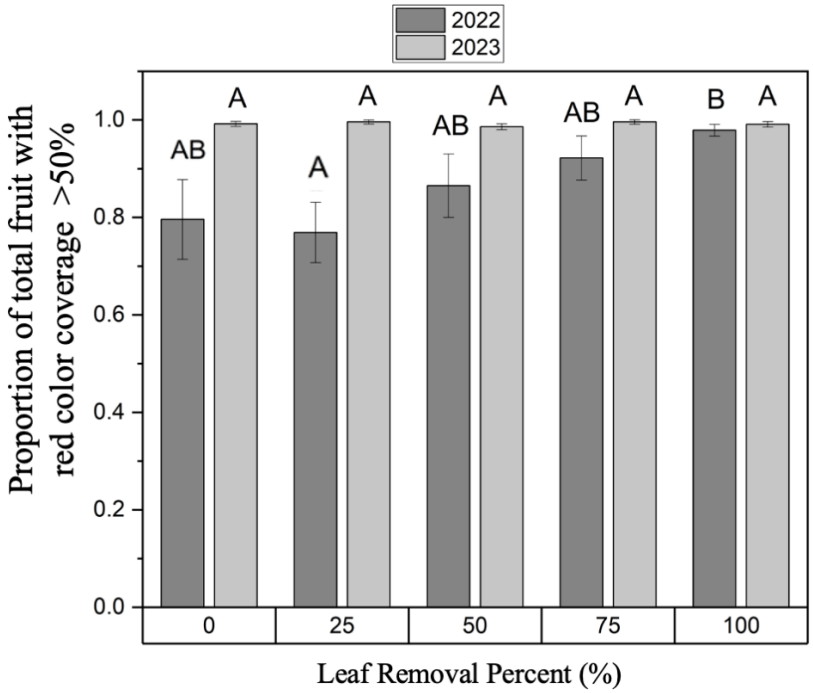


Figure 2. The proportion of 'WA 38' fruit with more than 50% red color coverage for five defoliation severities (0, 25, 50, 75, and 100% leaf removal). Error bars denote the standard error of the mean (N=5), and lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

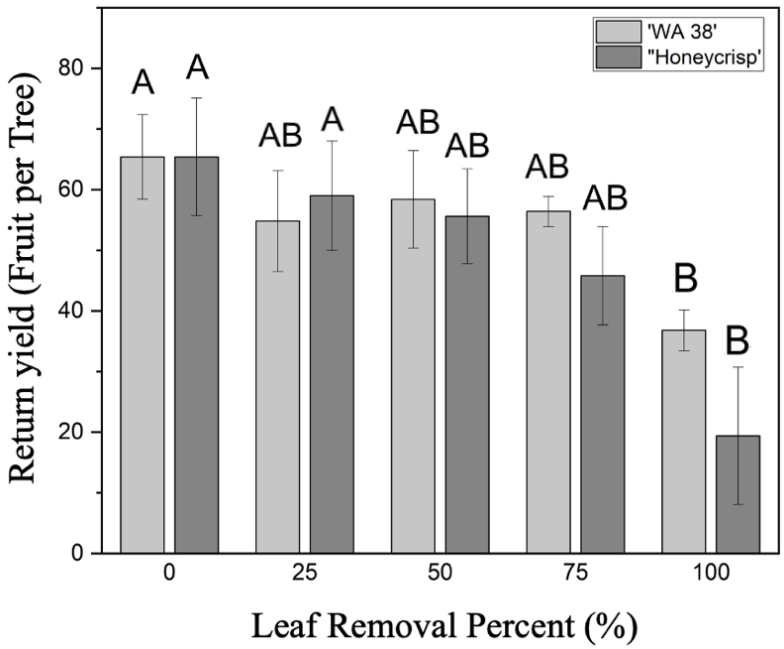


Figure 3 Return yield (fruit per tree) in 2023 following leaf removal in 2022 for 'Honeycrisp' (light grey) and ‘WA 38’ (dark grey). Crop loads were equal among treatments in 2022. Error bars indicate the standard error of the mean (N=5) for each leaf removal treatment group.

Table 5. Return yield in 2024 for ‘Honeycrisp’ and ‘WA 38’ apples under different defoliation treatments (0%, 25%, 50%, 75%, and 100% leaf removal). Crop loads were equal among treatments in 2023.

| | Treatment | Honeycrisp | WA 38 |
|---|---|---|---|
| **‘Honeycrisp'** | **0** | 32.8 | 26.4 |
| | **25** | 29.6 | 33.8 |
| | **50** | 23.8 | 20.4 |
| | **75** | 11.8 | 20.2 |
| | **100** | 4.6 | 13 |

Table 6. Vegetative growth in 2023 for ‘Honeycrisp’ and ‘WA 38’ apples under different defoliation treatments (0%, 25%, 50%, 75%, and 100% leaf removal). Lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test (α = 0.05).

| Cultivar | Treatment | Length (cm) | Groups | Length (cm) | Groups |
|---|---|---|---|---|---|
| ‘Honeycrisp' | **0** | 14.2 ± 3.0 | ab | 13.9 ± 1.0 | abc |
| | **25** | 15.3 ± 1.3 | a | 16.6 ± 1.1 | a |
| | **50** | 11.1 ± 1.5 | ab | 16.1 ± 2.8 | ab |
| | **75** | 13.9 ± 0.5 | ab | 9.4 ± 1.2 | bc |
| | **100** | 9.8 ± 0.9 | b | 8.8 ± 1.4 | c |
| | **p-value** | 0.034 | | 0.005 | |
| ‘WA 38' | **0** | 18.6 ± 4.4 | a | 27.7 ± 3.7 | a |
| | **25** | 27.8 ± 6.4 | a | 24.0 ± 4.1 | a |
| | **50** | 18.6 ± 3.2 | a | 25.0 ± 1.2 | a |
| | **75** | 22.5 ± 4.0 | a | 22.0 ± 2.1 | a |
| | **100** | 19.8 ± 3.4 | a | 22.4 ± 2.4 | a |
| | **p-value** | 0.56 | | 0.649 | |

Table 7. Estimated revenue gains for six case study commercial trials of Envy, Cripps Pink, Fuji, WA 38, Gala, and Honeycrisp apples in Washington State using different deleafing machines.

| Cultivar | % gain of premium colored fruit (> 40% red color coverage) compared to check | Estimated % lost to sunburn | Extra packed boxes per bin (@ 22 boxes per bin) | Estimated revenue at 60 bins/acre ($)** |
|---|---|---|---|---|
| **Envy** | 8.5 | 0.0 | 1.87 | 5055 |
| **Cripps Pink** | 1.0 | 0.0 | 0.23 | 385 |
| **Fuji** | 2.1 | 0.0 | 0.47 | 730 |
| **WA 38*** | 0.00 | 0.0 | 0.00 | 0 |
| **Gala***** | 13.1 | 2.0 | 2.45 | 2937 |
| **Honeycrisp***** | 15.6 | 3.0 | 2.78 | 7499 |

*Red color threshold for WA 38 was 50% red color coverage.

**Price per box was $45 for Envy, $28 for Cripps Pink, $26 for Fuji, $28 for WA 38, $20 for Gala, and $45 for Honeycrisp based on FOB pricing in December 2024 and may vary depending on year and time in storage.

***Gala and Honeycrisp trials were done in 2022 when color development was exceptionally poor. These gains may not be realized in a better coloring year.

**Timing Experiment**

Goal: To assess how differing leaf removal timings near harvest affects sunburn and red color development for Honeycrisp (hard to color) and WA 38 (easy to color) apples.

Treatments: Control, 50% leaf removal 7 days before harvest, and 50% leaf removal 14 days before harvest.

Conclusion: Leaf removal increased red color compared to the control, even 7 days before harvest. In years where temperatures are high, leaf removal a week prior to harvest can still have a positive effect on red color development for hard to color cultivars like Honeycrisp.

**Table 8.** Average red color coverage distribution on 'Honeycrisp' apples analyzed by a AWETA Commercial Fruit Sorting Line (AWETA, Nootdorp, Netherlands) in 2022 and 2023. Fruits were collected from trees with 50% leaf removal at 7 or 14 days before harvest plus an untreated control. Percentages of red color coverage were categorized into five ranges: 0-20%, 20-40%, 40-60%, 60-80%, and 80-100%. Lettering indicates mean separation within each year among treatments determined using a Tukey's HSD test ($\alpha = 0.05$).

| Year | Treatment (DBH) | Proportion of Red Color Coverage (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0-20 | 20-40 | 40-60 | 60-80 | 80-100 |
| 2022 | 0 | 70.2 ± 6.3 a | 15.8 ± 3.8 a | 7.4 ± 1.4 a | 3.7 ± 1.5 a | 2.8 ± 1.5 a |
| | 7 | 62.5 ± 13.8 a | 8.14 ± 3.3 a | 8.9 ± 4.9 a | 15.5 ± 7.4 a | 4.5 ± 3.4 a |
| | 14 | 46.1 ± 9.4 a | 24.3 ± 6.9 a | 13.7 ± 4.4 a | 12.7 ± 3.7 a | 3.1 ± 2.6 a |
| | p-value | 0.279 | 0.118 | 0.511 | 0.238 | 0.885 |
| 2022 | 0 | 26.6 ± 4.3 a | 25.0 ± 3.5 a | 16.3 ± 4.3 a | 18.3 ± 3.5 a | 13.8 ± 2.8 a |
| | 7 | 10.4 ± 3.9 b | 19.6 ± 1.7 a | 18.8 ± 2.2 a | 28.3 ± 2.8 ab | 22.9 ± 3.8 ab |
| | 14 | 13.3 ± 4.1 ab | 14.3 ± 3.2 a | 18.0 ± 2.8 a | 21.4 ± 3.7 a | 32.9 ± 6.3 b |
| | p-value | 0.033 | 0.068 | 0.854 | 0.139 | 0.035 |

Table 9. The average red color coverage distribution on 'WA 38' apples was analyzed by the AWETA Commercial Fruit Sorting Line (AWETA, Nootdorp, Netherlands) in 2022 and 2023. The table displays the percentages of red color coverage categorized into five ranges: 0-20%, 20-40%, 40-60%, 60-80%, and 80-100%.

| Year | Treatment (DBH) | Proportion of Red Color Coverage (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0-20 | 20-40 | 40-60 | 60-80 | 80-100 |
| 2022 | Control | 87.5 ± 3.9 | 12.5 ± 3.9 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| | 7 | 93.3 ± 4.1 | 6.3 ± 3.7 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| | 14 | 90.1 ± 5.2 | 8.3 ± 4.9 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| | p-value | 0.684 | 0.609 | - | - | - |
| 2023 | Control | 0 ± 0 | 0 ± 0 | 0.4 ± 0.4 | 1.6 ± 1.2 | 97.9 ± 1.6 |
| | 7 | 0.4 ± 0.4 | 0 ± 0 | 0 ± 0 | 0.4 ± 0.4 | 99.1 ± 0.1 |
| | 14 | 0.4 ± 0.4 | 0 ± 0 | 0 ± 0 | 0.4 ± 0.4 | 99.2 ± .005 |
| | p-value | 0.618 | - | 0.397 | 0.465 | 0.679 |

The results of this study suggest that the timing of 50% leaf removal before harvest has minimal impact red color development or sunburn incidence in 'Honeycrisp' and 'WA 38' apples. Both cultivars benefited from increased light exposure through defoliation, with a higher proportion of fruit displaying enhanced red color coverage. However, whether leaf removal was performed 7 or 14 days before harvest, no significant differences in fruit quality, including red color intensity and sunburn incidence, were observed. This indicates that growers have flexibility in scheduling leaf removal operations within this timeframe.

For 'Honeycrisp,' which is more prone to sunburn, it is important to balance improving red color with minimizing sunburn risks, as this cultivar exhibited a trend toward higher sunburn incidence with leaf removal, particularly at 50% removal 14 days before harvest. 'WA 38,' on the other hand, displayed greater tolerance to sunburn and can potentially benefit from more aggressive defoliation strategies to enhance red color without compromising fruit quality.

These findings provide useful insights for growers aiming to optimize fruit color while minimizing the risk of sunburn. While 50% leaf removal at different intervals before harvest showed no significant impact on sunburn or color for 'WA 38,' careful management is still needed for 'Honeycrisp.' Complementary practices such as reflective ground covers, strategic pruning, and canopy management may further enhance light penetration and improve fruit quality. Future research should investigate the long-term effects of repeated 50% leaf removal on tree health, productivity, and overall cost-benefits to develop sustainable orchard management strategies that ensure both high fruit quality, tree vitality, and on-farm profitability.

**Deleafing location and combining reflective material experiment**

Goal: To assess whether whole tree deleafing increased sunburn risk compared to lower canopy deleafing like a commercial machine would as well as assess the use of reflective material in deleafed orchards and its effects on red color and sunburn incidence.

Treatments: Either Extenday or grass and then combined with a control, whole tree leaf removal, or just lower canopy leaf removal.

Conclusion: Extenday continues to have strong positive effects on red color and combining reflective material and deleafing can strongly improve red color development leading to better packout and revenue for the grower. Growers should be mindful of the type of reflective material and environmental conditions during deployment to reduce the risk for photooxidative sunburn that can sometimes develop.

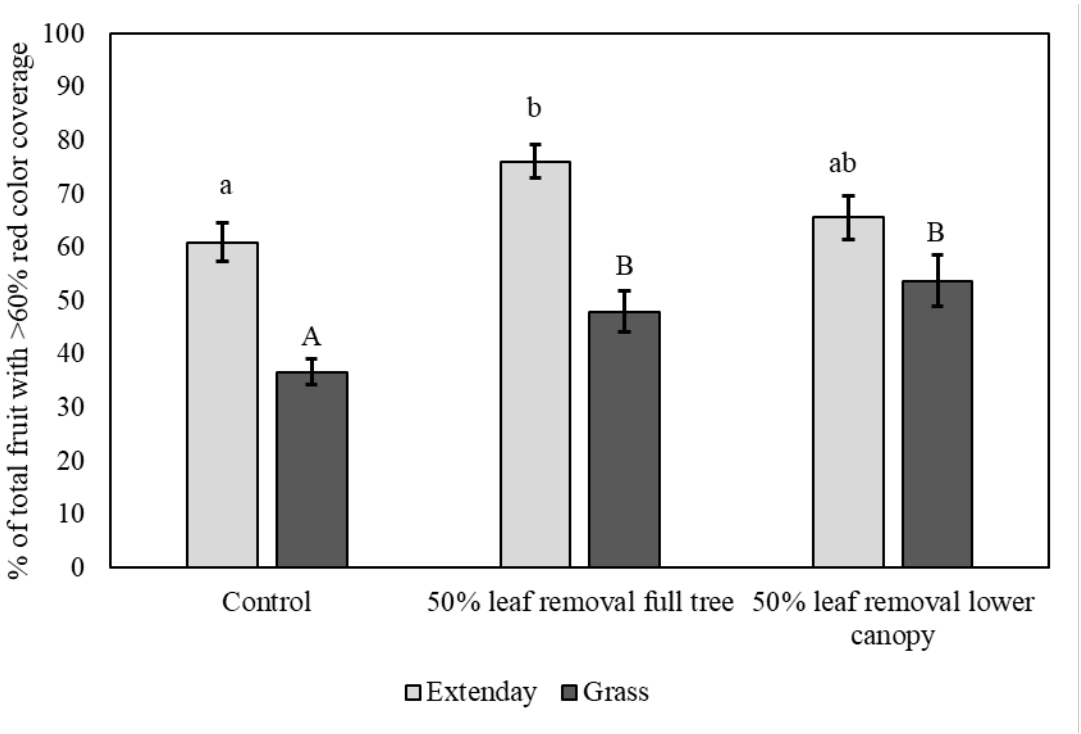


Figure 4. The percentage of fruit with greater than 60% red color coverage for Honeycrisp apple with either Extenday placed four weeks prior to harvest or just grass and then either 50% leaf removal on the full tree, 50% leaf removal on just the lower canopy, or an untreated control. Error bars denote standard error and letters denote significant differences between deleafing treatments.

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EXECUTIVE SUMMARY

**Project title:** Measuring the impact of leaf removal on spur and tree health

**Key words:** Deleafing, return yield, red color, sunburn, Honeycrisp, WA 38

**Abstract:** Leaf removal before harvest can be useful for enhancing red color for bicolor apple cultivars. Red color increases the appeal to consumers. Despite the potential benefits of leaf removal for enhancing red color, there may also be risks to fruit quality. Removing leaves increases sun exposure, which can lead to sunburn damage, reducing the percentage of marketable, non-sunburned fruit and affecting overall fruit quality. It is important to maximize fruit coloration but minimize risks to both the current and subsequent seasons' crops. In the industry, leaf removal has been used to enhance red color but has not thoroughly addressed the impact on tree health and productivity in subsequent years. Here, two experiments were conducted to research the impact of leaf removal on fruit quality. In one experiment, five different leaf removal levels were applied (0%, 25%, 50%, 75%, and 100% of the leaf canopy) 14 days before harvest (N=5) to assess their effects on fruit coloration, sunburn, return yields, and shoot vigor in ‘Honeycrisp’ and ‘WA 38’ apples. In the second experiment, red color development and sunburn was evaluated for leaf removal treatments either 7 or 14 days before harvest compared to an untreated control. As the percentage of leaf removal increased, the percentage of marketable, non-sunburned fruit decreased, indicating that sunburn increased as leaf removal increased for both cultivars but to a lower extent for ‘WA 38’. Red color coverage increased with an increase in leaf removal, with 100% removal resulting in the highest percentage of red color coverage in both ‘Honeycrisp’ and ‘WA 38’. When leaf removal severity was greater than 50%, the yields the following year were lower but shoot vigor was unaffected. Leaf removal at either 7 or 14 days before harvest improved red color compared to the control, but there were no differences between these two treatments, indicating that the window for leaf removal can be as little as 7 days before harvest. ‘WA 38’ was easier to color than ‘Honeycrisp,’ and deleafing in a poor color year was good for both cultivars but in a good color year like 2023, deleafing was only useful for hard to color cultivars like ‘Honeycrisp’

# **Project Title:** Phase 3 Evaluation of WSU Apple Breeding Selections

**Report Type:** Continuing Project Report

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**Cooperators**: Agrofresh Inc., Legacy Fruit, Columbia Fruit. Growers: Stemilt Inc. and Allan Brothers. Apple Breeding Program Advisory Committee: Aylin Moreno (Taggares Fruit), Paul Cathcart (Columbia Reach), Dale Goldy (Gold Crown), Dave Gleason (Kershaw), Dena Ybarra (WTFRC commissioner), Jeff Cleveringa (Columbia Fruit), Jeff LaPorte (Chelan Fruit), Lauren Gonzalez (GS Long), Sarah Franco (Allan Bros.), Suzanne Bishop (Allan Bros.), Tim Welsh (Columbia Fruit), Rob Blakey (Stemilt), Anne Morrell (Columbia Fruit), Erick Smith (Taggares Fruit Company), Craig Anderson (Gilbert Orchards), Matt Miles (WTFRC commissioner), Technical consultants: Stefano Musacchi, Carolina Torres, Bernardita Sallato,

**Project Duration:** 3 Years

**Total Project Request for Year 1 Funding:** $ 53,478.00
**Total Project Request for Year 2 Funding:** $ 56,127.00
**Total Project Request for Year 3 Funding:** $ 59,791.00

**Other related/associated funding sources:** Stemilt and Allan Brothers provide farm crew assistance for pruning, thinning, and field maintenance, Agrofresh donates Smartfresh, and Stemilt assists with SmartFresh and postharvest fungicide application. Columbia Fruit and Columbia Reach assisted with packing line assessment. Washington Fruit donated apple boxes and trays.

**Agency: WSU apple breeding program royalties**
**Amount awarded:** ~$500,000 per year (2023-2026)
**Notes:** Funding supports all other aspects of the apple breeding program (Phase 0 to Phase 2), including all program staff, a full-time farmworker position at WSU Columbia View orchard, and graduate student assistantships. Funds to supplement Phase 3 evaluations are provided as necessary for consumer tastings, equipment, and consumables.
**Funding Duration:** 2021-2024

**Agency Name:** WSDA Specialty Crop Block Grant Program
**Amount awarded:** $220,045
**Notes:** Establishing rootstock and production system recommendations for new Washington apple selection (WSU 'L') Evans, Musacchi, Sallato. This project will collect complementary information for an elite P3 selection that will be released.
**Funding Duration: 2021-2024**

**Agency Name:** Washington Research Foundation
**Amount awarded: $99,932**
**Notes:** Rootstock and systems trial for WA 64 apple. Evans, Musacchi, Sallato. This project continues the development of production recommendations for WA 64.
**Funding Duration: 2024-2025**

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| Item | 2023 | 2024 | 2025 |
|---|---|---|---|
| Salaries | | | |
| Benefits | | | |
| Wages | $28,523.00 | $30,079.00 | $32,259.00 |
| Benefits | $11,409.00 | $12,120.00 | $13,204.00 |
| RCA Room Rental | $12,746.00 | $13,128.00 | $13,528.00 |
| Shipping | | | |
| Supplies | $500.00 | $500.00 | $500.00 |
| Travel | $300.00 | $300.00 | $300.00 |
| Plot Fees | | | |
| Miscellaneous | | | |
| | | | |
| | | | |
| | | | |
| Total | $53,478.00 | $56,127.00 | $59,791.00 |

**Footnotes:** Wages/Benefits: calculated based on expected staff wage adjustments. RCA room rentals: 2 rooms, including room operation costs and warehouse fees, adjusted yearly. Supplies: consumables for fruit quality analysis (KOH, distilled water, iodine, etc.). Travel: in-state travel

## Justification

New and improved apple varieties are essential for a successful Washington apple industry. The WSU apple breeding program (WABP) aims to produce a portfolio of new, improved, unique varieties specially selected for the environment of central Washington and available to Washington's growers. Developing improved apple varieties leads to *sustainable production and enhanced postharvest efficiency to promote sustainability and long-term economic viability by increasing apple packouts*.

Currently, four selections are planted in three grower-collaborator sites. The advantage of this arrangement is the ability to observe the growth habits and characteristics of advanced selections in a commercial production setting. Having the WTFRC manage P3 provides an independent and industry-oriented evaluation that, with the input of industry representatives in the apple breeding program advisory committee (BPAC), ensures that the data collected and information provided align with stakeholders' interests. The project results, including single pick potential, harvest window, storability, and resistance to biotic and abiotic stress, are presented to the BPAC annually. Field visit opportunities are included throughout each season.

## Objectives

1. Evaluate and determine the commercial potential of advanced selections of the WABP

## Significant Findings

1. Although selection P has good shelf-life potential, it was *discontinued* due to its bi-annual bearing and small fruit size
2. Q, R, and S grew to reach the top wire within the first year on both sites
3. Selection Q has good firmness retention, losing only about 2 lb. after long-term storage
4. Selection R has inconsistent flavor and lower firmness retention unless treated with 1-MCP
5. Selection S, a yellow-colored apple, stored well in 2023, presenting low storage disorder incidence and maintaining flavor and texture long term.
6. The clusters of WA 64, also known as selection L, are mostly singles and doubles, but there are differences between sites
7. WA 64 performed well in packing line assessments, achieving high packouts (71% to 92%)
8. WA 64 is not sensitive to high $CO_2$ concentration (0.5% O2/ 5.0% CO2) and performs well under low oxygen storage (0.6% $O_2$ and 0.5% $CO_2$).

## Methods

Bud and Bloom observation: Field observations start as the trees begin to bloom, occurring at least twice a week, considering the weather pattern and its influence on blooming. The full bloom date is determined for each Phase 3 (P3) selection and the standard varieties near the P3 plots. Starting at this stage, every field visit includes general observations on disease incidence, tree growth habits, and health. Standard management practices (rodent activity monitoring, powdery mildew sprays, row mowing, etc.) are conducted and discussed with field managers. Pest and disease incidence and monitoring are documented during the entire season.

Fruitlet development and pre-harvest: Field activities for this stage start after June drop. Orchard visits occur at least every other week until a month before the predicted harvest. Observations on fruit sets and self-thinning are documented. The orchard crew will perform hand-thinning and summer pruning when appropriate, as if the selections were being produced commercially. A specific pruning recommendation plan is put forward for each selection by the PI, with consultation from the grower, BPAC members, and other specialists (i.e., Stefano Musacchi).

Harvest: Starch degradation, color, background color, and flavor development are assessed during pre-harvest visits. Once the harvest date is established, the harvest is conducted in one to three picks, depending on selection and crop load. The selections are typically strip-picked. Apples are harvested using picking bags and placed in blue crates (30 lb.). The apples with cracks, insect damage, chemical damage, splits, severe sunburn damage, bitter pit, and bird peck are classified as culls in the field. These apples are collected during harvest and weighed separately; the reason for cullage is assessed on individual fruit, and the data is used to calculate the percentage of fruit loss in the field.

The storage samples are weighed in the field and separated into two storage conditions: Refrigerated air (RA, 33°F) and controlled atmosphere (CA, 34°F 1% $CO_2$, 2% $O_2$), with and without 1-MCP treatment. This fruit is drenched with postharvest fungicide at a Stemilt drencher location and stored at the Research CA rooms at Stemilt. Stemilt administers the 1-MCP treatment within one week of harvest.

Quality at harvest is assessed within 48 hours of harvest. Evaluation includes starch degradation (Cornell 1-8), firmness (lb.), soluble solids (% Brix), titratable acidity (% m.a.), color (% of red coverage), background color, size (in.), weight (gr.), DA index, and presence/absence of internal and external defects/disorders.

Post-harvest: Quality assessment occurs after 3 and 6 months of storage for apples in RA and 6 and 9 months for apples in CA. Apples with and without 1-MCP treatment are evaluated at the same time points. Quality analysis assessment is conducted after seven days at room temperature to determine the potential quality for consumers after shipping, handling, and purchase. Box size distribution data will be generated from individual fruit weights. Fruit will be distributed at meetings and events as available; industry taste panel and informal consumer acceptance evaluation data will be collected.

**New:** Per BPAC recommendation, apple quality will be evaluated after 2 weeks at room temperature for fruit stored at 6 months in RA and 9 months in CA, starting with fruit harvested in 2024. This analysis will assess the potential quality for consumers in a scenario where the time from fruit handling to consumption exceeds one week.

Advanced Phase 3

When a selection is considered a good contender for commercialization (typically after at least four years in P3), it will receive the following additional evaluations:

- commercial packing line handling: glossiness, bruising, stem puncture, cullage, size and packout data is collected. Fruit is evaluated in the laboratory after the packing line run on the same day, after 7 days in RA storage, and 7 days in RA + 7 days at room temperature.
- formal consumer taste panels: coordinated with Kate Evans (co-PI and WSU apple breeder) and performed in locations or events with diverse consumer demographics (i.e., Spokane mall, Apple Blossom Festival). The protocol utilized was generated by Carolyn Ross (Professor and Director of the Sensory Evaluation Facility, WSU Pullman).

WA 64 (selection L)

In addition to packing line handling and formal taste panel, this selection was tested for $CO_2$ sensitivity and low oxygen storage. The $CO_2$ sensitivity test was performed using the USDA CA chambers (0.5% O2/ 5.0% $CO_2$, 37°F), and low oxygen storage experiment was performed in two SafePods (control: 2.0% $O_2$ and 1.0 % $CO_2$, treatment: 0.6% $O_2$ and 0.5% $CO_2$), both set at 37°F.

## Results and Discussion

Selection P


This selection was grafted in Quincy and Prosser in 2017 and 2018, respectively. It is a bicolored apple that develops good red color coverage on a fruiting wall (Prosser) or spindle system (Quincy). The apples are crisp and juicy and have a unique tart-sweet flavor. It has low field cullage and a long shelf-life.

Fruit is typically harvested in two or three weekly consecutive picks from mid-September to early October. Firmness at harvest is around 18 lb. with good firmness retention throughout storage. Soluble solids concentration and titratable acidity at harvest are 14 (%brix) and 0.9 (% m.a.), respectively.

Size distribution is variable and affected by crop load. Most apples in the first year belonged to the 64 to 80 box size. Trees with moderate crop load typically produce apples peaking in the 80 to 100 box size range. Over the past four years, hand-thinning alone has not been aggressive enough to adjust high crop load, resulting in small fruit (box size 100-150) on heavy-cropped trees. Poor thinning in Quincy in 2022 resulted in the majority of the fruit being classified as size 150 (80%). Prosser top three sizes in 2022 were 125 (21%), 138 (22%) and 150 (40%). In 2023, an "off year" in both locations, fruit size peaked at 150 in Prosser (84%) and Quincy (60%).

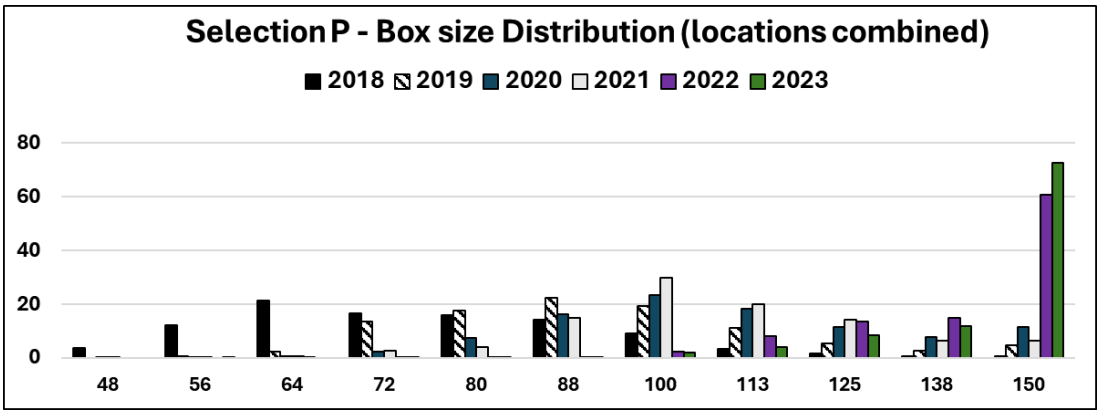


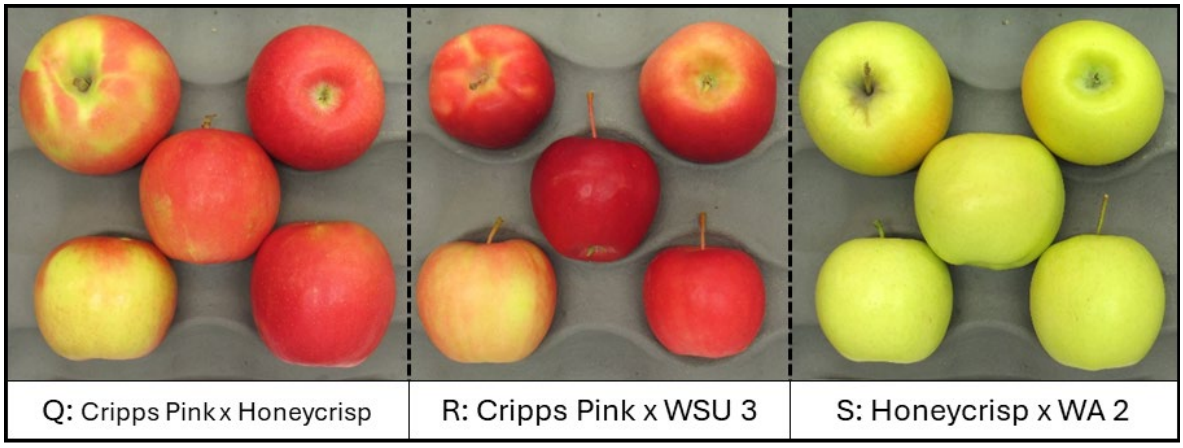

Figure 1. Box size distribution of selection P for Quincy and Prosser combined from 2019 to 2022. In 2018, only Quincy produced fruit.

Table 1. The total number of apples evaluated for fruit size distribution by location and year.

| Location | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 |
|---|---|---|---|---|---|---|
| Prosser | 0 | 202 | 1264 | 377 | 2906 | 133 |
| Quincy | 1289 | 1758 | 3768 | 2860 | 7118 | 1700 |
| Total | 1289 | 1960 | 5032 | 3237 | 10024 | 1833 |

Alternate bearing is prominent and can be observed in both sites, but it is more noticeable in Prosser, with heavy-cropped trees producing very little crop in the following year. In 2023, fruit production did not affect the fruit size, with both locations producing significantly fewer apples than in 2022 but still peaking in size 150 (Table 1). Considering these production issues and taking into consideration feedback from the BPAC, this selection **has been discontinued** and was not harvested in 2024.

Selections Q, R, and S



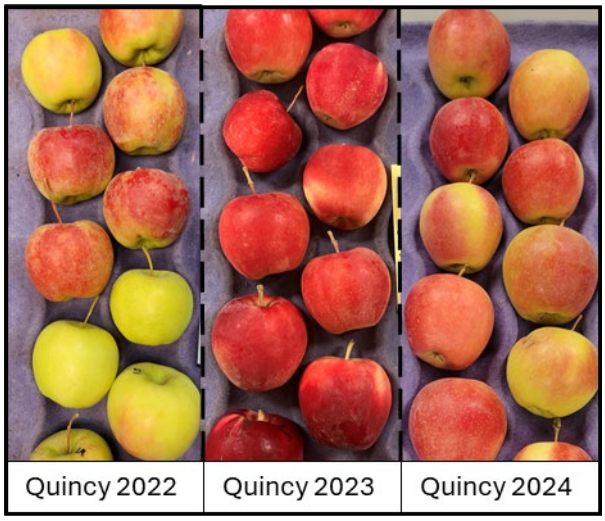

These three selections were top worked in Quincy in 2020, and Sagemoor in 2021. Most of the trees reached the top wire within one year. Tree growth is similar on both sites in the first year, with some blind wood in the middle section and heavily cropped treetop. Both locations were defruited in the first year and hand-thinned in the following years. At the Quincy site in 2023, the farm crew performed hedging and summer pruning in accordance with Stefano Musacchi's recommendations. In Sagemoor winter pruning was performed following selection specific recommendations by Bernadita Sallato.

Fruit was harvested in 2022 from Quincy and from both sites in 2023 and 2024. In 2022, all ABP selections and apple varieties harvested by the WTFRC crew generally had less color and higher bruising incidence. Also, we observed stagnation of starch degradation for a few weeks, followed by rapid depletion, which might have resulted in the fruit being picked at advanced maturity. In 2023 fruit had better color and lower bruise incidence. Quality analysis for 2024 is ongoing, thus the data provided on the following sections are based in the two previous seasons.

Selection Q

Large to medium size fruit, with box size peaking at 72 (range 72-88). Fruit typically has a short stem, and it was considered easy to pick. This selection was harvested in three consecutive picks, with starch averages at 4.3, 4.7, and 5.8. In 2022, it bruised relatively easily. Little variation was observed for firmness over time. At harvest it ranged between 15.3 and 16.8 lb, with a maximum loss averaging 2lb. Incidences of bitter pit, soft scald, superficial scald, and split were below 1% per pick. Internal browning incidence was only observed in fruit stored in CA.

Selection R




Fruit presented high color variability during harvest, with fruit from Sagemoor being more consistent. The elongated stems made the fruit more difficult to detach during harvest. Pre harvest drop was observed in Quincy before the last pick (starch above 5.0 on Cornell scale). This selection's top three box sizes were 150, 113, and 125, respectively.

Firmness of fruit harvested from Quincy was on average 18 lbs. at harvested on both years and decreased overtime, with significant changes in fruit stored for 6 months in RA (lost of 5 to 8 lb.). Fruit harvested from Sagemoor had higher firmness at harvest (21 lb. average) but underwent significant losses after 3 and 6 months in RA (5 to 6 lbs.). However, the use of 1-MCP delayed firmness loss significantly maintaining firmness levels similar to those observed at harvest. Greasiness was high, especially for fruit stored for 6 and 8 months in RA storage.

In 2022, apples from Quincy had internal browning on all three picks after 6 months in storage. In 2023 the incidence was lower and started after 9 months. Split incidence increased with maturity. Bitter pit and soft scald incidence were below 2% in 2022 and not observed in 2023. No superficial scald was found in 2022 but was recorded in 2023 in both locations (below 2.5%). Flavor is inconsistent (sweet, sour, tart) with some apples rated as starchy even after 6 months in storage.

Selection S


This is the only single-colored apple selection in P3. In both sites, the color at harvest varies from pale yellow to golden, with a darker color and blush on the sun-exposed side of the apple. Size distribution is variable and yet to be defined, considering that fruit was only harvested from second and third-leaf trees. Annual bearing was observed in Quincy, where 2023 was a low production year.

There were three consecutive picks in Quincy 2022 (second leaf). A wide variation in starch degradation at harvest (Cornell 2 to 7) was observed on the first pick. The second and third picks had

advanced starch degradation at harvest (7.3 ± 0.6). Overall, greasiness was low, with a higher incidence in fruit stored for 6 months in RA. Stem puncture and soft scald were below 2%. Split incidence was higher in the last pick (2.5%). No bitter pit or superficial scald was found.

In 2023, the fruit in Quincy was harvested in one single pick, about two weeks earlier compared to 2022, and starch degradation was at 2.4 ± 1.1. A lower bruise incidence was observed (11% overall, with 9% of small bruises). No soft scald was recorded, split was below 1.5%, and limb rub 11.4%. Sunburn incidence was lower in Quincy (6%) than in Sagemoor (16%), where fruit is more exposed to sunlight. Blush was also more prominent in Sagemoor (56%) than in Quincy (13%). Flavor and texture are consistently rated as good during storage.

Internal browning decreased significantly from 2022 to 2023. In 2022 most of the timepoint/treatment combinations had internal browning and cavities, starting at 3 months in RA and increasing by pick and time in storage. In 2023, internal browning and cavity were first observed after 6 months in storage at a much lower rate, indicating that the high incidence of these disorders in 2022 was due to advanced maturity at harvest.

## WA 64 (Selection L) – Sunflare™


WA 64 is the new release from the WSU apple breeding program and has received the commercial name of Sunflare™. Commercial planting availability and the first commercial harvest are predicted for 2026 and 2029, respectively.

This selection was grafted in 2015 on both Prosser and Quincy locations, on M9.337 and G.41 rootstocks, respectively. Tree structure (type III) and harvest timing are similar to cv. Golden Delicious, with bloom time similar to cv. Gala in Quincy.

WA 64 is a bicolored symmetrical apple that colors well when exposed to sunlight, typically achieving 50% to 70% red/pink blush with a yellow background. It is slow to brown, easy to pick, and pre-harvest drops have not been observed. Its unique trait is high firmness retention during storage, which, combined with the low incidence of disorders and diseases in the field and during storage, grants this selection a long shelf-life potential.

Self-thinning was observed and recorded as qualitative data in the P3 sites for the few years of production. In 2023, sections of 30 trees in each location were marked with ribbons during bloom, and the number of clusters was recorded. The number of fruitlet clusters and cluster classification (singles, doubles, triples, and quadruples or higher) were recorded after the June drop but before hand-thinning. The tree sections were selected to have 50 to 60 bloom clusters.

In Quincy fruitlet distribution was similar in both years, with more than 60% of clusters set as singles, and triples and quadruples combined at 10% (Figure 2.). In Prosser the crop load was more evenly distributed between categories, with an increase in quadruples from 2023 (12%) to 2024 (21%). The clusters in Prosser were hand-thinned to singles and doubles, and production levels were similar to previous years (data not shown).

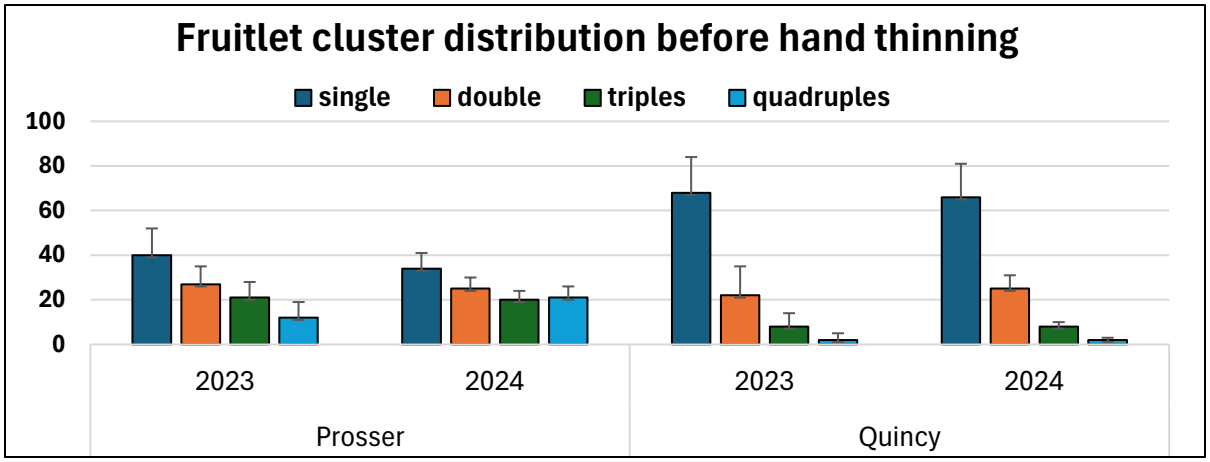



Figure 2. Distribution (%) and standard deviation of WA 64 fruitlet cluster by category (singles, doubles, triples, and quadruples or higher) in 2023 and 2024 for Prosser and Quincy.

*Fruit flavor after harvest*

This data was collected to determine whether WA 64 apples can be eaten immediately after harvest, meaning for no delays in packing and shipping. WA 64 harvested from Prosser not treated with 1-MCP was stored in RA for one week. The apples were tasted at room temperature during four events, 10 to 18 days after harvest. A total of 97 participants evaluated the appearance, flavor, and texture of WA 64 apples on a scale from 1 (poor) to 5 (outstanding). The combined scores for categories 4 and 5 were 93% for appearance, 94% for flavor, and 96% for texture. No starchy or poor flavor were detected.

*Packing line assessments*

Packing line handling evaluations, including glossiness, bruising, stem puncture, decay, storage disorders, and fruit flavor, were conducted in 2022 and 2023. In 2022, two bins of WA 64 were harvested from Quincy, and the evaluations occurred in March and August of 2023 using fruit stored in RA and CA, respectively (Figure 3A). In 2023, 2 bins of apples were harvested from each site, and one bin per site was stem clipped. One set (stem clip vs. non-stem clipped) was stored in RA and evaluated in May; the other in CA was evaluated in August 2024 (Figure 3 B and C). The Cripps Pink grading program was used for all packing line assessments.

Packed fruit (≥ 25 % red color + small or no defect) was above 70% for all packing line assessments, achieving as high as 92% for fruit from Prosser. Quincy is a challenging site for color development, and the variability is reflected in the increase of the “25 to 49% + no defect” category in 2023. WA 64 from Prosser typically has better color and size than Quincy, thus delivering higher packouts. Stem clipping provided slightly higher packouts in fruit from Quincy (3 B), with less fruit allocated for processing and lower cullage. Fruit from Prosser that was not stem clipped had higher packouts but slightly higher cullage.

Stem clipping will be investigated further on fruit harvested in 2024, along with bruising susceptibility, glossiness retention, and lenticel breakdown incidence. All the data collected regarding these characteristics, as well as the reason for cullage, will be released once the 2024 packing line assessment has been completed.

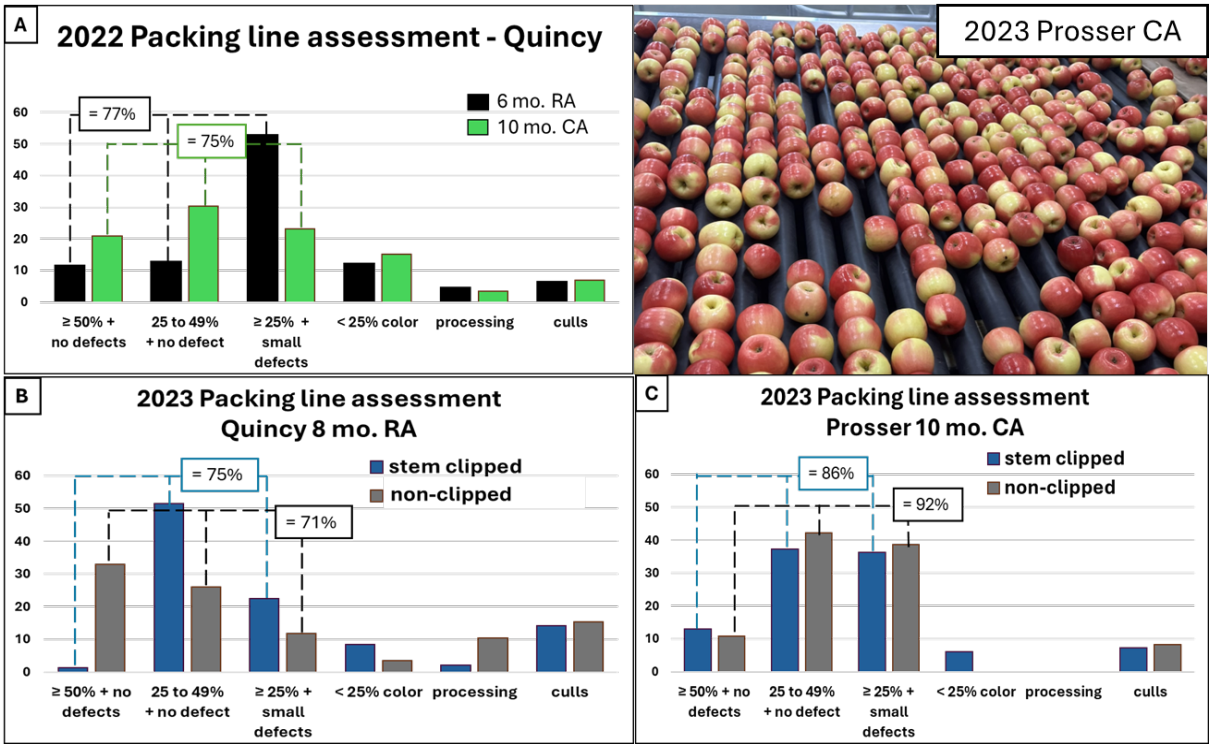



Figure 3. Packout results for the WA 64 packing line assessment conducted with fruit from Quincy (A and B) or Prosser 2023 (C). In 2022, the fruit was not stem clipped. Sample size is one bin per treatment, totaling six bins.

*Storage trials*

$CO_2$ sensitivity: WA 64 apples were stored for 6 months in CA chambers at the USDA with high $CO_2$ concentration. Evaluations occurred monthly for external symptoms, and fruit was kept at room temperature for two weeks after the end of the experiment. No internal or external $CO_2$ injury was found.

Dynamic controlled atmosphere: Fruit from Quincy and Prosser were transported to Union Gap and stored for 6 months at the Storage Control Systems cold room (37°F) in two SafePods (control: 2.0% $O_2$ and 1.0 % $CO_2$, treatment: 0.6% $O_2$ and 0.5% $CO_2$). In summary, fruit quality parameters were equivalent between treatments and similar over time. Better ratings for flavor were observed on fruit with lower oxen levels after 6 months in storage and a week at room temperature. The respiration coefficient and the low oxygen limit will be reported at a later date.

*Outreach*

- The article WA 64 – Tree Characteristics and Horticulture (authors: Bernardita Sallato, Sara Serra, Manoella Mendoza, Kate Evans, and Stefano Musacchi) is available at the WSU Tree Fruit website
- WA 64 Session in the WA State Tree Fruit Association 2024 Annual Meeting
- Both P3 plantings will continue to host the WA 64 field days in conjunction with the WSDA sites (Sunrise and Roza).
- Two Fruit Matters articles about fruit quality and storability will be written in 2025, based on the data collected by WTFRC in P3

**Project Title:** Life Cycle Assessment for Apple Production in the Pacific Northwest.

**Report Type:** Continuing Project Report

**Primary PI: Greg Thoma**
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**City/State/Zip:**

**Other funding sources (see attachment)**

**Cooperators**: Chad Kruger (WSU), Andrew Bierer (USDA ARS), Brent Milne (McDougall & Sons, Inc.), Derek Tweedy (Domex Superfresh), Greg Pickel (G.S. Long), Suzanne Bishop (Allan Brothers F), Marty Matlock (Nokose, Inc.)

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 161,531
Total Project Request for Year 2 Funding: $ 192,195
Total Project Request for Year 3 Funding: $ 174,900

Budget 1
Primary PI: Greg Thoma
**Organization Name:** Resilience Services, PLLC.
Contract Administrator: Greg Thoma
Telephone: 479-445-5277
Contract administrator email address: gjthoma@gmail.com

| | **2023** | **2024** | **2025** |
|---|---|---|---|
| Salaries (fully burdened) Thoma & Matlock | $32,500 | $48,500 | $55,000 |
| Benefits | | | |
| Wages | | | |
| Benefits | | | |
| RCA Room Rental | | | |
| Shipping | | | |
| ISO Review Panel | | | 15,000 |
| Travel | $4,800 | | |
| Plot Fees | | | |
| Miscellaneous | | | |
| Total | $37,300 | $48,500 | $70,000 |

2024 expenses to be invoiced:
Matlock salary: $8500
Thoma salary: $40,000
ISO review ($15,000) budgeted in 2024 is pushed to 2025 due to delays in receiving survey data which pushed back the ISO reporting schedule.
Travel: $0

Budget 2

**Co PI 2:** Hans Blonk
**Organization Name:** Blonk Sustainability Tools
Contract Administrator: Hans Blonk
Telephone: 0031628848241
Contract administrator email address: hans@blonksustainability.nl

| | **2023** | **2024** | **2025** |
|---|---|---|---|
| Salaries | $65,200 | $92,400 | $92,400 **+$10,000 = $102,400** |
| Benefits | | | |
| Wages | | | |
| Benefits | | | |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | | | |
| Travel | $2,500 | $2,500 | $2,500 |
| Survey Dissemination | | | |
| Miscellaneous | | | |
| Total | $67,700 | $94,900 | $94,900 **+ $10,000 = $104,900** |

**Actual costs 2024**

Salaries: €85,486.50 or $92,641.72, based on an average exchange rate of 1.0837 for 2024. Based on inflation and anticipated workload for the tool development, the third-year budget is increased by $10,000 above the originally submitted budget.

Budget 3

**Co-PI 3**: Georgine Yorgey (WSU lead Co-PI), Suzette Galinato (WSU Co-PI)
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**Contract Administrator:** Stacy Mondy
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**Contract administrator email address:** arcgrants@wsu.edu
**Station Manager/Supervisor:** Chad Kruger (Tree Fruit Research and Education Center Director, Center for Sustaining Agriculture & Natural Resources Director)
**Station manager/supervisor email address:** cekruger@wsu.edu

| | 2023 | 2024 | 2025 |
|---|---|---|---|
| Salaries (1) | $14,901 | $22,962 | |
| Benefits | $4,972 | $10,064 | |
| Wages (2) | $28,800 | $9,318 | |
| Benefits | $2,938 | $951 | |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies (3) | $1,920 | | |
| Travel (4) | $3,000 | $500 | |
| Plot Fees | | | |
| Miscellaneous | | $5000 | |
| | | | |
| Total | $56,531 | $48,795 | $ 0 |

**Total Spent, as of 2024: $76,611**

## Objectives

We are conducting a lifecycle assessment to evaluate the environmental impacts of apple production from orchard establishment through harvest and cold storage (or alternate supply chain stage where the apples are ready for delivery to the consumption stage). The analyses will include the upstream (e.g., suppliers) and downstream (e.g., waste management) processes associated with apple orchard and warehouse operations (e.g., production of raw, auxiliary, and operating materials), including all relevant inputs, emissions into the air, water, and soil, and disposal of all elements of production (e.g., pruning wood and end-of-life trees). This will enable the apple industry to respond with cost-effective adaptive strategies to sustain production and profitability into the future, address buyer concerns, take advantage of government programs, and prepare for potential federal regulatory oversight (e.g., reduction in GHG emissions) being developed. The results of this LCA will also offer insights into the entire production system from which the sector could construct a public policy or public relations narrative regarding the impacts of tree fruit production on climate change and other environmental impacts.

***The primary project goals are to provide a baseline assessment of the environmental sustainability*** of Northwest apple production and to ***develop a scenario analysis tool*** that will support the evaluation of management decisions over the orchard life cycle and provide the standard against which future improvements can be documented. These objectives are being achieved through stakeholder-engaged efforts to define the sector's most relevant data and sustainability metrics.
At scale, we envision a continuum of orchard stages. As new practices and technologies emerge, the scenario tool can inform decisions regarding the next establishment phase's management. Since environmental sustainability metrics are vital components of the scenario analysis tool, baseline life cycle impact assessment results will be a key deliverable from this project and implemented in the tool.
Specific objectives of this project are:

- Design and test a comprehensive life cycle data collection survey to provide data for a baseline sustainability assessment [complete] (e.g., Carbon and water footprint, energy consumption, eutrophication, etc.) and the development of a scenario tool for the evaluation of alternate management scenarios (e. g., biomass to energy versus composting of end-of-life trees) [year 3 objective].
- Provide an evaluation of current sustainability metrics of a range of management alternatives of NW apple production – that is, ***a baseline suite of metrics against which future progress can be evaluated***. [85% complete]
- Develop an LCA model for environmental impact assessment and scenario testing.
- Engage stakeholders in the development of a scenario analysis tool with which producers can simulate alternate management practice effects on environmental sustainability metrics that can be used to identify strengths and weaknesses of alternate management systems to identify environmental hotspots as opportunities for improvement. [ongoing]

## Significant Findings

Workflow 1: Survey implementation

- An extensive list of over 100 questions for apple orchard growers was created based on a literature review, expert judgment and previous LCA experience. This list formed the basis for focused, in-depth interviews. Subsequently a shortened survey was deployed via Qualtrics and available for most of 2024. A total of 62 completed surveys (including focus group interviews) have been received and used to generate the inventory model in the Simapro LCA software platform.

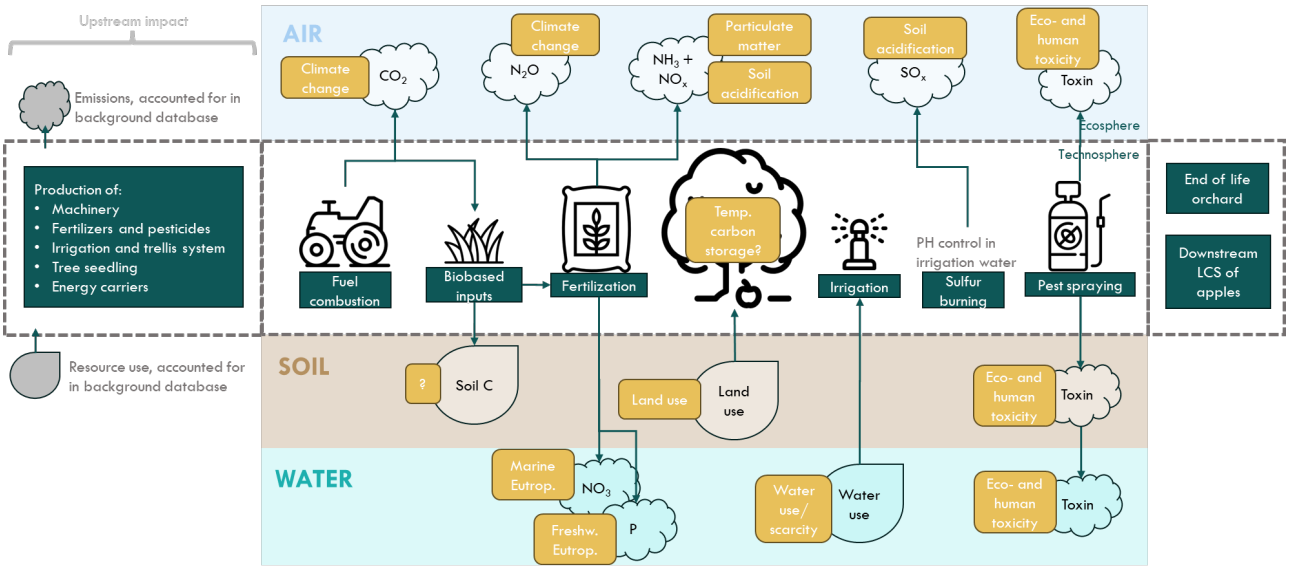



*Figure 1. Schematic overview of processes considered in the LCI and associated environmental impacts.*

Workflow 2: Life cycle inventory and report

- A parameterized lifecycle inventory (LCI) model has been created in the SimaPro software platform. The model includes an accounting of inputs (including upstream processes), outputs, and emissions of the establishment and production phases of apple cultivation. The model is being used for the full lifecycle impact assessment and sensitivity analyses using data collected from Qualtrics and focused surveys. A schematic overview of the production activities within the scope of the model, and the associated environmental impacts, are depicted in Figure 1.

- A full LCA report is in development and anticipated to be available for internal review in early March 2025 followed by an ISO external panel review. The report describes the LCA process and methodologies employed in detail.

Workflow 3: LCA tool developments

- All learnings regarding management practices and LCA calculations form the basis for the development of the scenario analysis tool in year 3 of the project. A virtual meeting soliciting stakeholder guidance for tool features and development was held 12/19/2024.

**Methods**

Quantifying the impacts of current Pacific Northwest apple production practices on the environment is important to understand the environmental impacts associated with apple production and supply chains in the region, to position the apple industry to be in compliance with buyer demands, and to engage with the USDA Climate-Smart Commodities Program. The LCA from this project will provide a baseline environmental profile and can assist in identifying opportunities for greenhouse gas mitigation and other sustainability efforts, in turn allowing the apple industry to make impactful, data-driven decisions. Further, the effort will support communication to educate the Pacific Northwest apple industry, retail partners, and consumers about the sustainability characteristics of

apple production. Finally, the research will assist the industry in generating a credible, science-based narrative showcasing its efforts as good caretakers of the land and resources.

An LCA relies on the concept of a functional unit, which allows comparison across different production systems. In this assessment, the functional unit will be reported in terms of 40 lb boxes and on a 1000 lb basis of apples for the fresh market, ready for delivery to the retail sector. Apples may be directly sold after harvest or stored in cold storage for up to several months. Differences in the storage period will be averaged for the LCA; however, in the sustainability assessment tool to be developed, the length of storage will be parameterized to enable an understanding of the potential effects on the sustainability characteristics. Further, alternative packaging and transport options will be included in the tool as scenario alternatives.

Broadly, this project has relied on stakeholder-engaged life cycle inventory data collection, which is being used in standard LCA software to calculate carbon and water footprints and other sustainability indicators. A lifecycle inventory model is constructed as a set of linked unit processes. Each unit process accounts for a specific activity in the supply chain (e.g., drip or other irrigation systems, or application of crop protection chemicals) and captures the full production chain of the system under study.

Figure 2 shows the workflows for the project. The overarching structure is highly integrated from the outset. The gradient shading is intended to indicate the degree of completeness of the activity. There is, of course, a linear flow that is depicted by the three workflow columns. Close coordination between the three workflow columns has been achieved through weekly or biweekly team meetings and is contributing to the overall project's success. Our efforts in year two were focused on completing the survey data collection for multiple production blocks as well as for the nursery, end of life, and packing house stages. Surveys were refined interactively and distributed widely.

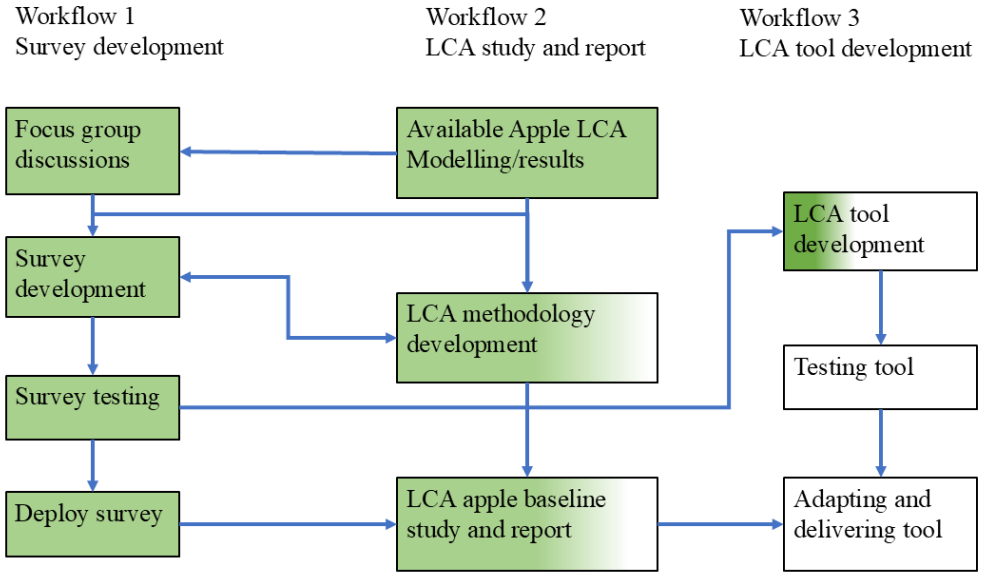



Figure 2. Workflow for the project. the gradient highlight indicates approximate level of completion of

**Status update**

The work done this past year has been focused on data collection and inventory modeling for the LCA. We have developed a good understanding of the most important data points to collect via the survey and insight into common management practices to ensure the model is complete and representative, to the extent possible given the data availability.

The development and use of the parameterized LCI model was an important step in automating large-scale survey data integration into the LCA calculations. The LCI model is the foundation for the LCA.

**Current Inventory Results**

The Qualtrics survey platform has been active for approximately a year, and we currently have 49 online survey responses and 13 focus group interviews representing up to 29 companies and 25 unique ZIP codes with the following distribution of blocks:

- CONV Gala/Fuji/Red Delicious/Granny: 34
- ORG Gala/Fuji/Red Delicious/Granny: 9
- CONV Honeycrisp: 4
- ORG Honeycrisp: 12

Separate surveys for pre- and post-orchard production stages include:

- Nurseries:
  - 1 complete survey received
  - 4 additional possible
- Packinghouses:
  - 3 complete surveys and 3 partial surveys received
  - 3 additional possible

Finally, we have some expert opinion information from informal interviews regarding the end-of-life management of removed trees. They use a 2-step process: excavation and grinding. For a mature apple orchard (over 20 yr old), the total estimated fuel use is 180 gal/ac. For a younger, dwarfing orchard, it is 90 gal/ac of fuel.

Table 1 presents a summary of survey and interview results. We do not present footprint results for the carbon footprint in this continuation report as the process is nearing completion and it is anticipated that some refinement of the modeling will occur during the final reconciliation stage of the modeling.

**Table 1. Summary of survey responses. Note that values spanning both survey and focus group columns are averages of all reported (combining survey and focus group)**

| | | survey average | focus group interview average |
|---|---|---|---|
| **in-row spacing** | feet | 4.3 | 3.9 |
| **between row spacing** | feet | 12.9 | 11.9 |
| **expected orchard lifetime** | years | 28.1 | 20.4 |
| **acres replanted annually** | % of all acres | 3.5% | |
| **acres topworked annually** | % of all acres | 2.3% | |

| | | | |
|---|---|---|---|
| **yield** | MT/acre | 21.0 | 27.4 |
| **annual irrigation** | acre-feet/acre | 3.51 | 3.53 |
| **trellis type** | % V trellis | 33% | |
| | % vertical trellis | 46% | |
| | % no trellis | 21% | |
| **synthetic N (average of those reporting reasonable non-zero values)** | lb N/acre | 44.1 | 37.4 |
| **N from biobased sources (average of those reporting additions)** | lb N/acre | 25.9 | 34.1 |
| **synthetic P (average of those reporting reasonable non-zero values)** | lb P2O5/acre | 24.2 | 48.3 |
| **biobased P (average of those reporting reasonable non-zero values)** | lb P2O5/acre | 19.3 | 56.3 |
| **synthetic K (average of those reporting reasonable non-zero values)** | lb K2O/acre | 40.2 | 63.0 |
| **biobased K (average of those reporting reasonable non-zero values)** | lb K2O/acre | 12.1 | 58.5 |
| **diesel use (average of reported)** | gallons/acre | 35.7 | |
| **propane (average of reported)** | gallons/acre | 37.0 | |
| **gasoline (average of reported)** | gallons/acre | 16.3 | |
| **electricity (average of reported)** | kWh/acre | 1562.0 | |

Table 2 presents the outline for the ISO report to be completed during the first quarter of 2025. Internal stakeholder review of the report is anticipated for early March, and external ISO panel review to begin in April.

Table 2 US Apple ISO Report Outline

| | Section |
|---|---|
| **1. Goal & Scope** | |
| | **1.1 Introduction** |
| | **1.2 Goal** |
| | **1.3 Scope / Audience** |
| | **1.4 Data collection** |
| **2. Methodology** | |
| | **2.1 Methodological standards and approach** |
| | **2.2 Environmental impact assessment method** |
| | **2.3 Modelling of apple cultivation & handling** |
| | **2.3.1. Nitrogen modelling** |
| | **2.3.2 P modelling** |
| | **2.3.3 Pesticide modelling** |
| | **2.3.3 Built infrastructure modelling** |
| | **2.3.5 Nurseries** |
| | **2.3.6 End of Life** |
| | **2.3.7 Warehouse** |
| | **2.4 Carbon stock changes** |
| | **2.5 Allocation** |
| | **2.6 Cut-offs** |

| | |
|---|---|
| | **2.7 Data quality requirements** |
| **3. Life Cycle Inventory** | |
| | **3.1 Survey overview** |
| | **3.2 Inventory data** |
| | **3.3 Data sources and data quality** |
| **4. Life Cycle Impact Assessment** | |
| | **4.1 Carbon, water, and energy footprints** |
| **5. Life Cycle Interpretation** | |
| | **5.1 Contribution analysis** |
| | **5.2 Sensitivity analysis** |
| | **5.3 Uncertainty analysis** |
| **6. Conclusion** | |
| | **6.1 Discussion** |
| | **6.2 Conclusion** |

**Continuing efforts**

Focus points for next year (year 3 out of 3 of this project) are the following:

- Completing the LCIA and report, submitting for internal review, revision and subsequently to an external ISO panel.
- Finalize processing the survey responses to populate the LCI model allowing block specific LCA results.
- Sensitivity and uncertainty analysis based on the survey results, using the LCI model.
- Submit the LCA report to an ISO conformance review. The report will present the goal and scope, methodology, data inventory, results, conclusions, and discussion regarding the environmental impact of Pacific Northwest apple production.
- Implementing the requirements for the LCA tool, which is to be developed in year 3.

**Scenario assessment tool**

The tool will be built on and include baseline life cycle impact assessment results and will support simulation of management practices on environmental sustainability metrics. It will allow users to identify strengths and weaknesses of alternate management systems to identify environmental hotspots as opportunities for improvement.

We met with stakeholders in December 2024 to discuss the tool requirements and development process (Figure 1). The consensus was to develop an excel spreadsheet-based tool that would consider the capacity differences between the vertically integrated operations and the smaller independents. The goal is to provide utility to a range of operators. While the vertically integrated operations (i.e., through to the retail receiving gate) are likely to have someone who can be

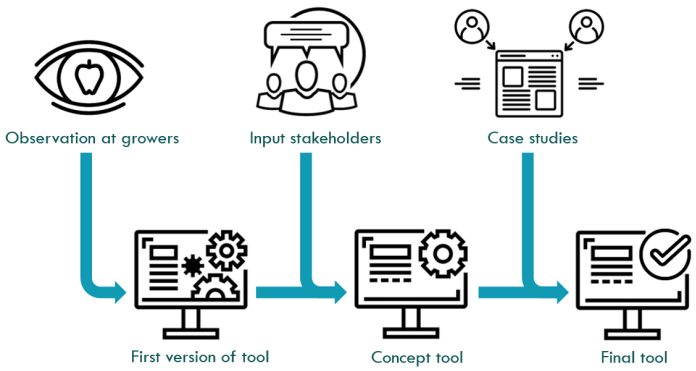



Figure 1. Schematic of the proposed stakeholder-engaged process for creating the scenario analysis tool.

assigned to use the tool, we know from many other situations that small, independent farms and ranches generally struggle to use complicated tools / models – both from a technical skillset perspective and from a "too many competing priorities" perspective. To support achieving this balanced tool the process outlined in Figure 1 is planned.

A mock-up of the user interface is shown below. There will be data input screens that emulate the survey used for construction of the lifecycle inventory of the LCA and graphical and tabular presentation of results, including comparison of alternate scenarios.

## Input

### Cultivation

| General information | Unit | 2021 | 2022 | 2023 | PNW average |
|---|---|---|---|---|---|
| Gross area apple cultivation | ha | 0 | 0 | 0 | 5 |
| Lifetime trees | year | | | | |
| Tree density | trees / ha | 3000 | | | |
| Leaving pruning wood on land | (choice) | | | | |
| Leaving mowed grass on land | (choice) | | | | |

| Use and diesel consumption of machinery | Unit | 2021 | 2022 | 2023 | PNW average |
|---|---|---|---|---|---|
| Tractor, large (> 30 hp) and car | hour/ha/year | | | | |
| Tractor, small (< 30 hp) | hour/ha/year | | | | |
| Scissor lift | hour/ha/year | | | | |
| Forklift | hour/ha/year | | | | |
| Pump (e.g. irrigation pump, fertigation u | hour/ha/year | | | | |
| Other powered implement (> 30 HP) | hour/ha/year | | | | |
| Other powered implement (> 30 HP) | hour/ha/year | | | | |
| Diesel | L/ha | | | | |
| Share of contract work (excl. planting) | % | | | | 0% |

| Fertilizer use | Unit | 2021 | 2022 | 2023 | PNW average |
|---|---|---|---|---|---|
| Synthetic fertilizer: Amount of N | kg/ha/year | | | | |
| Synthetic fertilizer: Amount of P | kg/ha/year | | | | |
| Synthetic fertilizer: Amount of K | kg/ha/year | | | | |
| Total use of organic manure (for transport) | kg/ha/year | | | | |
| Animal manure: Amount of N | kg/ha/year | | | | 0 |
| Amount of lime (CaCO3) | kg/ha/year | | | | 0 |

Calculate the carbon footprint of apple cultivation based on primary data

# Apple Carbon Footprint Tool

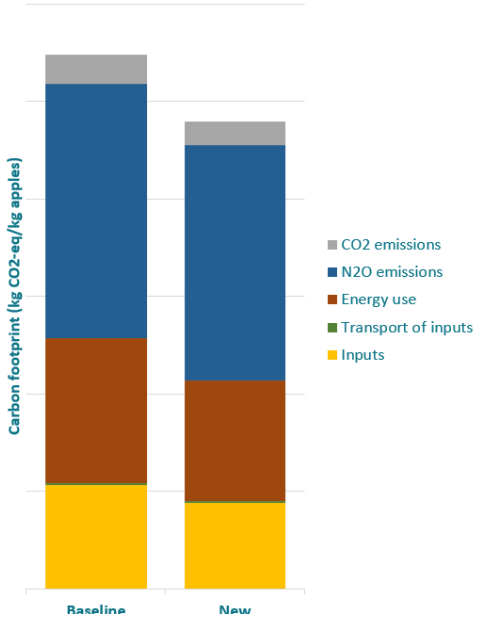
Below you can see the carbon footprint of the baseline scenario, and the new situation. You can see both an aggregated and detailed break-down of emissions.

| Value chain stage | Baseline | | New | |
|---|---|---|---|---|
| **Inputs** | **0.106** | | **0.088** | |
| Synthetic fertilizers | | 0.074 | | 0.061 |
| Organic fetilizers | | 0.009 | | 0.008 |
| Pesticides | | 0.023 | | 0.019 |
| Lime | | 0.001 | | 0.000 |
| **Transport of inputs** | **0.002** | | **0.002** | |
| Transport of inputs | | 0.002 | | 0.002 |
| **Energy use** | **0.149** | - | **0.124** | - |
| Electricity | | 0.048 | | 0.040 |
| Diesel | | 0.101 | | 0.084 |
| **N2O emissions** | **0.261** | | **0.241** | - |
| N2O emissions synthetic fertilizers | | 0.117 | | 0.097 |
| N2O emissions organic fertilizers | | 0.003 | | 0.003 |
| N2O emissions crop residues | | 0.142 | | 0.141 |
| **CO2 emissions** | **0.030** | - | **0.025** | - |
| CO2 emissions lime | | 0.017 | | 0.014 |
| CO2 emissions urea | | 0.013 | | 0.011 |
| **Total** | **0.548** | | **0.480** | |

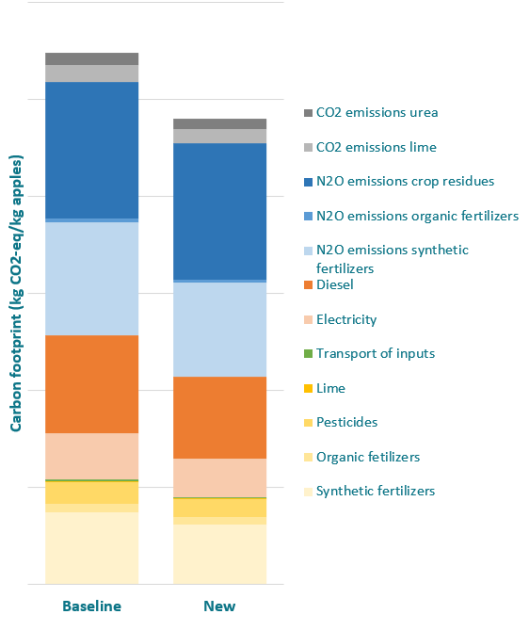
Example values only

| Difference | -12% |
|---|---|

Carbon footprint of 1 kg apples - aggregated




Carbon footprint of 1 kg apples - detailed



# **Project Title:** Towards next generation maturity indices: apple biomarker discovery; AP-22-101A

**Report Type:** Continuing Project Report - Year 2

**Primary PI:** Dr. Loren Honaas
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**Cooperators**: AllanBrothers Inc., Stemilt LLC.

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $ 266,267
**Total Project Request for Year 2 Funding:** $ 155,303
**Total Project Request for Year 3 Funding:** $ 160,510

**Other related/associated funding sources:**
**Funding Duration:** annual congressional appropriation
**Amount:** $85,000
**Agency Name:** USDA ARS
**Notes:** 3-year total = $255,000: Personnel $180,000, Consumables/Supplies $30,000, Equipment (including computational resources): $45,000

**Funding Duration**: The funding source has expired but resources are still available.
**Amount**: $86,000
**Agency Name**: WSU Ficklin Start-Up Funds
**Notes**: These funds were used to purchase high-performance computing resources on WSU's Kamiak computing cluster. These resources will provide the computing power necessary for model development.

**Funding Duration**: 2017-2022
**Agency Name**: US National Science Foundation (NSF) Award #1659300
**Amount**: $150,000
**Notes**: A portion of this award was used to fund 600 Terabytes of storage for execution of scientific workflows and storage of results. We will use that infrastructure for this project.

**Funding Duration**: 2020-2025.
**Amount**: $100,000
**Agency Name**: Auburn Harkess Start-Up Funds
**Notes**: These funds are being used to purchase molecular genomics reagents and equipment for apple DNA and RNA isolation and sequencing.

**Budget 1**
**Primary PI:** Dr. Loren Honaas
**Organization Name:** USDA ARS TFRL
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**Contract administrator email address:** chuck.myers@ars.usda.gov, sharon.blanchard@ars.usda.gov
**Station Manager/Supervisor: N/A**
**Station manager/supervisor email address: N/A**

| Item | 2022 | 2023 | 2024 |
|---|---|---|---|
| **Salaries** | 44,775 | 45,894 | 47,042 |
| **Benefits** | 18,806 | 19,276 | 19,758 |
| **Wages** | | | |
| **Benefits** | | | |
| **Equipment** | | | |
| **Supplies** | | | |
| **Travel** | | | |
| **Miscellaneous** | | | |
| **Plot Fees** | 5500 | 5700 | 5900 |
| **Total** | 69,081 | 71,410 | 72,700 |

**Footnotes:** Plot fees for WSU SRO blocks that provide validation samples.

**Budget 2**
**Co PI 2:** Dr. Stephen Ficklin
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**Contract administrator email address:** anastasia.mondy@wsu.edu
**Station Manager/Supervisor: N/A**
**Station manager/supervisor email address: N/A**

| Item | 2022 | 2023 | 2024 |
|---|---|---|---|
| **Salaries** | 61509 | 63969 | 66528 |
| **Benefits** | 19677 | 20464 | 21282 |
| **Wages** | | | |
| **Benefits** | | | |
| **Equipment** | 46,000 | | |
| **Supplies** | | | |
| **Travel** | | | |
| **Miscellaneous** | | | |
| **Plot Fees** | | | |
| **Total** | 127,186 | 84433 | 87810 |

**Footnotes:** Postdoc will be co-advised by all project PIs. Salaries and benefits are estimated to be inflated by 4% per year per WSU guidelines.

**Budget 3**
**Co-PI 3:** Dr. Alex Harkess
**Organization Name:** Auburn University and HudsonAlpha Institute for Biotechnology
**Contract Administrator:** Mercedes McKoy
**Telephone:** 334-844-3951
**Contract administrator email address:** MLF0015@auburn.edu
**Station Manager/Supervisor: Optional**
**Station manager/supervisor email address: Optional**

| Item | 2022 | 2023 | 2024 |
|---|---|---|---|
| **Salaries** | | | |
| **Benefits** | | | |
| **Wages** | | | |
| **Benefits** | | | |
| **Equipment** | | | |
| **Supplies** | | | |
| **Travel** | | | |
| **Miscellaneous** | 70,000 | | |
| **Plot Fees** | | | |
| **Total** | 70,000 | 0 | 0 |

**Footnotes:** Miscellaneous funds are for RNA-Seq: this is global gene activity analysis of a majority of the total validation samples, estimate analysis of 350 total samples from 45 cultivar/years.

**Budget 4 N/A**
**Co-PI 4:** Dr. James Mattheis
Co-PI requests no funding.

**Objective:**
Develop and improve methods for biomarker discovery.

A. Use novel analytics and modeling approaches to strengthen biomarker discovery approach
B. Generate new global-scale gene activity data from current and new multi-year samples for rapid validation
C. Investigate disagreement between technologies for gene activity estimates to enhance translation to NGMIs

**Year 4 goals:**

1. Analysis of remaining validation sample sets
2. Finish prototype NGMI model
3. Summarize commercial prototype NGMI trials emphasizing storage outcomes

**Significant findings/results from 2024:**

1. 2023-2024 validation samples in the queue for analysis at HudsonAlpha
2. Storage trial outcomes reveal a key opportunity to test biomarker panel
3. Modeling paper (in review) suggests data requirements: 2,500 - 3,000 datasets
4. Full stability analysis of current NGMIs shows minimal overfitting
5. Prototype "test kits" + nanotech sensing for NGMI show promise
6. Prototype "translator algorithm" allows models to run on new data types
7. Enhanced NGMI aims to use weather forecast to predict *future* fruit maturity (1 week out)

**Methods (Significant findings indicated in parentheses)**

***Analysis of validation samples***

*(Significant finding #1)*

We have been building a catalog of samples that we can use to test and/or improve our pome fruit maturity prediction models for the last 7 years. These include 32 genotypes in total (14 cultivars and 18 accessions from an original RosBreed apple population at Washington State University's Sunrise Orchard; rosbreed.org), combining for at least 113 unique orchard year combinations (e.g. orchard a vs. b; orchard year 1 vs. 2). Altogether, a grand total of 1,686 unique biological samples have been collected and retained in our sample catalog, over half of which have been sequenced and hundreds more are queued for analysis. Each unique sample has corresponding at-harvest fruit quality data (weight, color, internal ethylene content, defects, firmness, Brix˚, TA, starch), as well as postharvest outcomes, and other meta data. In the 2023 and 2024 harvest seasons (funded by this project), 519 unique biological samples were added to the catalog (~1/3rd of all samples we have in hand), including samples from research blocks and commercial samples from industry partner AllanBros Inc.

Raw sequencing data are processed as described in Hadish *et al.* (2022) & Honaas *et al*. (2021). Gene activity profiles are used to predict various fruit physiological traits (internal ethylene concentration, starch, harvest date, disorder incidence, etc.). Model predicted values are plotted against the actual values and Root Mean Square Error (RMSE) and Pearson's $R^2$ were used to assess model performance.

***Commercial storage trials***

*(Significant finding #2)*

In order to test whether our prototype maturity index will provide actionable information for growers, we conducted storage trials of commercial lots of apple fruit. Our commercial partner provided fruit from select lots of nominally equivalent fruit (i.e. similar storage and marketing plan) and agreed to provide their storage outcome data. At receipt we collected fruit physiological data and peel samples of this fruit (stored at -112˚F until RNA was extracted as per Hadish *et al.,* 2024). We stored additional samples of the fruit in air at 33˚F for 6-8 months followed by a simulated supply chain of 7 days at 68˚F, after which we conducted a full fruit quality assessment as described in (Honaas et al 2021). We

compared commercial cull percentages of the same lots to the percentage of fruit with disorders that developed in our storage trials and the correlation in disorders/cullage with at harvest starch clearing and internal ethylene concentrations (Figure 4).

***Evaluation of trait prediction model performance as a function of input data***
*(Significant finding #3)*
As we reported previously, we have been using very large, publicly available data sets to estimate how models that predict traits improve as more data are added. Following the same approach as we previously reported, we expanded this experiment to include other plant species and other traits (Hadish *et al.* preprint 2024).

**In silico *tests of model performance***
*(Significant finding #4)*
The models we are building to predict apple fruit maturity are prone to overfitting. That is, the models can tend to find patterns in the data that seem to be predictive because they are correlated, when in fact they are just correlated by chance and will therefore have poor accuracy in real world testing scenarios. As described in Hadish *et al.* (2024) we conducted a full stability analysis of the maturity models to test for overfitting using a computational approach.

***Building a point-of-contact (POC) or near POC test***
*(Significant finding #5)*
Our current model of POC test uses a BioEcho kit to extract RNA, RT-LAMP assay to convert RNA to amplified cDNA, and a Cas12a+Gold Nanoparticle colorimetric assay to quantify marker expression level as an indicator of maturity. The BioEcho kit yields sufficient RNA, yet low 260/280 wavelength ratios as calculated with a Nanodrop™. Modifications are being made to the protocol in collaboration with BioEcho, and have resulted in higher 260/280 ratios; however, further optimization is required. We chose 17 potential markers from the internal ethylene prediction models to find the relationship between qPCR to RNA-Seq values and how it fits our models. To validate our primer design and prepare for qPCR, we have used PCR to test 5 out of the 17 markers so far, 4 were positive. We have successfully tested the RT-LAMP protocol and have designed/ordered the conjugated gold nanoparticles for the colorimetric assay to be ready for testing in the new year.

***An algorithm to convert POC test data into a form that models can use***
*(Significant finding #6)*
The patterns that we discover in the data that are predictive of fruit maturity are derived from technology (RNA-Seq) that is fundamentally different than what would be used in a POC test (e.g. qPCR). It may therefore be necessary to convert the data from a POC test into a form that can be used as a model input. The relationship between RNA-Seq and qPCR data has been investigated for use in other studies, though it often involves transforming the RNA-Seq values (Li *et al.* 2019). Since RNA-Seq values are locked into the prototype models we have developed, we cannot transform the source data. Thus, we aim to transform POC expression values for initial testing.

To achieve this, we selected 16 genes of interest (GOIs) from a maturity-predictive gene list generated by our models for testing using Real Time Quantitative Polymerase Chain Reaction (RT-qPCR), a potential type of POC test. Primers were designed for the selected genes following our previously published primer development methods (Hadish *et al.*, 2024; Waite *et al.*, 2023) for RT-qPCR tests. qPCR generates gene expression values called Relative Expression (RE), which is calculated using the Cq values of the GOIs and the geometric mean of two reference genes. We then ran a Pearson correlation analysis between RNA-Seq normalized counts (TMMs - the data type used to create our models) and RE values for each gene. Genes with correlations higher than 0.75 were selected for data transformation (9 out of 16). We then tested four different methods for transforming RT-qPCR RE values in order to convert them into a value that can more easily be used as input by the fruit

maturity prediction model. The correlations between the transformed qPCR RE and model TMM expression was evaluated using Pearson's r (Table 1).

Method #1: Multiply the Relative Expression (RE) of a gene of interest (GOI) by the geometric mean of the RNA-Seq normalized counts (TMM) of the reference genes, following similar methods using reference gene expression for qPCR (Pfaffl 2001, Pfaffl 2004).

$$Method\ 1\ (M1) = RE_{GOI} * (TMM_{Reference\ gene\ 1} * TMM_{Reference\ gene\ 2})^{\frac{1}{2}}$$

Method #2: Multiply the value generated using Method #1 by 50.

$$Method\ 2 = M1 * 50$$

Method #3: Multiply the value generated using Method #1 by an appropriate scaling factor (5, 10, 50) on a gene by gene basis, based on each gene's expression level in RNA-Seq data, so that the transformed qPCR RE was closer to the same relative order of magnitude as the RNA-Seq TMMs.

$$Method\ 3a = M1 * 5$$

$$Method\ 3b = M1 * 10$$

$$Method\ 3c = M1 * 50$$

Method #4: Subtract the reference genes' TMM from each GOI's TMM for each sample, and then multiplying the result by the RE value.

$$Method\ 1\ (M1) = RE_{GOI} * (TMM_{GOI} - (TMM_{Reference\ gene\ 1} * TMM_{Reference\ gene\ 2})^{\frac{1}{2}})$$

***Improving lead time for the NGMI***

*(Significant finding #7)*

An important component of our commercial concept is to allow growers to set custom thresholds for NGMI values - just as growers use current maturity indices. In order to provide more lead time, we are exploring how to use weather forecasts to predict future NGMI values. This by-and-large leverages our existing data set where weather, pick date, fruit quality, gene activity and more can be used in concert to investigate how model values change based on weather patterns leading up to harvest dates.

**Materials and Methods Work Cited**

Hadish *et al.* 2024 - https://doi.org/10.1371/journal.pone.0297015
Honaas *et al.* 2019 - https://doi.org/10.1016/j.postharvbio.2018.09.016
Hadish *et al.* preprint- https://doi.org/10.1101/2024.12.09.627626
Li *et al.* 2019 - https://doi.org/10.1186/s12864-019-5661-x
Waite *et al.* 2024 - https://doi.org/10.1093/g3journal/jkad120
Pfaffl 2001 - https://doi.org/10.1093/nar/29.9.e45
Pfaffl 2004 - "Quantification strategies in real-time PCR." *AZ of quantitative PCR* 1 (2004): 89-113.

**Results and Discussion (Significant findings indicated in parentheses)**

***NGMIs can help predict fruit storability***

*(Significant findings #1-4)*

To illustrate what we are measuring for our prototype NGMIs, we created a plot that shows the activity pattern, over time, for the top 50 genes from our harvest week prediction model (Figure 1).

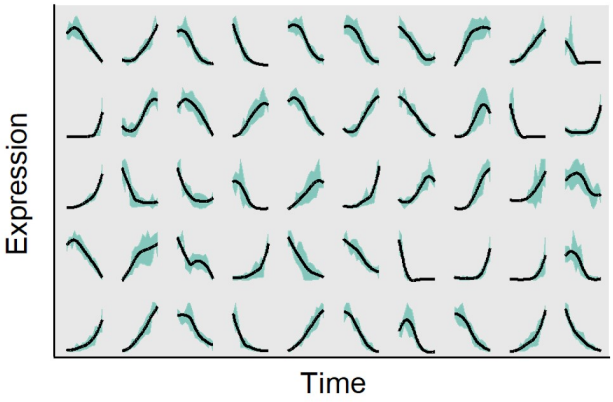


**Figure 1. Prototype NGMIs integrate information from many gene activity signatures to render a prediction.** Shown are normalized gene expression (i.e. gene activity) profiles in the 10-week apple time course experiment for the 50 genes most predictive of harvest week in our prototype model. Each line represents the change in gene expression of a single gene over time. Time is on the x-axis and normalized gene activity is on the y-axis for each gene. Shaded regions (teal) show standard error.

Using only the combined information of these gene activity patterns, we can estimate, for instance, the harvest date of the fruit to within at most ~11 days across all 14 tested cultivars; for some cultivars this prediction is often to within just a few days. In another model, we can accurately estimate the level of fruit internal ethylene concentration (IEC) across the same set of samples to within 1 part per million - this is generally the range of IEC values (as measured by Gas Chromatography/MassSpectrometry; GC/MS) that suggests autocatalytic ethylene production (Figure 2).

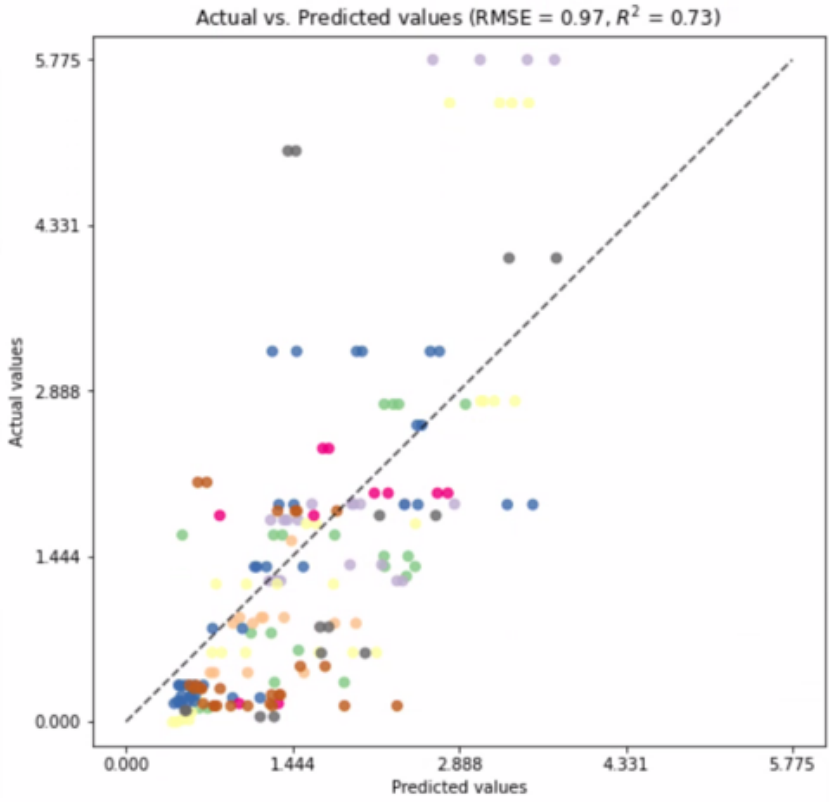

**Figure 2. Prototype NGMIs predict apple Internal Ethlyene Concentration (IEC) across years, orchards, and new cultivars.** Accuracy plot of actual IEC (measured via GC/MS) with NGMI predicted IEC. RMSE indicates the average error in PPM. Fruit were picked at regular intervals, centered on commercial harvest. This imposed a contrast of maturity that exceeds the harvest window, ensuring that we captured commercially relevant maturity changes. These contrasts were accurately predicted by the preliminary model. Points are colored by cultivar.

This past year we have explored how adding data improves our models using our pome fruit data - we are clearly in a high return phase of model development because we see substantial model accuracy increases as data are added. A key question is "How much data is needed to maximize model accuracy?" To estimate how models will work with several times as much data as we currently have for apple, we fetched publicly available data for several plant species and built models to predict plant age (a proxy for a trait like fruit maturity). These results suggest that model performance plateaus at around 2,500-3000 samples (each consisting of massive gene activity data and plant physiological data; Figure 3).

In addition to building a massive data set to ensure high model accuracy, another key part of the strategy to build a robust NGMI is to take advantage of natural apple diversity and build models with many cultivars - this comparative genomics approach should reduce overfitting by filtering out cultivar specific noise. Our recent stability tests of several models show that overfitting is limited, that is, the models are likely to be reliable and accurate across a wide range of real-world testing scenarios. This suggests that our approach is working, but also that robust models require a large amount of data that capture a lot of real-world noise.

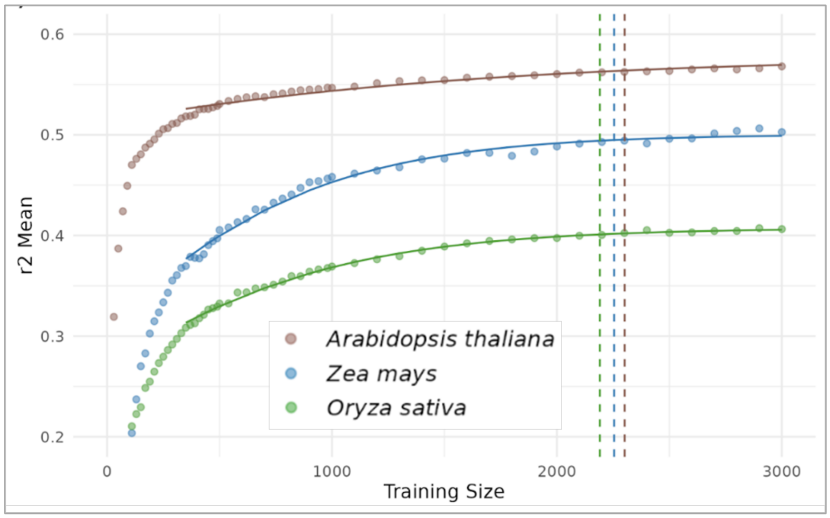


**Figure 3. Models that use gene activity to predict plant age reach max accuracy at around 2,250 data sets.** Publicly available data was used for this analysis. The order of traces in the legend is the same as in the plot. This suggests that to predict complex traits, like fruit maturity, the training data size is large. Because the pattern is similar across species we hypothesize that the data requirement is similar in pome fruit.
From Hadish *et al. preprint*.

Based on years of storage trial data in a related project on Envy™ apple, we have shown that internal ethylene concentration (IEC) of apple fruit is a better predictor of apple fruit maturity, and by extension a better predictor of fruit storability than starch clearing. This is consistent with many previous reports (Watkins and Mattheis 2019; references therein). Because IEC is a more reliable maturity index, we have built models that predict IEC in apple fruit using only gene activity signatures from fruit peel. In our experiments, these models are accurate to predict IEC across all 14 tested cultivars, indicating that this set of biomarkers may be a key component to an eventual NGMI. In the most recent commercial trials, we captured a contrast where starch clearing was a less reliable predictor of storability, yet IEC was a much better predictor for each pack (Figure 4A vs 4B). This key contrast will allow us to examine whether NGMIs would have provided better packout estimations than starch clearing. We hypothesize that because we can predict IEC with high accuracy, that we can estimate storability using the NGMI predicted IEC values instead of the direct IEC measurement.

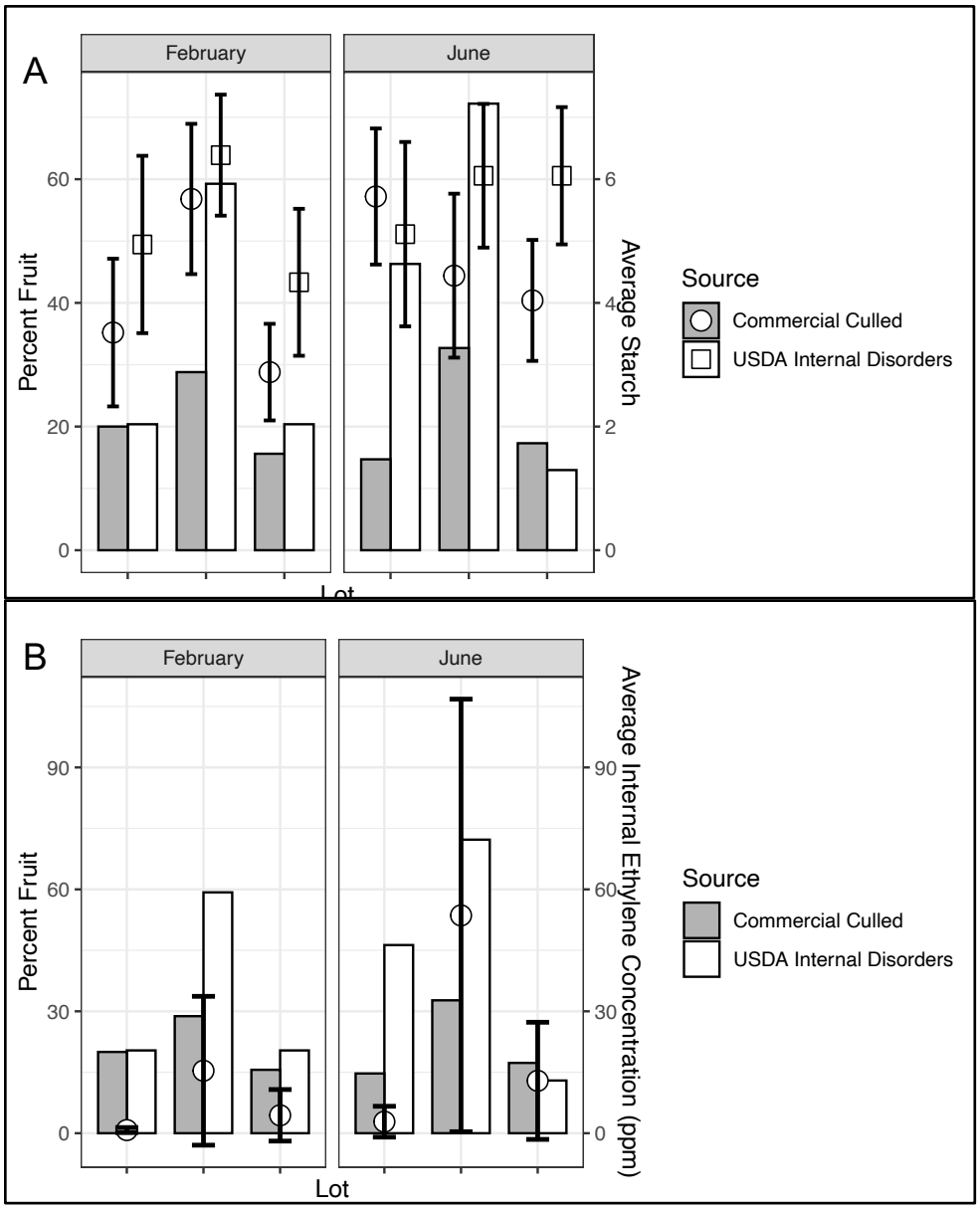


**Figure 4. Internal Ethylene Concentration predicts fruit storability better than starch clearing.** In the storage trials summarized above, fruit was stored at USDA in air in order to provoke loss of quality and therefore estimate the risk for loss of quality in a commercial scenario. **A)** Starch clearing for the pack in February was well correlated with packout and disorder incidence but was not for the pack in June. **B)** By contrast, especially within each pack, IEC was a reliable predictor of postharvest fruit quality as measured by cullage (commercial storage) and internal disorder incidence (USDA-TFRL).

### *Working towards a commercializable test*

In parallel with development of next generation maturity index models, we have been exploring how to build a fast and easy test for the panel of biomarker genes used to predict apple fruit maturity. While the models are built on super-resolution gene activity data sets for all ~40,000 apple genes, the tests used in a commercial setting will need to measure 10s of gene activity signatures via a fast and easy testing method. Kit based sample preparation methods are a key step towards adoption by industry because they provide reliable, affordable, and consistent results.

In our commercial concept, we envision a colorimetric assay (Figure 5 - see NGMI "puck") where several genes are tested and then analyzed via spectrometry. Prior to running such a test, peel samples must be collected from a representative batch of fruit, those tissues must be stabilized and then extracted, and finally amplified for colorimetric analysis via spectrometry; we have made substantial progress in the sample processing aspects of the project (synergy with WTFRC project AP-24-103A which uses the same testing approach). For sample collection and processing, we have been working with partner BioEcho who has provided us with a prototype kit that is custom made for our application. Last, we have begun development of RT-LAMP+Cas12a+Gold Nanoparticle quantification assays in order to quantify target mRNAs (the gene activity markers our models are based upon). Altogether, this approach is centered around commercially available reagents and equipment that will facilitate a pilot test of our NGMI technology in a real-world setting. Below are sections describing progress in each of these areas.

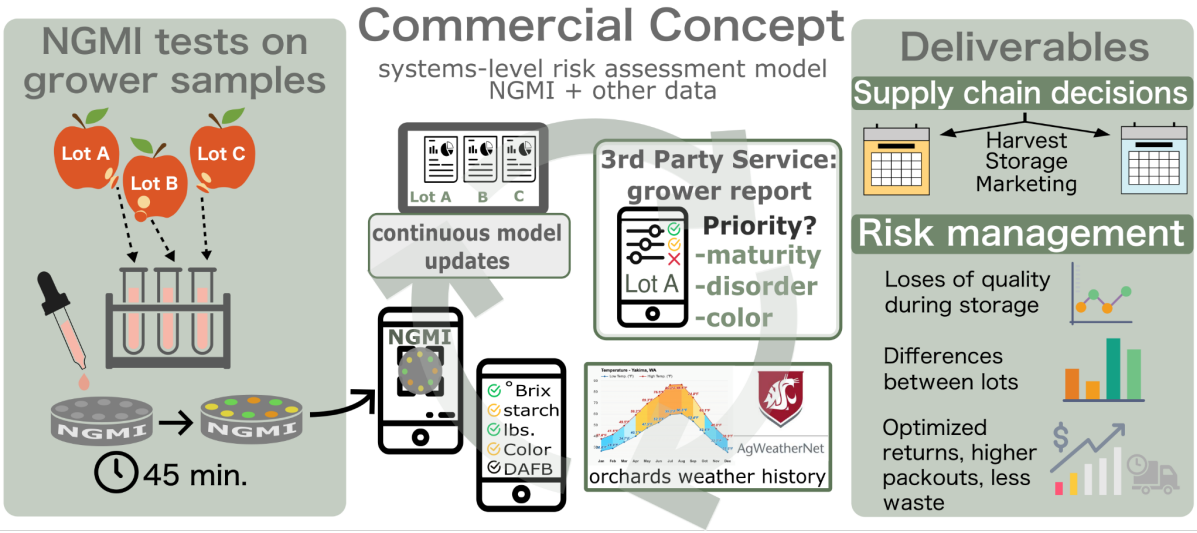


**Figure 5. Commercial concept for a Next Generation Maturity Index.**

*(Significant finding #5)*

***mRNA stabilization and extraction***

Our current RNA extraction method produces large quantities of high-quality RNA, which is required to generate high quality RNA-Seq data for use in model development. However, this extraction method is not conducive to the high throughput, low-cost requirements of a POC test. Fortunately, RNA for a RT-LAMP/PCR based POC test has lower quality and quantity thresholds compared to RNA-Seq. To develop a sufficient, low-cost RNA extraction method, we have been working with BioEcho to develop a rapid, kit-based extraction method that yields sufficient quantities and quality of RNA for RT-LAMP/PCR type POC. So far, extractions using kit prototypes have yielded enough RNA; however, the RNA has contaminants, as indicated by the 260/280 wavelength ratio acquired on a Nanodrop™. Despite the low 260/280 ratio, we saw both ribosomal bands on an RNA gel for samples with a concentration greater than 10 ng/uL indicating that the RNA was non-degraded and could be used for future applications. We continue to collaborate with BioEcho to improve the quality of RNA collected using their kits by testing modifications made to the protocol. Doing this, we have seen an increase in 260/280 ratio for samples that have been more finely ground when frozen.

***Target mRNA amplification***

The targets of our current biomarker panels are mRNAs - the relative number of mRNA copies of several target genes are considered together to produce a prediction about fruit such as the relative maturity of fruit lots (see Figure 1). For measurements of the target gene mRNAs, a common practice

is to amplify the targets using a PCR, or PCR-like, approach - amplification improves signal to noise ratio and allows tests to be run on small mRNA samples. For NGMIs specifically, we aim to use RT-LAMP to amplify the target genes because it is both faster than qPCR and requires less-expensive equipment. We use a six-primer approach and assay kit designed by a third party (Thermo Fisher Scientific, Waltham, MA). Such commercially available assays are fast and reliable.

Before we can use RT-LAMP for our POC test, we need to pick and test markers that are predictive of fruit maturity. We designed PCR primers for 17 genes of interest that were chosen from a list of overlapping predictive genes in the foundational and validation data sets in the internal ethylene model. The primers for 5 out of the 17 genes have been tested so far on 18 Envy™ samples collected at the time of harvest. 4 out of the 5 primer pairs yielded a single band for all 18 samples and are ready for quantification using qPCR. The RT-LAMP protocol has been successfully tested for the same 5 markers.

***RT-LAMP product detection***

The last step in the sample analysis process is quantification of the RT-LAMP assay products, which we aim to do via a Cas12a+Gold Nanoparticle detection system (Figure 6). This assay produces a result that can be read by a commercially available and inexpensive spectrometer. We are working towards a fast and low-cost point-of-contact test kit to quantify marker gene expression using Loop-Mediated Isothermal Amplification (LAMP) and Cas12a+Gold Nanoparticle assays.

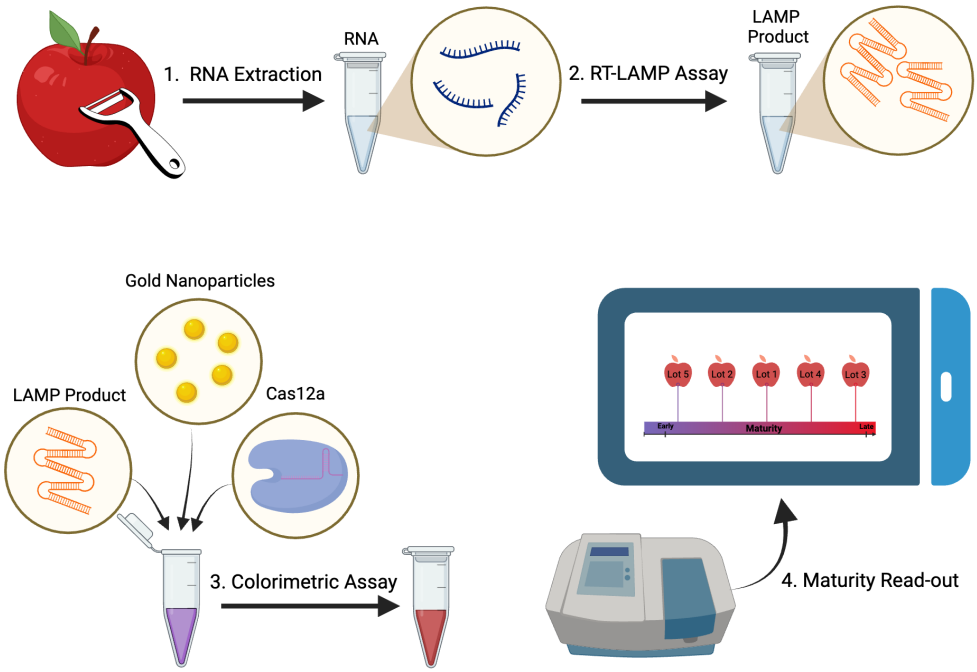


**Figure 6. Sample processing schematic for NGMI prototype kit.** We aim to build a kit that includes sample processing, extraction, and biomarker quantitation.

We chose RT-LAMP instead of the popular Polymerase Chain Reaction (PCR) method because it is an easier, faster, and a cheaper alternative to qPCR. We combine the LAMP assay with a Reverse Transcription (RT-LAMP) step that converts RNA to cDNA to simplify and hasten the process after RNA extraction (Figure 6, Step 2). Furthermore, the RT-LAMP assay is preferable because it does not require the expensive real-time thermocycler machine necessary for qPCR. After cDNA is amplified using RT-LAMP, we plan to quantify it with a Cas12a+Gold Nanoparticle colorimetric assay (Figure

6, Step 3). The addition of Cas12a enzymes and Gold Nanoparticles to our RT-LAMP assay result in a measurable color change in the tube from purple to red. This color change is sensitive to the amount of amplified marker present in the reaction and can be read using a spectrophotometer to determine the amount of each target gene activity in each sample (Figure 6, Step 4).

*(Significant finding #6)*

***Progress towards actionable information from a POC test***

A practical component to interpreting the results of a POC test, where RT-LAMP products are quantified via a colorimetric assay, is the calculation of a trait prediction based on the assay results. While the model we have is accurate, it requires digital data that is time consuming and expensive to generate. To bridge this gap, we are exploring ways to convert our RT-LAMP data to a form that approximates the digital data so it can be interpreted by the trait prediction model. Our initial results with qPCR suggest that an objective approach to transform the data maintains a high correlation (Table 1), and ***tests of the model with the transformed data show that the accuracy of prediction is similar to the RNA-Seq data.***

Methods 1-3 did not change the agreement between the two technologies, indicating that these types of scaling based transformations do not fundamentally alter how the data will be interpreted by the models. Correlations using Method 4 varied from the untransformed correlations, indicating that this type of transformation may have a gene specific effect that would need to be evaluated further for its usefulness moving forward. While this may not be necessary for the final test, it does provide important information on how the fundamentally different methods to measure gene activity (RNA-Seq vs qPCR vs RT-LAMP) influence how trait predictions, like fruit maturity, are impacted.

| Gene ID | Untransformed | Methods 1-3 | Method 4 |
|---|---|---|---|
| Gene 1 | 0.879 | 0.911 | 0.594 |
| Gene 2 | 0.880 | 0.899 | 0.815 |
| Gene 3 | 0.705 | 0.763 | 0.815 |
| Gene 4 | 0.952 | 0.970 | 0.893 |
| Gene 5 | 0.841 | 0.847 | 0.762 |
| Gene 6 | 0.894 | 0.885 | 0.784 |
| Gene 7 | 0.882 | 0.893 | 0.838 |
| Gene 8 | 0.873 | 0.891 | 0.923 |
| Gene 9 | 0.917 | 0.871 | 0.912 |

**Table 1. The transformation of qPCR data to a form that approximates digital gene activity data (a.k.a. RNA-Seq) does not substantially change correlation values.** This suggests that different methods for measuring gene activity, even though they each have distinct assumptions and technological biases, are effectively interchangeable. Tests of the model using this new data show similar accuracy to the original data.

*(Significant finding #7)*

***Increasing lead time for maturity predictions***

Weather history derived from AgWeatherNet (https://weather.wsu.edu/) has been added to our meta data files over the past year. This means that for every trait prediction model we have, we now have corresponding weather data (see Figure 7). This potentially allows us to predict *future* trait values based upon the weather *forecast.* It would therefore allow us to estimate the time/date that a particular lot of fruit would cross a grower-defined threshold. Furthermore, as weather forecasting accuracy increases nearer to the indicated threshold date, growers could be notified of changes to the predicted date at which time the fruit would cross the user-defined threshold value. We envision this could provide increased lead time for estimation of pome fruit maturity and by extension fruit storability.

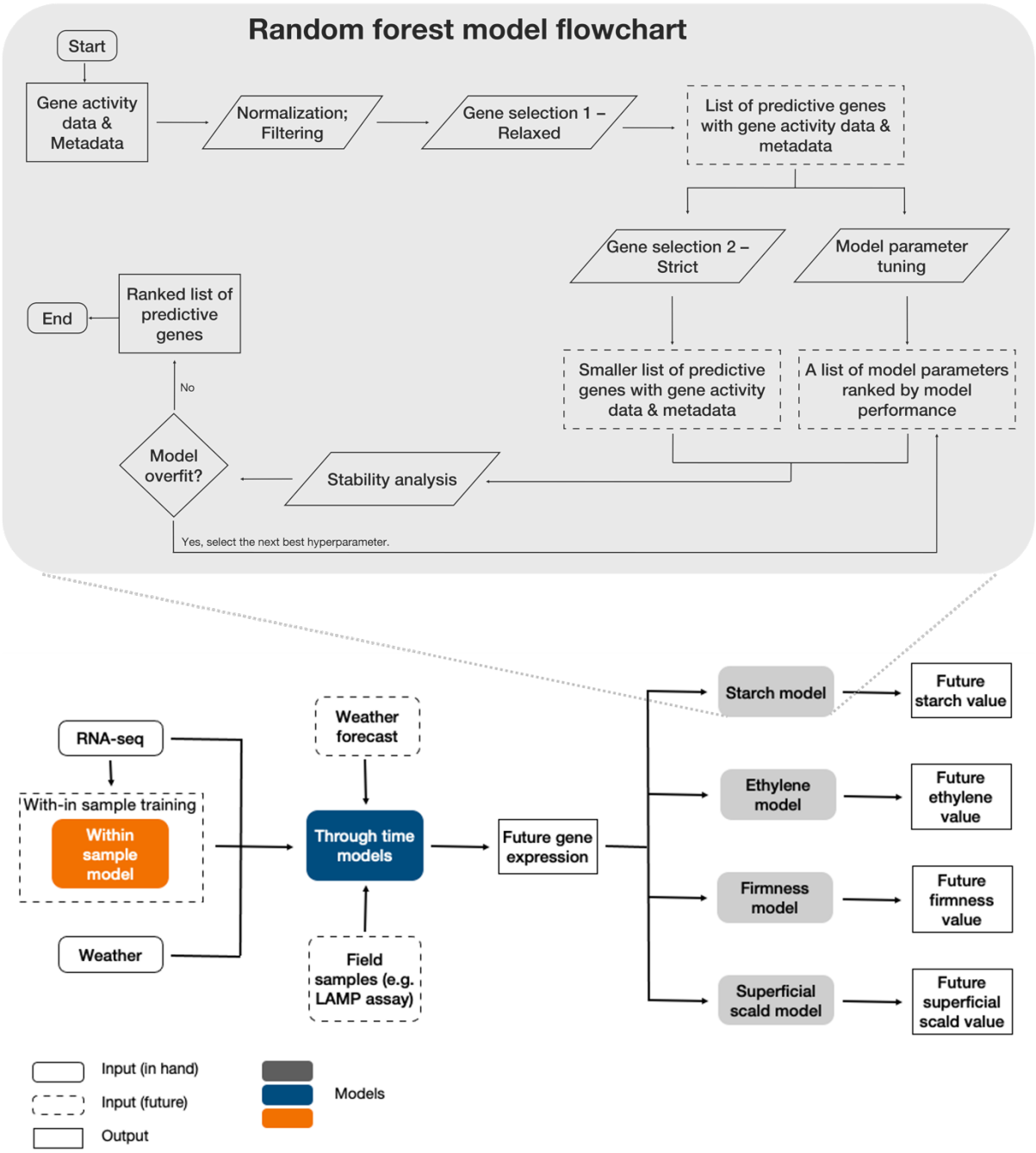


**Figure 7. Schematic summary of our modeling approach.** For any given model (e.g. starch clearing or IEC) we can use the weather forecast to predict future values of the prediction. We hypothesize this is a means to give growers more lead time for information that impacts supply chain decisions.

## Results and Discussion Works Cited

Hadish et al 2024 preprint - https://doi.org/10.1101/2024.12.09.627626
Watkins and Mattheis 2019 - https://doi.org/10.1201/b22001

# Project/Proposal Title: Evaluation of an alternative postharvest fungicide applicator

**Report Type:** FINAL Project Report Year 3NCE

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**Address:** 620 Market Street
**Address 2:** WSU Extension
**City/State/Zip:** Prosser, WA 99350

**Cooperators**: Jason and Jordan Matson, Matson Fruit, Faith Critzer, University of Georgia; Clark Kogan, Statscraft LLC, Pace International LLC.

**Project Duration:** 3 Years
**Total Project Request for Year 1 Funding:** $132,793
**Total Project Request for Year 2 Funding:** $110,993
**Total Project Request for Year 3 Funding:** $4,500

**Other related/associated funding sources:** None

**WTFRC Collaborative Costs:** None

**Budget 1**
**Primary PI: Achour Amiri**
**Organization Name: WSU**
**Contract Administrator:** Kevin Rimes
**Telephone:** 509-786-2226
**Contract administrator email address:** kevin.rimes@wsu.edu

| Item | 2021 | 2022 | 2023 |
|---|---|---|---|
| **Salaries** | 69,064 | 63,989 | 0 |
| **Benefits** | 21,563 | 18,520 | 0 |
| **Wages** | 6,483 | 5,411 | 0 |
| **Benefits** | 807 | 541 | 0 |
| **Equipment** | 0 | 0 | 0 |
| **Supplies** | 29,160 | 21,060 | 4,500 |
| **Travel** | 5,716 | 1,472 | 0 |
| **Miscellaneous** | 0 | 0 | 0 |
| **Plot Fees** | 0 | 0 | 0 |
| **Total** | 132,793 | 110,993 | 4,500 |

## OBJECTIVES

1. Optimize coverage of fruit in alternative sprayer with fluorescent tracer and water sensitive paper (Hoheisel; yr 1).

2. Comparison of efficacy against postharvest decay organisms between drench and alternative fungicide application (Amiri; yr 1and 2).

3. Quantification of indicator organisms (*E. coli* and coliforms) in water and on fruit treated with fungicides applied in drench and alternative applications (Hoheisel; yr 1 and 2).

4. Communication of findings with the apple and allied industries and engage regulatory bodies for approaches for implementation of alternative fungicide application on farm (Amiri, Hoheisel; yr 1, 2 and 3).

### Significant Findings

- A novel field drencher (FD) was optimized for spray coverage.

- Coliform counts were higher in the field sprayer whereas *E. coli* recovery was higher in the commercial packinghouse drencher (CPD).

- Residue levels of thiabendazole (TBZ) were similar between the field drencher and commercial packinghouse drencher (CPD) but levels of fludioxonil (FDL) were higher on apples treated with the field drencher.

- Spores of *Penicillium* spp. (blue mold) were neither detected on apples nor in fungicide solutions of field sprayer or warehouse drencher in 2021 but increased in the CPD tank as the number of bins increased to 600 bins.

- Total microflora recovered from apples treated with fungicides through the FD was significantly reduced compared to the control and fruit treated via the CPD.

- Overall, decay incidence after 8 months of storage was lower in apples treated via the FD compared to those treated with CPD.

## Results and Discussion

**Objective 1.** Optimize coverage of fruit in alternative sprayer with fluorescent tracer and water sensitive paper.

The overall deposition was higher in the non-recycling FD (255.4 µg/mL) but not significantly different ($P = 0.27$) from the packinghouse drencher (CPD) (175.9 µg/mL) (**Figure 1**). In the non-stacked bins drenched through the filed drencher (FD), deposition was higher on the top versus bottom sections of two out of four bins (**Figure 2**). In the stacked bins treated through the CPD, deposition was significantly ($P < 0.001$) higher on the top bin section of the 3rd high bin on the truck, whereas deposition was overall uniform among the other zones and stacked bins (**Figure 2**).

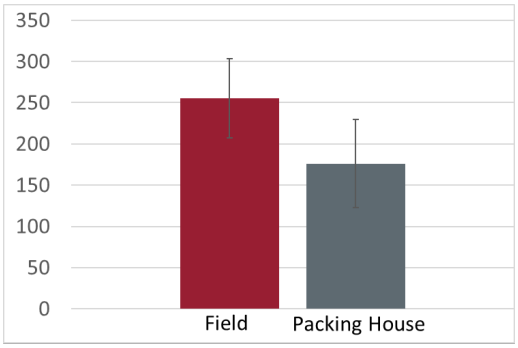


**Figure 1**. There is no significant difference (P-value: 0.2696) between the mean of the tracer concentration from the field and packing house, averaging across the experiment.

Deposition within the packing house was uniform except for the upper most collection zone and location receiving more (Figure 3, left). This is obviously due to the shower-down nature of the application. Nonetheless, it is positive that the lowest collection area (Lower, bottom zone) had similar deposition to other areas and is likely due to the extremely high flow rate in the packing house. The field drencher (Figure 2, right) is not stacked but goes under the spray bar with bin 1 going in first. After the last bin is sprayed, the driver waits 30 seconds and backs out with the bin 4 being the first under the spray bar. In this analysis there was a difference in deposition with the third bin receiving slightly less. We need to inspect possible differences in driving or patterns that could explain this difference. It contrasts with the regularity of time sprayed per bin (12 sec) which showed no significant difference in spray time among bins. Additional differences can be seen between the top and bottom zone of the bin, however, the impact of this would need to be assessed with efficacy data from storage rots. Meaning, there may be adequate deposition in the lower portion to control, but if not, rate should be increased to achieve more deposition in the bottom.

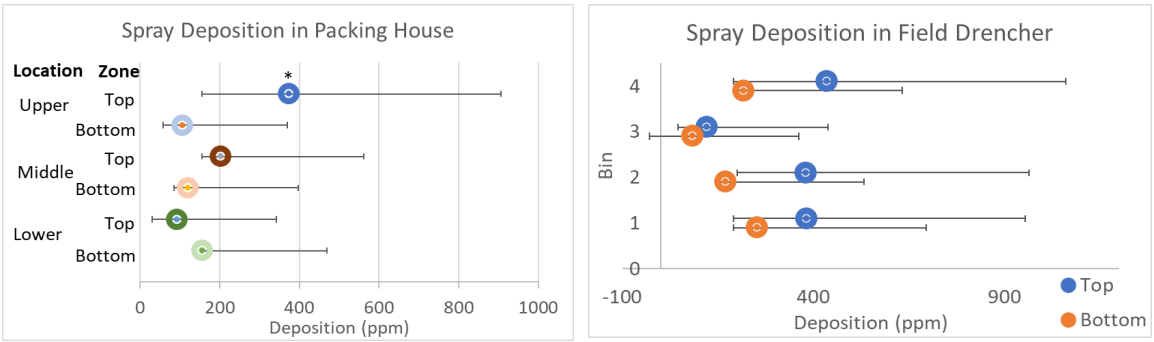


**Figure 2**. Spray deposition in the packing house and field drencher. In the packing house, bins are stacked (location) and there are two zones within a bin. Only the upper top collection area showed significant difference ($p<0.001$). In contrast, the field drencher is not stacked, but goes under the spray bar from bin 1 to 4. Significant differences were seen between the top and bottom of the bin ($p>0.0197$) and bin order ($p>0.047$).

**Objective 2. Comparison of efficacy against postharvest decay organisms between drench and alternative fungicide application**

2.b. Quantification of spores of fungal pathogens on fruit treated via two drench applicators

In 2021, the mixture of FDL and TBZ applied through the FD and CPD significantly ($P$ = 0.03) reduced the total microbiota in four and three Honeycrisp lots, respectively, compared to the control (**Table 1**). The most frequent fungal pathogen was *Alternaria* spp. which was equally recovered regardless of the application method. The number of propagules of *Penicillium* spp. recovered from Honeycrisp apples at harvest ranged from 0.0 to 0.05 CFU/cm$^2$ and was similar between the FD and CPD. In 2022, the total microbiota was significantly lower on apples treated with PYR via the FD compared to the CPD (four lots) and the control (three lots). The density of *Alternaria* spp. was higher on treated compared to the untreated apples and increased in three lots drenched through the FD. The density of *Penicillium* spp. was significantly higher on apples drenched via the CPD in three out of four Honeycrisp lots in 2022 (**Table 1**).

*Penicillium* spp. was not isolated from the fungicide solutions of FDL + TBZ applied through the FD or the CPD in 2021, whereas other fungi, i.e., *Alternaria* spp. and *Mucor* spp. were detected at ≤ 1 CFU/mL regardless of the drencher type (**Table 1**). In 2022, the density of *Penicillium* spp. ranged from 0.5 CFU/mL in Hc1918 lot to 16.8 CFU/mL in Hc1156 in the PYR solutions applied through the FD (**Table 1**). The population of other fungi, i.e., *Alternaria* spp. and *Mucor* spp., ranged from 4.8 to 23.3 CFU/mL among the four Honeycrisp lots. The fungal population in the PYR solution drenched through the CPD in 2022 was positively correlated with the number of bins drenched through and increased up to 60.8 CFU/mL for *Penicillium* spp. ($R^2$ = 0.94) and 40.2 CFU/mL for other fungi ($R^2$ = 0.62), after 600 bins had been drenched (**Table 2**).

**Table 1.** Number of colonies of *Penicillium* spp. and other fungi recovered from the surface of the fruit treated through field (FD) and warehouse (WH) drenchers in September 2022.

| | | Penicillium | | | Other fungi | | |
|---|---|---|---|---|---|---|---|
| **Cultivar** | **Lot** | **Control** | **FD** | **WD** | **Control** | **FD** | **WD** |
| Honeycrisp | 1136 | 0.04 | 0.3 | 0.4 | 17.2 | 14.8 | 31.8 |
| Gala | 901 | 0.2 | 0.2 | 0.2 | 5.4 | 12.1 | 9.3 |
| Gala | 1124 | 0.04 | 0 | 0.3 | 2.4 | 6 | 4.4 |
| Gala | 1113 | 0.08 | 0 | 1.2 | 27.7 | 42 | 13 |

2.c. Fungicide residue levels

The overall residue levels of TBZ, FDL and PYR on Honeycrisp apples were not significantly different between the FD and CPD (**Figure 3A**). There were no significant differences between the four bins drenched through the FD across lots, therefore, the residue level values were averaged. In 2021, the residue levels of TBZ and FDL on Honeycrisp apples were not significantly different ($P$ = 0.66) between the top, middle, and bottom sections of the bins regardless of the application method (**Figure 3B**). In 2022, the overall residue levels of PYR were significantly higher ($P$ = 0.04) at the top bin section compared to the middle and top sections of the bins treated via the FD (**Figure 3B**). Similar to the spray deposition patterns observed with the CPD, residue levels were significantly higher at the top of the 3$^{rd}$ high bin in the stack on the semi-truck for TBZ (**Figure 3C**) and the top of the 3$^{rd}$ and 2$^{nd}$ high bins for PYR (**Figure 3D**), but not for FDL (**Figure 3C**).

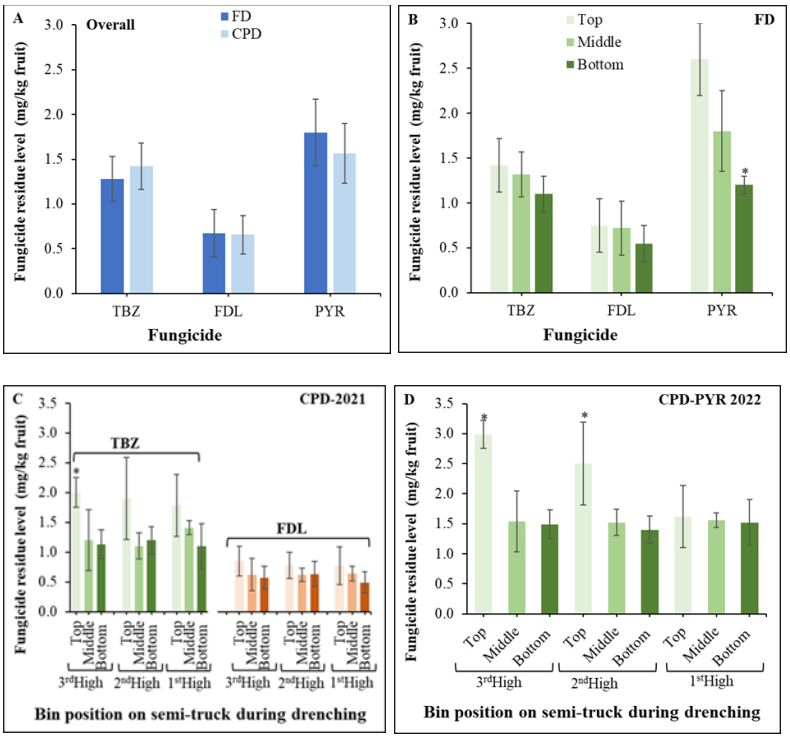


**Figure 3.** Residue levels of thiabendazole and fludioxonil (A) and pyrimethanil (B) on Honeycrisp apples collected from the top, middle, and bottom sections of bins treated through the field drencher or commercial packinghouse drencher in 2021 and 2022, respectively. An asterisk indicates significant difference based on Tukey's test at $P \leq 0.05$.

The concentrations of TBZ and FDL in 2021 and PYR in 2022 in the solutions of the FD tanks was similar between lots and ranged from 547 to 610 µg/mL for TBZ, 277 to 303 µg/mL for FDL, and 320 to 360 µg/mL for PYR (**Figure 4A,B**). The concentrations of the three fungicides in the CPD tanks decreased gradually as more bins were drenched resulting in a positive correlation between the number bins treated with the recycled solution and the concentration of TBZ ($R^2 = 0.92$), FDL ($R^2 = 0.84$), and PYR ($R^2 = 0.92$) in 2021 (**Figure 4C)** and 2022 (**Figure 4D**).

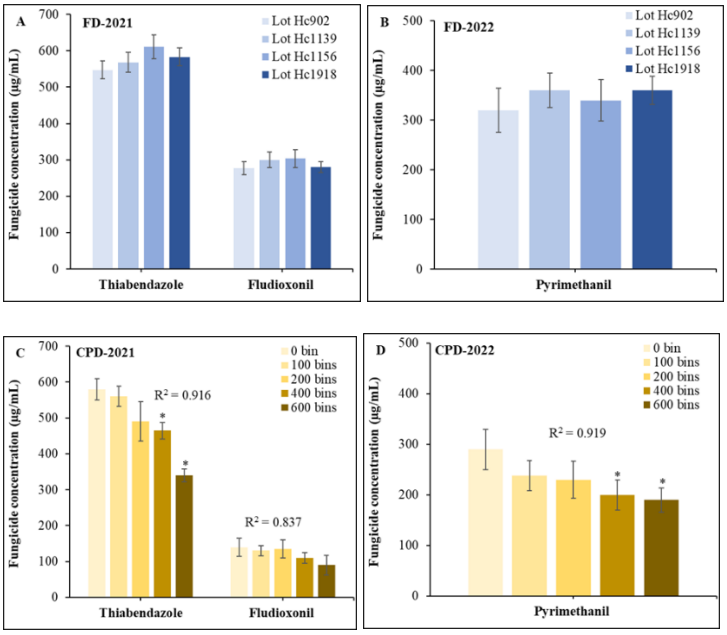


**Figure 4.** Residue levels of thiabendazole and fludioxonil (**A**) and pyrimethanil (**B**) in fungicide solutions applied through the field drencher (FD); residue levels of TBZ and FDL (**C**) and PYR (**D**) applied through the commercial packinghouse drencher (CPD) after several (0-600) bins had been drenched in 2021 and 2022, respectively. An asterisk indicates significant difference based on Tukey's test at $P \leq 0.05$.

2.d. Determination of decay incidence and decay types in cold storage on fruit treated at harvest with fungicides through alternative and drench approaches

Three hundred apples (100 apples/treatment) were collected from each lot and stored at 55°F for 2 weeks, then at 37°F in RA. Overall decay varied between lots and was either lower in field drencher after 9 months or equal to incidence recorded in warehouse drenched-fruit except in lot 1139 (Figure 5).
During the 2022-23 season, five lots of Honeycrisp and three lots of Gala that were not treated with any fungicide preharvest were treated at harvest with Penbotec. Four bins of each lot were treated using the field sprayer and 4 other bins from each lot were treated with the warehouse sprayer. Bins were stored at the collaborating were house in CA. The overall decay incidence after 8 months for Honeycrisp lots was significantly lower in two lots (1162 and 1918) and was numerically lower in the 3 other lots when treated with the field drencher (Table 2). For the Gala lots after 9 months of storage, incidence was equal in two lots and was significantly higher in apples treated with the warehouse drencher for lot 1124 (Table 2).

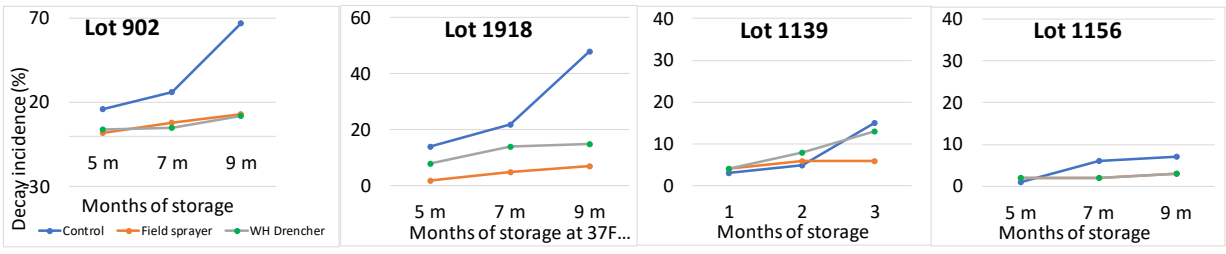


**Figure 5.** Overall decay incidence in four Honeycrisp lots untreated (control) or treated via field or warehouse drenchers in 2021-22 season and stored in regular atmosphere at 37°F.

**Table 2.** Overall decay incidence on Honeycrisp and Gala apples stored at the commercial storage cold room during the 2022-23 season under controlled atmosphere for 8 months.

| | | Decay incidence (%) | |
|---|---|---|---|
| Cultivar | Lot | Field sprayer | Warehouse Drencher |
| Honeycrisp | 903 | 1.5 | 1.83 |
| | 1156 | 2.5 | 2.5 |
| | 1162 | 0.9 | 1.4 |
| | 1918 | 7.1 | 15.2 |
| | 1136 | 3.2 | 3.4 |
| Gala | | | |
| | 901 | 0.3 | 0.24 |
| | 1113 | 0.8 | 0.6 |
| | 1124 | 0.4 | 2.1 |

**Objective 3. Quantification of indicator organisms (*E. coli* and coliforms) in water and on fruit treated with fungicides applied in truck and alternative applications**

Water samples were collected in the harvest of 2021 and 2022, while apple samples were collected in 2022. Approximately 94% (85-98%) of the apple samples in the packing house and 84% (70-93%) in the field are coliform free before any drench treatment (Figure 7a). However, post drench treatment, 6% (2-17%) of the apple samples in the packing house and 94% (84-98%) of the apple samples in the field are coliform free after treatment. This is a significant ($p>0.001$) decrease for the packing house with an 87% (75-94%) decrease. Although there is a 9% difference (0.8- 20.5%) for the field drencher, pre and post treatments are not significantly different to each other.

***Apple samples**:* Of the samples that tested positive for Coliform, some also showed *E. coli* populations. Nearly 100% (96-100%) of the packing house apple samples and 96% (86-99%) of the field apple samples were *E. coli* free on arrival. After the drench treatment, an estimated 93% (79-98%) of the packing house apple samples and 98% (92-99%) of the field apple samples were *E. coli* free. There was no significant different between pre- and post-spray application for either Drencher.

For the subset of apples that did have contamination, the colony forming units (CFU) were compared pre and post spray applications. The mean CFUs for Coliform contaminated post application apple samples for field and packing house drenchers was 548 (127-2371) and 23899 (8255-69190), respectively (Fig 8a). For the field drencher, there is a non-significant 0.9-fold decrease in the CFUs for contaminated apples. In contrast, there is a 36.9-fold increase in the coliform CFUs for apples that tested positive for coliform. The mean CFUs for *E. coli* contaminated post application apple samples for field and packing house was 254 (51-1278) and 2288 (706-7417), respectively (Fig 8b). For apples from the field drencher, that is only 1.0 fold non-significant change in *E. coli* CFUs. Whereas apples from the packing house were nearly 100% free of coliform before treatment, the drench application introduces on average 2288 *E. coli* CFUs.

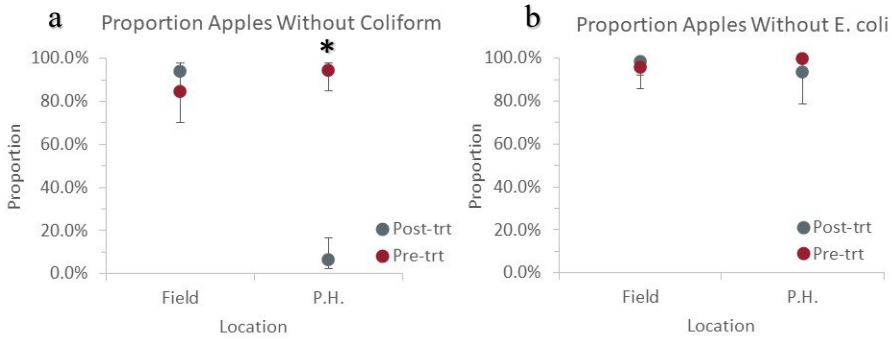


**Figure 5.** Proportion of apples without coliform (a) and *E. coli* (b) populations for apples pre- and post-drench treatment for Field and Packing House (P.H.) drenchers. There is a significant difference in apples with coliform (*=P-value>0.001) between the pre and post treatments in the packing house. While the field drencher showed no significant differences. And there was not a significant increase in apples with *E. coli* (b) pre or post drench for either treatment

***Water samples**:* There was an estimated mean of 17 (95% CI: 9-33) thousand coliform CFU in the typical field drencher water sample and a mean of 0.6 (95% CI: 0.3-1.3) million coliform CFU in the typical packing house drencher water sample. This is an estimated 35 (95% CI: 13-93) times the number of coliform CFU in the packing house compared to the field (Table 3). There was an estimated mean of 111 (95% CI: 24-523) *E. coli* CFU in the typical field drencher water sample and a mean of 2 (0.5-7.1) thousand coliform CFU in the typical packing house drencher water sample. This is an estimated 17 (95% CI: 2-131) times the number of E. coli CFU in the packinghouse compared to the field (Table 3).

**Table 3.** Average concentrations of total coliforms and generic *Escherichia coli* in the fungicide solutions of the field and packinghouse drenchers and on apple surfaces before and after drenching.

| Type of sample | Method of application | Sampling point | Indicator Organism | |
|---|---|---|---|---|
| | | | Total Coliforms | Generic *E. coli*[4] |
| Fungicide solutions | CPD | During Treatment | 5.71 ± 0.51 c | 3.03 ± 1.36 b |
| | FD | During Treatment | 3.59 ± 1.38 b | 1.26 ± 1.07 a |
| Apple surfaces | CPD | Pre-treatment | 2.02 ± 0.17 a | 2.00 ± 0.00 a |
| | | Post-treatment | 3.96 ± 1.18 b | 2.08 ± 0.36 a |
| | FD | Pre-treatment | 2.08 ± 0.28 a | 2.01 ± 0.08 a |
| | | Post-treatment | 2.40 ± 0.23 a | 2.00 ± 0.08 a |

## Discussion

The field drencher (FD) was optimized for spray coverage and carries approximately five times less fungicide solution than the traditional commercial packinghouse drencher (CPD). As used in this study, the FD applies approximately 1.5 gal of the fungicide solutions per bin, 50% less than the estimated 3.17 L through the CPD. Despite this difference, deposition patterns were

equal or better through the FD likely because bins are not stacked and that 90% of the fungicide solution is retained on the fruits and bins during FD drenching. Comparatively, the concentration of the active ingredient strongly correlated with the number of bins drenched via the CPD and fungicide (a.i.) loss was estimated to be 40, 36 and 35% for TBZ, FDL and PYR, respectively, between 0 and 600 bins. Apples treated through the CPD were collected after approximately 200 bins had been treated, and it is possible that residue levels may be lower on CPD-treated fruit at the end of the lifespan of the tank. As expected from the "shower-down" nozzle, more deposition on the top of bins occurred on some occasions, but it was not always significantly different from the middle and bottom of the bins.

Spray coverage results were further supported by the fungicide residue levels detected on apples. Thus, FDL and TBZ levels were not significantly different between bin sections in 2021, whereas apples at the bottom received less PYR in 2022. Residue levels of FDL, TBZ, and PYR were all below the maximum residue levels of 5, 10, and 15 ppm, respectively, for both FD and CPD. The lower fungicide residue levels at the bottom of the bins treated through the FD are unlikely to reduce their efficacy as the minimum residue levels required for appropriate control are met for all three fungicides. The FD is practical as it can be used to treat fruit immediately after harvest at the vicinity of orchards and therefore may protect fruit from infections that start on fresh wounds caused during harvest, transportation and handling at the storage facility. The FD is a portable system that can be transported between orchards but can also be used at vicinity of packinghouses. The spray turnout is only slightly higher through the CPD, which treats approximately 192 bins/h, when three bins are stacked, versus approximately 160 unstacked bins/h for the FD. Besides the mentioned benefits, future economic analyses and risk analysis accounting for changes in labor, waste management costs, decay, and food safety management are needed to accurately assess the economic benefits of the FD. It is a complex analysis in that operation of the FD requires more operational hours to move bins from the orchards to the drencher then to a semi-truck, however, the risk of potential introduction of fungal and food-borne pathogens must be assessed in the return on investment.

The two major postharvest pathogens known to spread through water recirculation are *Mucor* and *Penicillium* spp., the causal agents of blue mold and mucor rot, respectively. *Mucor* spp. was not isolated from the surface of apples treated with either drencher in this study, and the frequency of *Penicillium* spp. on fruit was relatively low at harvest confirming that infection by this pathogen occur mainly after harvest. However, there was evidence of increased fruit contamination with *Penicillium* spp. spores via the recycling CPD in 2022 as their density increased 7.5 to 15-fold compared to the control in three Honeycrisp lots. Meanwhile, there were significantly less spores of *Penicillium* spp. on apples of 75% of lots drenched with the FD compared to the CPD and the control. This may indicate that the combination of TBZ and FDL in 2021 had a better efficacy against *Penicillium* spp. that may be resistant to either fungicide or that spores that are PYR-resistant have accumulated in the CPD at the time the apples were drenched in 2022. The FD and CPD equally reduced the carpoplane population of *Alternaria* spp. on apples treated with TBZ+FDL in 2021, whereas in 2022, *Alternaria* spp. increased in three and two lots on FD- and CPD-treated apples, respectively, post-drenching. Since spores of *Alternaria* spp. originate from the orchard, it is unlikely that spores were spread though the FD tank solution but rather due to different apples within the bins carrying different spore loads. Moreover, the large volume applied through the CPD may detach more spores from the apple surface than the FD.

After eight months of storage in RA and ~78% relative humidity (RH), the FD provided a greater efficacy in reducing the overall disease incidence in 50% of apple lots compared to the CPD, whereas equal efficacy was seen in the other two Honeycrisp lots, a cultivar highly susceptible to postharvest diseases. The efficacy of the FD in mitigating postharvest diseases was particularly evident in 2022, when the disease pressure was higher, as significant reductions were observed compared to the CPD in all but one lot. In the larger commercial trial including Honeycrisps and Gala apples from seven lots stored in CA at RH > 90%, the overall disease incidence was lower in 57% of the lots treated with PYR through the FD, albeit not always significantly compared to the CPD. The most prevalent postharvest diseases encountered in 2021 and 2022 were blue mold and gray mold. While the incidence of gray mold was either equivalent between the FD and the CPD or significantly lower in fruit treated through the CPD, the incidence of blue mold was significantly reduced by the non-recycling FD in 75% of the lots treated in 2021 and 2022. In the controlled atmosphere (CA) commercial trial, the incidences of blue mold and Mucor rot were reduced in 71% of Honeycrisp and Gala lots treated through the FD at harvest. Like in RA conditions, the incidences of gray mold, Alternaria rot, and bull's eye rot, caused by the preharvest pathogens *Botrytis* spp., *Alternaria* spp., and *Neofabraea* spp., respectively, were higher in 50% of the fruit lots treated through the FD compared to the CPD after 10 months in CA.

Although the residue levels are above the minimum levels (0.5 to 2 ppm) needed to control sensitive isolates of the above pathogens, it is plausible that the relatively lower levels observed in the middle and lower sections of the bins treated through the FD may have not provided the anticipated efficacy against some of these preharvest pathogens, as opposed to a higher efficacy observed against wound pathogens like *Penicillium* spp. and *Mucor* spp. Furthermore, all lots used in this study were not treated with preharvest fungicides, which may be highly recommended to further enhance the efficacy of the FD against the preharvest pathogens. The FD has already been used by packers in the PNW in the past three years and feedback was positive in terms of reducing postharvest losses. Additional commercial trials testing different cultivars and fungicides are necessary to verify these observations, which may warrant additional adjustments in the volume of fungicide applied and the nozzle types utilized in the FD.

Differences observed in populations of *E. coli* and total coliforms on apples before and after FD and CPD drenching suggest that the CPD has a greater risk of cross-contamination compared to the FD, similar to the risk of spreading spore of plant pathogens. While pathogenic strains could not be employed in the present study, results support the notion that the FD reduces the risk of cross-contamination, including from foodborne pathogens, thereby enhancing overall food safety. Further evaluating the cross-contamination risk by modeling the transfer of inoculated surrogate organisms with phenotypic markers in both systems would be beneficial to help inform risk assessments tied to food safety. Additionally, postharvest water that comes into contact with crops must have no detectable *E. coli*/100 mL based upon water quality criterion in the U.S. Produce Safety Rule. While both drenchers had populations of *E. coli* recovered that are contributed from fruit and bins, the FD fungicide solution is not be recirculated, contrary of the CPD water, which is recycled until it reaches the end of life based on the number of bins treated. With a population of 3.03 ± 1.36 MPN/100 mL *E. coli* recovered in CPD fungicide solutions; it is obvious that water will not meet the water quality criteria specified in the PSR as the indicator concentration increases with each subsequent pass through the system. These findings have significant implications for regulatory compliance, as continued use of recirculated fungicide solutions in CPD systems could lead to failure to meet the PSR's microbial water quality standards.

Since total coliform and generic *E. coli* accumulated in both drenchers, the addition of antimicrobial agents compatible with fungicides without affecting their efficacy, could effectively reduce bacterial levels in both systems. PAA as used in this study may have further reduced total coliform and generic *E. coli* better in the FD. Future work to optimize sanitizer use and examine compatibility with fungicides within a single use and recirculated drencher is needed.

**Executive Summary**

**Project title:** Evaluation of an alternative postharvest fungicide applicator

**Key words:** Fungicides, non-recycling drencher, postharvest decay, food safety.

**Abstract:** Recycling drenchers used to apply postharvest fungicides in pome fruit may spread microorganisms, i.e., plant and foodborne pathogens, that increase fruit loss and impact food safety. A non-recycling field drencher (FD), which drenches unstacked bins of fruit, was compared to a commercial recycling packinghouse drencher (CPD) for fruit coverage, fungicide residues, postharvest diseases control and spread of plant pathogens, total coliforms and generic *Escherichia. coli*. A mixture of fludioxonil (FDL) and thiabendazole (TBZ) was used in 2021, while pyrimethanil (PYR) was applied in 2022 to alternate fungicides. The overall spray coverage assessed with pyranine was not significantly different between the FD and CPD. The residue levels of FDL and TBZ were similar between the two methods on Honeycrisp apples at the top, middle, and the bottom of the bins, whereas the residue levels of PYR were significantly lower at the bottom of the bins treated through the FD. The density of plant pathogens and overall disease incidence were similar on apples drenched through both systems in 2021 and significantly lower in FD-treated apples in 2022. The incidence of blue mold, the most important postharvest disease caused by *Penicillium* spp., was significantly lower in apples treated through the FD in both years. The levels of total coliforms and generic *E. coli* were significantly higher in fungicide solutions collected from the CPD compared to the FD. Total coliforms increased significantly on apples treated via the CPD but not on apples treated through the FD. Findings from this study suggest that the new non-recycling drencher has potential as an alternative to recycling packinghouse drenchers in reducing the spread of plant and foodborne pathogens.

**Proposal Title:** Efficacies of biocontrol agents against postharvest decay in apples

**Report Type:** Continuing Project Report

**WTFRC Project Numb**er:

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**Cooperators:**

Stemilt Growers LLC.; Columbia Fruit Packers Inc.; Decco U.S. Postharvest

Cooperators from the apple industry will provide in-kind support in the forms of in-kind time commitment, technical input, and apple fruit donation for the proposed studies.

Decco U.S. Postharvest will help with NEXY samples and other biocontrol materials, testing procedures and packhouse trials.

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $80,400
**Total Project Request for Year 2 Funding:** $90,087
**Total Project Request for Year 3 Funding:** $93,353

**WTFRC Collaborative Costs:**

| **Item** | 2023-2024 | 2024-2025 | 2025-2026 |
|---|---|---|---|
| Salaries | | | |
| Benefits | | | |
| Wages | $2,330.00 | $5,051.00 | $5,712.00 |
| Benefits | $932.00 | $2,008.00 | $2,285.00 |
| RCA Room Rental | | $3,760.00 | $3,872.00 |
| Shipping | | | |
| Supplies | $250.00 | $275.00 | $300.00 |
| Travel | $350.00 | $375.00 | $400.00 |
| Plot Fees | | | |
| Miscellaneous | | | |
| | | | |
| | | | |
| | | | |
| Total | $3,862.00 | $11,469.00 | $12,569.00 |

**Footnotes:**

**Budget 1**

**Primary PI:** Meijun Zhu
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| Item | 2023-2024 | 2024-2025 | 2025-2026 |
|---|---|---|---|
| Salaries | $36,836.00 | $38,309.00 | $39,841.00 |
| Benefits | $9,548.00 | $9,929.00 | $10,327.00 |
| Wages | $5,522.00 | $5,743.00 | $5,973.00 |
| Benefits | $132.00 | $137.00 | $143.00 |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | $21,000.00 | $21,000.00 | $21,000.00 |
| Travel | $1,500.00 | $1,500.00 | $1,500.00 |
| Plot Fees | | | |
| Miscellaneous | $2,000.00 | $2,000.00 | $2,000.00 |
| | | | |
| | | | |
| | | | |
| Total | $76,538.00 | $78,618.00 | $80,784.00 |

**Footnotes:**

## OBJECTIVES

1. *In vitro* screening of inhibitory effects of commercial biocontrol agents alone or combination against *Penicillium expansum* and *Botrytis cinerea.*
2. Evaluate the efficacy of the selected biocontrol agents or combinations on rots of pre-wounded apples artificially inoculated with *P. expansum* and *B. cinerea* during storage.
3. Assess the efficacy of the selected biocontrol treatments on apples artificially infected by the major decay pathogens in commercial cold storage without wounding

## SIGNIFICANT FINDINGS

## METHODS

### *1. Decay pathogen strain selection*

Four *P. expansum* strains, including NRRL # 974, NRRL # 976, NRRL #35695, and NRRL #62431, were used in this study to evaluate blue mold. A 4-strain spore/conidial cocktail was used in addition to individual strains to elucidate the impact of strain variability.

### *2. Pathogen spore/conidial inoculum preparation*

Each strain was plated onto potato dextrose agar (PDA) plates and incubated at 25°C for 7 days. To harvest *P. expansum* conidia, 10 ml of sterile 1 × PBS, pH 7.4 with 0.1% Tween 80 (PBST) was added onto each PDA plate. The plates were swirled, and the suspension was filtered through sterile 8-layer cheesecloth to remove mycelium. The conidial concentration of each strain spore suspension was determined using a hemocytometer.

### *3. Biocontrol agent selection and treatments*

*BioSave* 10LP (*Pseudomonas syringae* strain ESC-10), Stargus, Serifel, Cease, and Botrystop were obtained from their respective manufacturers. Each BCA working solution was prepared using sterile water according to the manufacturer's recommended application rates.

### *4. In vitro antifungal activity*

#### *4.1 Inhibition of spore germination*

The spore germination inhibition assay was conducted based on previously published methods with modifications (Tian et al., 2002). *P. expansum* spores were suspended in yeast extract peptone dextrose (YPD) broth at a concentration of $2\times10^6$ spores/mL. An equal volume of 2× BCA working solution was added, mixed, and incubated at room temperature for 14 hours. After incubation, 10 µL of the solution (~300 spores) was pipetted into a hemocytometer chamber and examined under a Fisher Scientific Microscope Model J0812-2861-327 (400×) to assess germination rates. Germination rates (%) were calculated as the number of germinated spores divided by the total number of spores, multiplied by 100. Each treatment was performed in triplicate, and the experiment was repeated three times. Germination was defined as a germ tube length twice the diameter of the conidium.

#### *4.2 Decay pathogen spore viability assay*

Spore viability was determined using a previously described method with slight modification (Cheon et al., 2016). The spore suspension of the selected decay pathogen was mixed with an equal volume of biocontrol agent followed by an incubation at RT. The spore suspension was then 10-fold serially diluted. The appropriate dilutions were plated onto PDA and incubated for 3-5 days at 72 °F/22 °C when the spore

viability was evaluated. Each treatment was performed in triplicate, and the experiment was repeated three times.

*3) Inhibition of mycelial growth*

The ability of the BCAs to inhibit the mycelial growth of *P. expansum* was determined per previous published method by Mahmud et al. (2017) with slight modifications. BCA strains were applied to the center of the PDA agar plates, while decay pathogens were inoculated to equidistant points (4 cm from the center). Plates were incubated at 25 ℃ for up to 14 days. Growth inhibition (%) was calculated as [(diameter of control plate - diameter of treatment plate) / diameter of control plate] × 100. Each treatment was performed in triplicate, and the experiment was repeated three times.

***5. The compatibility at cold temperature***

*5.1 In vitro testing in nutrient broth*

To assess cold storage stability, each BCA strain was inoculated into Tryptone Yeast Glucose (TGY) broth at an initial concentration of ~6 $\log_{10}$ CFU/mL and incubated at 0°C (32°F) for up to 28 days. Samples were collected on days 0, 1, 4, 7, 14, 21, and 28. The respective dilutions were plated on TGY or PDA. The experiment was repeated three times, with three biological replicates per treatment.

*5.2 Stability of apples during commercial cold storage*

BCA strains identified as cold-stable in the broth testing were further evaluated for stability on apples under commercial refrigerated air (RA) and controlled atmosphere (CA) storage. Unwaxed Granny Smith apples at commercial maturity were treated with the respective BCA strain at ~7 $\log_{10}$ CFU/apple. Treated apples were stored under commercial RA or CA conditions for 18 weeks, with sampling conducted every three weeks.

## RESULTS AND DISCUSSION

***1. In vitro anti-fungal activity***

To evaluate the efficacy of different BCAs against *P. expansum*, mycelial growth inhibition was assessed for single strains and a four-strain cocktail. Fig. 1 shows that all BCAs significantly reduced mycelial growth. BCA strains except BioSave 10 are more effective against strain 1 compared to strain 2 and 4-strain cocktail. Cease, Serifel, and Stargus exhibited 64% inhibition against strain 1, while their efficacy against strain 2 was 56% for Stargus, 53% for Serifel, and 54% for Cease (Fig. 1A), indicating strain variety. This observation aligns with Lambrese et al. (2021), who reported differential inhibition of *P. expansum* strains by *K. radicincita*.

Surprisingly, BioSave 10 showed limited efficacy, with only 6% inhibition in the mixed culture and a maximum of 38% against strain 2 (Fig. 1A). This low inhibition aligns with Cirvilleri et al. (2005), who reported 5% inhibition of *P. digitatum* by *P. syringae*. This may be due to the presence of stronger defense mechanisms in mature mycelia, hindering the activity of *P. syringae* in BioSave 10. The lower activity against the four-strain cocktail might be also attributed to the increased robustness and activity of the mixed culture.

To assess the efficacy of different BCA against *P. expansum*, the germination rates of fungal spores after being exposed to various compounds were further assessed. All BCAs tested demonstrated significant inhibition of *P. expansum* spore germination. Biosave 10 exhibited the highest inhibition (97.33%), followed by Serifel (70.52%), Stargus (70.43%), and Cease (66.50%) (Fig. 2A). Botrystop also exhibited a spore germination inhibition activity compared to the control (Fig. 2B), but to a lesser extent than the other treatments shown in Fig. 2A, resulting in ~35.63% inhibition. Fig. 2C provides a visual representation of germinated and ungerminated spores.

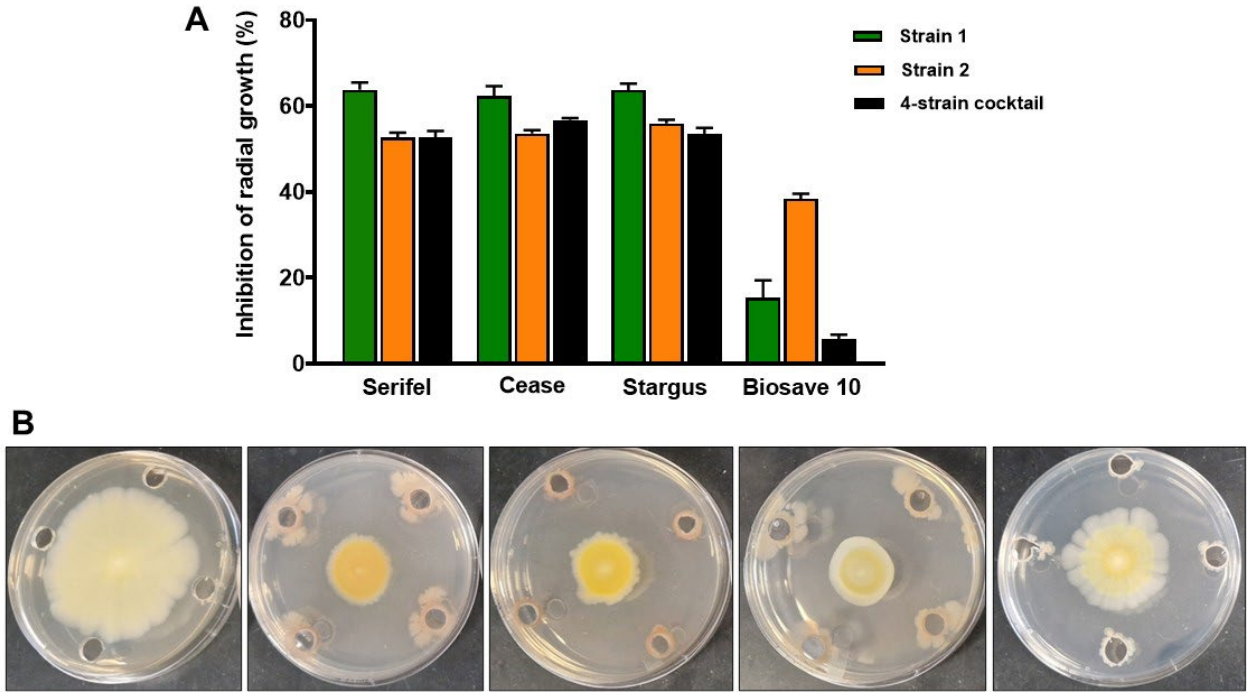


**Fig. 1. Inhibition of radial/mycelial growth of *Penicillium expansum* by commercial biocontrol agents (BCA).** BCA products were applied according to the manufacturer's recommended application rates. A. Quantitative inhibition of radial growth. B. Representative images of mycelia growth of *P. expansum* under different treatments (from left to right: Control, Serifel, Stargus, and Biosave 10). Data are presented as Mean ± SEM, n = 9.

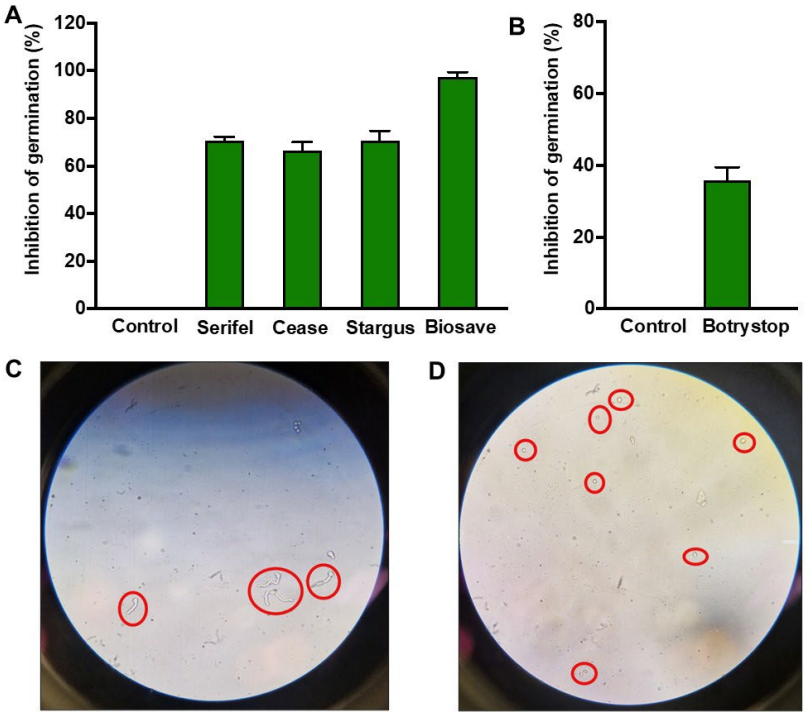


**Fig. 2. Inhibition of spore germination of *Penicillium* expansum by commercial biocontrol agents.** Products were applied based on the manufacturer's recommended application rates. A. Bacterial strains. B. Fungal strain. C. A representative image of germinated spores after 14 h of incubation, taken from the control sample. D. A representative image of ungerminated spores. Data is presented as Mean ± SEM, n = 9.

The efficacy of BCAs in reducing *P. expansum* spore viability was further assessed. Fig. 3A shows that all BCAs significantly reduced spore viability, with Cease and Biosave demonstrating the highest reduction (~70%), followed by Stargus and Serifel. Serifel exhibited the lowest reduction among the tested BCAs showed in Fig. 3A (~55%). Botrystop also reduced spore viability compared to the control (Fig. 3B), albeit to a lesser extent than the other BCA treatments shown in Fig. 3A. These findings suggest the potential of using Cease, Biosave, and Stargus for the biological control of blue mold.

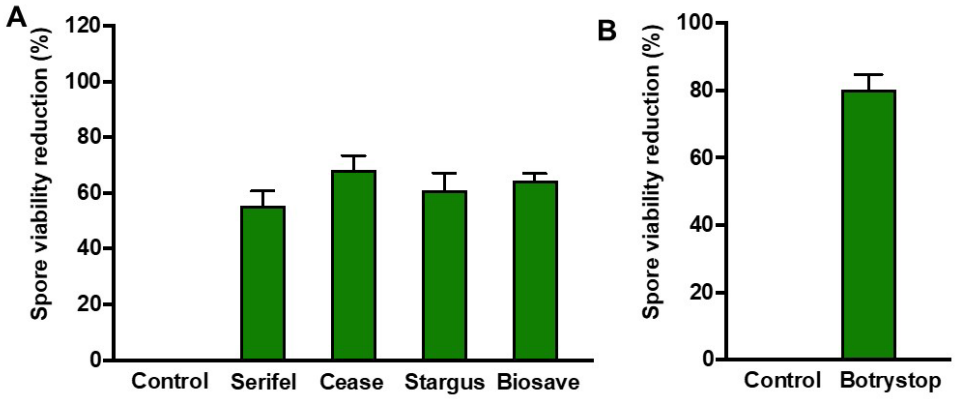


**Fig. 3. Spore viability reduction of *Penicillium expansum* by commercial biocontrol agents.** Products were applied based on the manufacturer's recommended application rates. A. Bacterial strains. B. Fungal strain. Data is presented as Mean ± SEM, n = 9.

## *2. The compatibility at cold temperature*

To evaluate the compatibility of these BCAs during extended commercial cold storage, the survival of Serifel, Cease, Stargus, Biosave 10, and Botrystop was assessed over 28 days at 0°C. As shown in Fig. 4A, Serifel and Cease maintained relatively high viability throughout the storage period. Biosave 10 demonstrated a significant increase over time, reaching 8 log CFU/mL by the end of storage. In contrast, Botrystop showed a marked decline in viability, with populations dropping below the detection limit (<1 log CFU/mL) by the end of the period. These findings indicate that Biosave 10 exhibited excellent stability and Serifel and Cease showed good stability during cold storage at 0°C, making them suitable for long-term storage and practical application in postharvest disease management. Conversely, Botrystop's poor survival under these conditions suggests it is not appropriate for postharvest use.

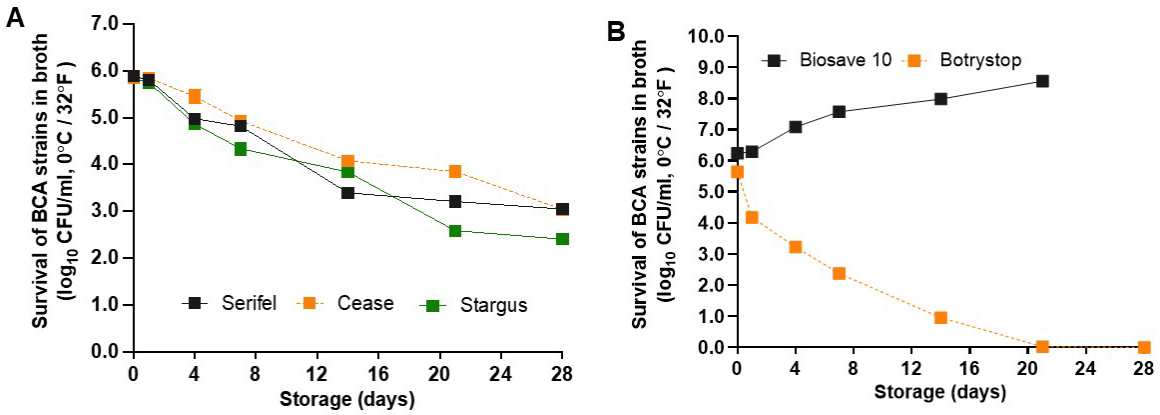


**Fig. 4. Survival of biocontrol strains in nutrient broth during four weeks of cold storage at 0 °C.** Inoculation level of ~6 log CFU/mL. A. Cease, and Stargus. B. Botrystop and Biosave 10. Data is presented as Mean ± SEM, n = 9, experiment was repeated thrice independently with 3 replicates per each repeat.

The stability of Cease and Biosave 10 on Granny Smith apples was further assessed under commercial RA and CA storage conditions at 1°C (33°F) for 12 weeks. On apples stored under RA, Cease exhibited a gradual decline in population density over time (Fig. 5A). Biosave 10, on the other hand, started with a slightly higher initial population and maintained higher levels during the first 3 weeks of RA storage (Fig. 5A). Under CA, both strains showed a survival pattern similar to that observed under RA conditions (Fig. 5B). This storage study is ongoing, but preliminary results indicate that consistent with the broth testing, Biosave 10 consistently demonstrates higher survival rates than Cease on apples, regardless of storage conditions.

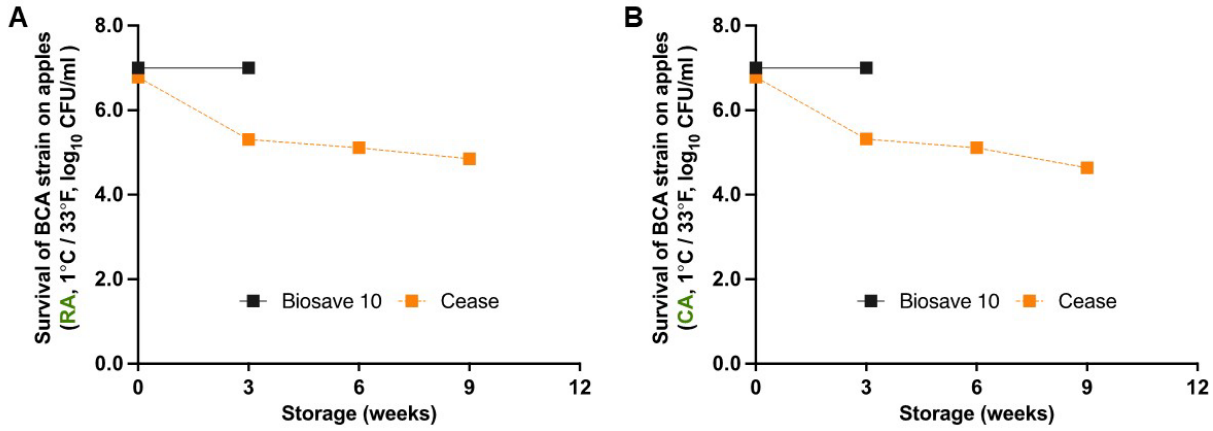


**Fig. 5. Survival of biocontrol strains on Granny Smith apples during commercial refrigerated air (RA) and controlled atmosphere (CA) storage.** Inoculation level of ~7 Log CFU/apple. A. RA, B. CA. Data is presented as Mean ±SEM, n = 20.

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Cirvilleri, G., Bonaccorsi, A., Scuderi, G., Scortichini, M., 2005. Potential Biological Control Activity and Genetic Diversity of Pseudomonas syringae pv. syringae Strains. J. Phytopathol. 153, 654-666.

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# **Project Title:** Genomic approaches to understand the etiology of postharvest decays

**Report Type:** Continuing Project Report

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**Cooperators**: N/A

**Project Duration:** 2-Year

**Total Project Request for Year 1 Funding:** $71,285
**Total Project Request for Year 2 Funding:** $109,025

**Other related/associated funding sources:** None

**WTFRC Collaborative Costs:** None

**Budget 1**
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**Station Manager/Supervisor:** Chad Kruger

| Item | 2023 | 2024 | |
|---|---|---|---|
| Salaries1 | $43,680.00 | $0.00 | |
| Benefits1 | $15,492.00 | $0.00 | |
| Salaries2 | $0.00 | $31,500.00 | |
| Benefits2 | $0.00 | $10,205.00 | |
| RCA Room Rental | $0.00 | $0.00 | |
| Shipping | $0.00 | $0.00 | |
| Supplies3 | $11,300.00 | $0.00 | |
| Travel4 | $813.00 | $0.00 | |
| Plot Fees | $0.00 | $0.00 | |
| Miscellaneous | $0.00 | $0.00 | |
| | | | |
| | | | |
| | | | |
| Total | $71,285.00 | $41,705.00 | $0.00 |

**Footnotes:**

**1:** Salaries are for a Scientific Assistant in Amiri Lab to conduct work related to fruit inoculation, sampling, and RNA extraction in Year 1 (2023) at monthly salary of $5,200 for 12 months at 0.7 FTE. Benefits are calculated based on 35.5% rate with a 4% inflation each year.

**2**: Salaries are for a Scientific Assistant in Ficklin Lab to conduct work related to RNAseq analyses in Year 2 (2024) from samples collected in Year 1, at monthly salary of $5,200 for 12 months at 0.5 FTE. Benefits are calculated based on 35.5% rate with a 4% inflation each year.

**3.** Supplies include $5,000 for microbiological media, Petri dishes, pipette tips, filters, bottles and other miscellaneous to be used to grow and maintain inocula of fungi for inoculation. Supplies also include $6,300 for RNA extraction estimated at $35/sample and estimated 180 total samples.

**4.** Travel to experimental sites in Washington States, to meet collaborators in Pullman to discuss data, and to attend extension event and apple review days. Estimated 1,300 miles a year at $0.625/mile.

**Budget 2**
**Co PI 2:** Alex Harkess
**Organization Name:** Hudson Alpha Institute for Biotechnology
**Contract Administrator:** Nancy Westfall
**Telephone: 256-327-0400**
**Contract administrator email address:** nwestfall@hudsonalpha.org

| Item | 2023 | 2024 | |
|---|---|---|---|
| Salaries | $0.00 | $0.00 | |
| Benefits | $0.00 | $0.00 | |
| Wages | $0.00 | $0.00 | |
| Benefits | $0.00 | $0.00 | |
| RCA Room Rental | $0.00 | $0.00 | |
| Shipping | $0.00 | $0.00 | |
| Supplies | $0.00 | $0.00 | |
| Travel | $0.00 | $0.00 | |
| Plot Fees | $0.00 | $0.00 | |
| Miscellaneous1 | $0.00 | $67,320.00 | |
| | | | |
| | | | |
| | | | |
| Total | $0.00 | $67,320.00 | $0.00 |

**Footnotes:**
**1:** Funds are for RNAseq work and analyses to be conducted at the Hudson Alpha Institute for Biotechnology. We plan to collect 3 samples at 10 different sampling times from 3 different cultivars for a total of 200 samples and two pathogens (*P. expansum* and *B. cinerea*). RNAseq analysis estimated at $170/sample.

## Objectives

1) Understand gene activity in three major cultivars in relation to infection by *P. expansum* and *B. cinerea* during several preharvest phenological stages and during storage using RNA-seq analyses. Here, we focus on these two major pathogens with a possibility to extend the research to other pathogens in future studies.

2) Compare three major apple cultivars, i.e., Honeycrisp (highly susceptible), Gala (moderately susceptible), and Granny Smith (less susceptible) in term of gene expression to better understand differences seen between cultivars in WA commercial warehouses.

3) Use the new knowledge to identify gene activity that could potentially predict risk for decay development early enough to allow a timely deployment of appropriate strategies to reduce fruit loss.

## Significant Findings

1) Fruit of three cultivars, namely Honeycrisp, Gala, and Granny Smith, were inoculated weekly with spore suspensions of *Penicillium expansum* and *Botrytis cinerea* in the orchard 5, 4, 3, 2, 1 week preharvest, at commercial maturity (harvest = 0 week), and 1, 2, 4, 8, and 24 weeks postharvest.

2) When wounded and inoculated, the three cultivars expressed decay symptoms differentially after 2 and 5 weeks in regular atmosphere storage at 34°F for Gala and Granny Smith and at 37°F for Honeycrisp.

3) *B. cinerea* showed higher early infection rates compared to *P. expansum*.

4) After two weeks of storage, Granny Smith apples did not exhibit any symptoms of blue mold, irrespective of the inoculation stage. In contrast, Gala and Honeycrisp apples expressed the highest blue mold incidence at four weeks preharvest and one week postharvest, respectively. Afterward, the incidence of blue mold exceeded 70% after five weeks of storage, regardless of the cultivar and inoculation stage.

5) Internal ethylene production was significantly induced by inoculations with *P. expansum* and *B. cinerea* in comparison to the control across nearly all inoculation stages.

6) Overall, *Botrytis cinerea* induced higher ethylene production compared to *P. expansum*.

7) The cultivar Honeycrisp produced more ethylene than the two other cultivars when inoculated with either pathogen.

8) As anticipated, fruit firmness decreased over time and did not show significant differences between cultivars at comparable inoculation times.

## Methods

***Activity 1.*** **Fruit inoculation and sampling** [2023, Amiri]

To assess gene activity changes as result from infections by *P. expansum* (blue mold) or *B. cinerea* (gray mold), fruit from Honeycrisp, Gala, and Granny Smith will be used. These three cultivars have

been selected for this study for two main reasons: 1) because they have a range of susceptibility to postharvest decay, allowing comparison of gene activity that may relate to this trait, and 2) because high quality genomes of Gala (Sun et al. 2020), Honeycrisp (Kahn et al. 2022), Granny Smith (Honaas' AP-19-103 final report) are available, facilitating deeper insights into the genetics of decay susceptibility. Fuji, another important cultivar, was suggested by a reviewer, however, because there is not currently a Fuji genome, we decided to include the aforementioned cultivars owing to the expanded possibilities offered by their new genome resources.

*Activity 1.1*. *Isolates growth and inoculum preparation*. Three isolates of *P. expansum* and three isolates of *B. cinerea* will be grown on potato dextrose agar until profuse sporulation is observed and will be used to prepare spore suspensions by mixing equal volumes form each isolate, and the final concentration will be adjusted to $10^5$ spore/ml. Fresh inoculum will be prepared for each inoculation time.

*Activity 1.2*. *Fruit inoculation and fruit sampling*. Trials will be conducted in research blocks at the WSU Sunrise experimental orchard in East Wenatchee, WA. In 2023, fruit from each of the abovementioned cultivars will be inoculated with spore suspensions of *P. expansum* and *B. cinerea* 5, 4, 3, 2, 1 and 0 weeks before estimated commercial maturity (Table 1). At each sampling time, three fruits on trees, will be tagged, and punctured with a sterile syringe (2 mm x 3 mm) at four equidistant points of equatorial zone of each fruit. Fruit will be inoculated with a 20-µl aliquot of the spore suspension of each pathogen, separately. At each inoculation time, three wounded apples, from the same trees, will be inoculated with 20 µl of sterile water and will be used a mock (control) fruit. To avoid cross-contaminations and additional external inoculum, fruit will be covered with fruit protect bags until sampling is conducted. To account for potentially confounding circadian rhythm bias, inoculation and sampling will be conducted at 1-3 pm in the afternoon for each inoculation and sampling time. Inoculated and mock apples will be harvested in labeled Ziplock bags 48 hours post-inoculation and immediately transported in cooler to WSU-TFREC. Tissue samples will be taken as described below and will be frozen at -80°C (-112°F) until used for RNA extraction.

**Table 1.** Cultivars, number of fruit, and sampling times expected in this study

| | Weeks preharvest | | | | | | | | | | | | Weeks postharvest | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | | 4 | | 3 | | 2 | | 1 | | 0 | | 1 | | 2 | | 4 | | 8 | | Total | samples x 2 |
| **Cultivar** | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | Cont. | Inoc. | samples | pathogens |
| Honeycrisp | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 60 | 120 |
| Gala | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 60 | 120 |
| Granny Smith | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 60 | 120 |
| | | | | | | | | | | | | | | | | | | | | | **180** | **360** |

Cont. = control (non-inoculated fruit), Inoc. = Fruit inoculated with either *P. expansum* or *B. cinerea*

For fruit to be sampled and tested during storage, 24 additional apples from each cultivar, will be harvested at commercial maturity, similar to sampling done at 0-week preharvest (Table 1), and will be immediately transported to WSU-TFREC and stored at 35°F (for Gala and Granny Smith) and at 55°F for 10 days then at 37°F hereafter for Honeycrisp to mimic the preconditioning step commonly done at commercial warehouses for this cultivar. Six apples will be sampled at 1, 2, 4 and 8 weeks postharvest (Table 1) and three will be inoculated with spore suspensions of *P. expansum* or *B. cinerea* as described above for preharvest fruit and three apples will be inoculated with water and used as a control. Experimental postharvest fruit will be stored in similar conditions used for preharvest fruit before proceeding with RNA extraction. For both pre and postharvest fruit, quality assessment of starch, firmness, weight, soluble solids, and titratable acidity will be conducted on a fruit subsample from the same tree at each sampling time.

***Activity 2.*** **Tissue sampling, RNA extraction, and quality analyses** [2023-24, Amiri]

Sampling will be performed by removing 8 mm diameter plugs, including peel tissues surrounding the wound and 4 mm of mesocarp (flesh) tissue using a disposable biopsy punch, centered on the wound site, which will be immediately sectioned into two small disks with a sterile scalpel blade. Each biological replicate will contain 24 discs (3 fruits and 4 wounds per fruit). All sectioned discs will be frozen in liquid nitrogen and stored at −121°F. RNA will be extracted using protocols described previously (Honaas and Kahn 2017, Mellidou et al. 2014) and evaluated for quality and purity before storing at −121°F.

***Activity 3.*** **Sequencing and data analyses** [Harkess & Honaas 2024]

Following methods developed by Honaas in the scope of WTFRC project AP-19-103, RNA samples of sufficient high-quality (RIN≥8, $A_{260/280}$ ≥1.8, $A_{260/230}$ ≥2.0) will be analyzed with RNA-Seq at the HudsonAlpha Institute for Biotechnology core facility. Libraries will be constructed using Illumina's TruSeq RNA Library Prep Kit V2 (https://www.illumina.com/) and sequenced to a target sample depth of ≥35 million reads (a higher-than-normal target because the RNA samples are a mixture of fruit and fungal RNA – essentially a double sample). RNA Seq data will be mapped and quantified as described in Honaas et al. (2020), following additional recommendations for analyzing a mixed sample transcriptome as outlined in Honaas et al. (2016).

***Activity 4.*** **Use comparative genomics to discover genes and potential markers in Honeycrisp, Gala, and Granny Smith related to infections and decay development** [Ficklin, Honaas, Amiri, 2024**]**

Recent work by Honaas' team has explored methods to select genes that are related to postharvest fruit quality traits. This has led to the identification of genes that may be useful someday as risk assessment tools, but also that help us learn about the genetics that control important fruit quality traits. Honaas' current project (AP-22-101A) takes large gene activity tables called "gene expression matrices" or GEMs and uses a mix of conventional methods with machine learning techniques (Honaas et al. 2021) to iterate through the data to select genes associated with various traits, like at-harvest fruit maturity or susceptibility to postharvest disorders. The project described here will add gene activity data from three additional cultivars and orchards to Honaas' data but will also add a new synergistic dimension – fruit susceptibility to postharvest decays. Using similar approaches described in AP-19-103 and AP-22-101A, we will identify genes that are relatable specifically to susceptibility to postharvest decays as a function of maturity (sampling done over time) and cultivar and expand the scope of the larger biomarker project by leveraging additional expertise and genome-scale data related to postharvest pathology.

A major benefit will be a first glimpse into how apple fruit gene activity changes in response to postharvest pathogens as a function of maturity (Table 1), and across cultivars that show a range of susceptibility to postharvest decay. This will help us learn about targets for future investigations, as well as potential molecular mechanisms that may explain differences in susceptibility to postharvest pathogens across cultivars. All-together, this project will enhance our understanding of how at-harvest fruit maturity impacts susceptibility to postharvest decays, and why there might be differences in risk among cultivars.

**Expected outcomes.** This study will generate extended across-cultivar genomic knowledge related to pathogen infections. The data generated could help discover Honeycrisp, Gala, and Granny Smith polymorphisms potentially linked to decay development or resistance in a dynamic way pre and postharvest. One hoped long-term objective will rely on comparing data from major cultivars to gather new knowledge (genes or markers) to informs us if similar or different pathways are triggered by the

two most important pome fruit pathogens, *P. expansum* and *B. cinerea*. We will also learn about how the time of infections matters for decay occurring later in storage.

**Pitfalls and limitations.** Because seasonal variability and weather conditions may affect genomic response of fruit to pathogen infections, large and complex data expected form this study will have to be analyzed carefully and accurately. Samples from different cultivars and sampling times following fungal inoculations will be useful to acquire a new cross-cultivars knowledge about the genomics of pathogen infection pre and postharvest that on the long term will be very useful to identify genes and markers that may help predict decay outbreak.

## Results

### Decay incidence and severity

All main effects, including cultivar, pathogen, inoculation time, and most interactions had a significant effect on the incidence of blue mold and gray mold. During the preharvest season, disease incidence and severity generally increased as the inoculation time approaches harvest and differences among cultivars are most pronounced during this period. The pathogen *Botrytis cinerea* exhibited higher early infection rates in comparison to *Penicillium expansum* (Figure 1). The time point at 0 WPr (harvest = commercial maturity) emerges as a crucial transition point. Disease development patterns undergo significant changes with both pathogens demonstrating increased aggressiveness (Figure 1). At this stage, differences in cultivar resistance become less pronounced.

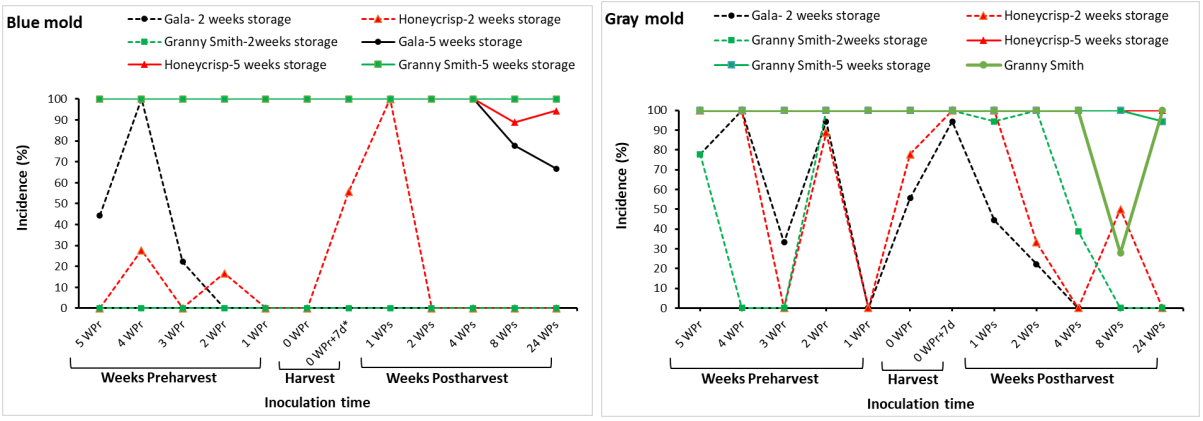


**Figure 1.** Incidence of blue mold (right) and gray mold (left) after two weeks (dashed lines) and 5 weeks (solid lines) in a regular atmosphere at on three apple cultivars inoculated with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

Gala showed higher blue mold severity than Honeycrisp during the preharvest period 5 weeks preharvest to 1 week preharvest, than an inverse situation was observed in storage with higher severity observed on Honeycrisp (Figure 2, left). Granny Smith was resistant to blue mold infection after 2 weeks in storage and exhibited the lowest blue mold virulence at almost all pre and postharvest inoculation stages when storage was extended to 5 weeks.

In terms of gray mold susceptibility, both Gala and Honeycrisp exhibited significantly greater vulnerability 5 and 4 weeks prior to harvest when compared to Granny Smith (Figure 2, right). As the

harvest period approached, the susceptibility of Gala to gray mold decreased, whereas that of Granny Smith increased, ultimately aligning with the susceptibility levels observed in Honeycrisp (Figure 2, right).

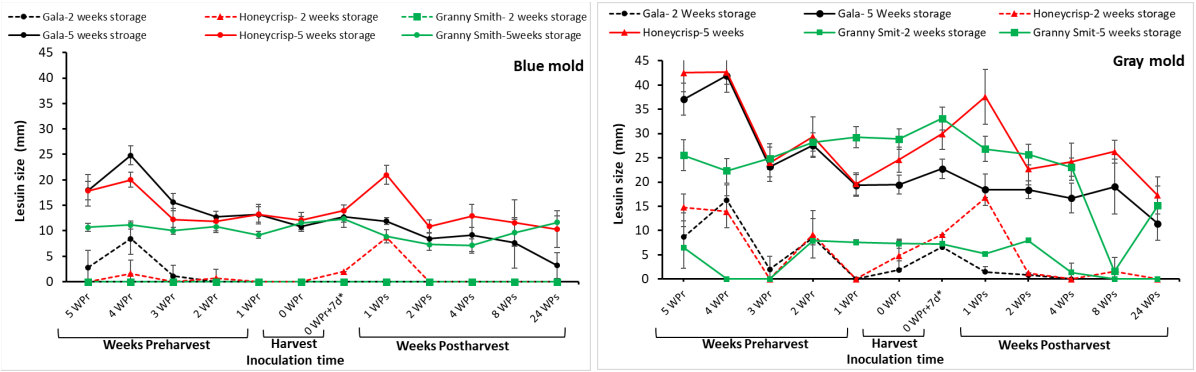


**Figure 2.** Severity of blue mold (left) and gray mold (right) after two weeks (dashed lines) and 5 weeks (solid lines) in a regular atmosphere at on three apple cultivars inoculated with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

## Fruit Quality Parameters

Seven fruit quality parameters were assessed at each weekly inoculation, including weight, firmness, Brix, titratable acidity (TA), internal ethylene production, color, and red over color. No significant differences were observed in weight, Brix, or titratable acidity across cultivars and pathogens. The firmness of the apples decreased over time yet exhibited similar trends among cultivars at the same sampling intervals (Figure 3).

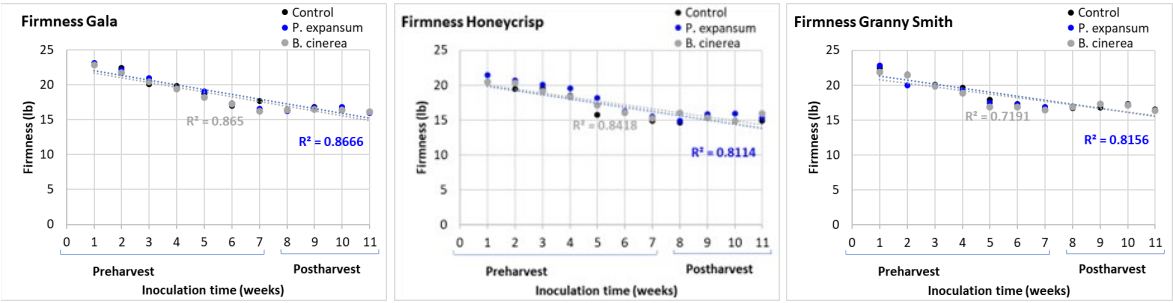


**Figure 3.** Firmness (lb) of Gala, Honeycrisp, and Granny Smith apples inoculated weekly at different maturity stages with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

Interestingly, internal ethylene production was significantly induced by inoculations with *P. expansum* and *B. cinerea* in comparison to the control at nearly all inoculation stages. Overall, *Botrytis cinerea* elicited a greater production of ethylene compared to *P. expansum*. Notably, the cultivar Honeycrisp produced more ethylene than the other two cultivars when inoculated with either pathogen (Figure 4).

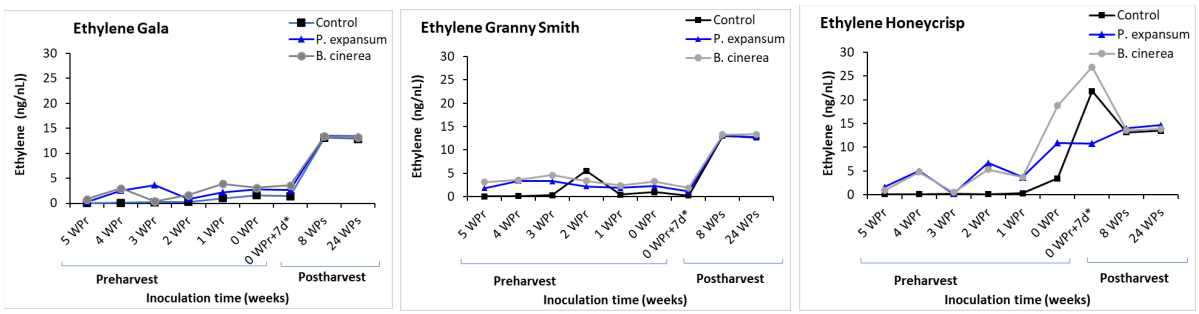


**Figure 4.** Firmness (lb) of Gala, Honeycrisp, and Granny Smith apples inoculated weekly at different maturity stages with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

## Significance to the industry

The objective of this study was to understand gene activity in three major cultivars in relation to infection by *P. expansum* and *B. cinerea* during several preharvest phenological stages and during storage using RNA-seq analyses. The ultimate goal will be to use the new knowledge to identify gene activity that could potentially predict risk for decay development early enough to allow a timely deployment of appropriate strategies to reduce fruit loss.

Although the ongoing genomic analysis will determine whether such differences are governed by specific genes, the variability in response to elicitation between the two pathogens and the three cultivars observed at the phenotypic level may provide opportunities to identify genetic markers that can assist in predicting the risks of gray and blue mold infections. If confirmed and validated, such tools will enhance the development of informed management strategies aimed at reducing decay and increasing packout.

# **Project Title:** UV-C and antimicrobial spray to control decay and food safety risks

**Report Type:** Continuing Project Report **Year**: 1 of 3

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**Cooperators**: Columbia Fruit Packers

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $71,136
**Total Project Request for Year 2 Funding:** $99,250
**Total Project Request for Year 3 Funding:** $80,519

**Other related/associated funding sources:** Awarded
**Funding Duration:** 2024 - 2027
**Amount:** $249,956
**Agency Name:** Washington State Specialty Crop Block Grant Program
**Notes:** N/A

**WTFRC Collaborative Costs:** None

**Budget 1**
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| Item | 2024 | 2025 | 2026 |
|---|---|---|---|
| Salaries | $28,924.00 | $30,081.00 | $15,642.00 |
| Benefits | $7,466.00 | $8,063.00 | $4,354.00 |
| Wages | | | |
| Benefits | | | |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | $9,000.00 | $9,000.00 | $7,000.00 |
| Travel | $582.00 | $582.00 | $582.00 |
| Plot Fees | | | |
| Miscellaneous | | | |
| | | | |
| | | | |
| | | | |
| Total | $45,972.00 | $47,726.00 | $27,578.00 |

**Footnotes:**
Salaries and Benefits: Salaries are requested for a Graduate Research Assistant (GRA) at 0.49 FTE for 2.5 years at 25.8% benefit rate. Salaries are calculated with an increase of 4% per year, and benefits are calculated with an inflation rate of 8%.
Supplies: Funds are requested for purchasing laboratory consumables (such as gloves, centrifuge tubes, and pipette tips), microbiology experiments (such as growth media, Petri dishes, inoculation loops), and chemical analysis (such as antimicrobial agents and assay solutions for phenolic content measurements).
Travel: Funds are requested to cover the mileage for transportation to WSU/cooperator facility for sample collection and facility visiting once a year adhering to university policies, delineated as $0.67 per mile × 868 miles = $581.56

*PI Wang and Co-PI Waite-Cusic will co-advise one GRA and share supplies throughout this project.

**Budget 2**
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| Item | 2024 | 2025 | 2026 |
|---|---|---|---|
| Salaries | $12,309.00 | $12,678.00 | $13,059.00 |
| Benefits | $9,355.00 | $9,635.00 | $9,925.00 |
| Wages | | | |
| Benefits | | | |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | $2,000.00 | $2,060.00 | $2,122.00 |
| Travel | | | |
| Plot Fees | $1,500.00 | $1,500.00 | $1,500.00 |
| Miscellaneous | | | |
| | | | |
| | | | |
| | | | |
| Total | $25,164.00 | $25,873.00 | $26,606.00 |

**Footnotes:**
Salaries and Benefits: Salaries are requested for a Faculty Research Assistant (FRA) at $49,236/year for 3 months, and 76% benefit rate. Salaries are calculated with an increase of 3% per year, and benefits are calculated with an inflation rate of 3%.
Supplies: Funds are requested for purchasing laboratory consumables and microbiological supplies such as growth media, Petri dishes, inoculation loops, and pipette tips.
Plot Fees: Annual plot fee at the Southern Oregon Research and Extension Center is $1500.

**Budget 3**
**Co-PI 4:** Claire Murphy
**Organization**: Washington State University Irrigated Agriculture Research and Extension Center
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**Contract administrator email address:** prosser.grants@wsu.edu
**Station Manager/Supervisor:** Naidu Rayapati
**Station manager/supervisor email address:** 509-786-9215

| Item | 2024 | 2025 | 2026 |
|---|---|---|---|
| Salaries | | $15,562.00 | $16,184.00 |
| Benefits | | $1,559.00 | $1,621.00 |
| Wages | | | |
| Benefits | | | |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | | $8,000.00 | $8,000.00 |
| Travel | | $530.00 | $530.00 |
| Plot Fees | | | |
| Miscellaneous | | | |
| | | | |
| | | | |
| | | | |
| Total | $0.00 | $25,651.00 | $26,335.00 |

**Footnotes:**

Salaries and Benefits: Salaries are requested in years 2 and 3, respectively, for a graduate student's summer hourly salary and a part-time hourly employee. Benefits are requested in years 2 and 3, respectively, which are tied to the graduate student's summer hourly salary and a part-time hourly employee.

Supplies: Funds are requested for purchasing laboratory consumables and microbiological supplies such as growth media, Petri dishes, inoculation loops, and pipette tips.

Travel: Funds are requested to cover the mileage for transportation to cooperator facility for sample collection and facility visiting adhering to university policies, delineated as $0.655 per mile × 808 miles = $529.24

## OBJECTIVES

The overall goal is to is to combine a short-time UV-C light treatment with an antimicrobial spray (sanitizers approved for organic use and plant-based agents) to create a practical and cost-effective strategy for reducing postharvest apple losses due to **decay-causing fungi** (***Penicillium expansum*, blue mold**) and **foodborne pathogens** (***Listeria monocytogenes***).

1. Define and optimize UV-C treatment to inactivate decay-causing fungi and foodborne pathogens.

2. Identify and evaluate plant-based antimicrobials and organic sanitizers to prevent or delay the growth of decay-causing fungi and foodborne pathogens.

3. Optimize integrated lab-scale UV-C and antimicrobial treatment to inactivate and delay the growth of decay-causing fungi and foodborne pathogens on the surface of apples while preserving product quality.

4. Assess the optimized UV-C and antimicrobial integrated treatment using a pilot-scale system followed by simulated bulk cold storage.

*The current project report (Year 1, Sep 2024 – current) covers research items from Objectives 1-3.*

## SIGNIFICANT FINDINGS

- Among the evaluated antimicrobial agents (essential oils, organic acids, organic-approved sanitizers), hydrogen peroxide ($H_2O_2$, 1-5%) has the most significant antifungal activity in inhibiting the growth of *P. expansum*.
- Similarly, hydrogen peroxide demonstrated the highest effectiveness inactivating *L. monocytogenes* among the evaluated chemicals.
- UV-C demonstrated dose-dependent antifungal efficacy against *P. expansum* spores.
- UV-C also demonstrated dose-dependent efficacy against *L. monocytogenes* in liquid suspension and on flat surfaces.
- The efficacy of UV-C on *L. monocytogenes* on apple surfaces was significant but non-linear as a function of exposure time.
- The non-pathogenic strain, *Listeria innocua*, can be a suitable surrogate for pathogenic *L. monocytogenes* cocktail for pilot-scale validation
- UV-C is effective in controlling postharvest decay caused by *P. expansum* on apple surfaces and extends apple shelf life in storage.
- UV-C treatment is less effective when the fungal infection is in the calyx or stem regions where the light does not reach.
- Hydrogen peroxide alone was less effective in controlling postharvest decay on apple surfaces.
- Combining hydrogen peroxide and UV-C treatment provides better results in decay control by addressing the limitations of each treatment.
- The sequence of UV-C treatment and hydrogen peroxide does not appear to affect effectiveness in the current study.

## METHODS

### PART I: Studies on Decay-Causing Fungi (*Penicillium Expansum*)

**Fungal colony growth rate analysis:**
To assess the normal colony growth rate of the *P. expansum* (**Figure 1**), small pieces of agar were inoculated onto Potato Dextrose Agar (PDA) under sterile conditions. Careful observation was done during 96 hours on isolated colonies. Measurements in orthogonal direction of the diameter of the colonies were taken every 24 hours in 48 hours.

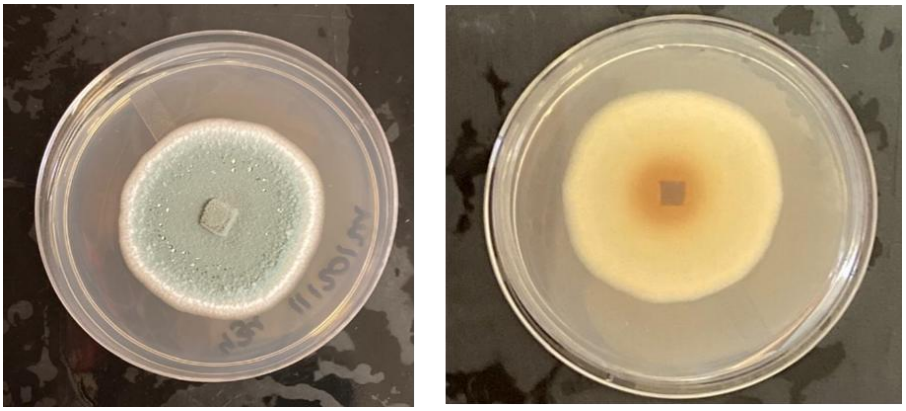

**Figure 1**: Colony of *P. expansum* (front and back)

**Antimicrobial screening against *P. expansum*:**
Various chemical antimicrobial agents approved for organic production, including essential oils, organic acids, and sanitizers, were tested for efficacy against *P. expansum*. The methods for testing include diffusion disk method, directly incorporating chemicals into the growth media, and spore suspension exposure. **Table 1**. Summarizes the antimicrobials and their respective concentration used to evaluate their antifungal efficacy.

**Table 1.** Antimicrobial agents and their concentrations evaluated against *P. expansum*

| | | |
|---|---|---|
| **Organic Acids** | Acetic Acid | 1%, 2%, 3%, 4% |
| | Lactic Acid | 1%, 2%, 3%, 4% |
| | Lactic + Acetic Acid | 3% lactic acid +3% acetic acid |
| **Essential Oils** | Tea Tree Oil | 0.5%, 1%, 2%, 3%, 4%, 100% |
| | Lemon Oil | 0.5%, 1%, 2%, 3%, 4%, 100% |
| | Thyme Oil | 0.5%, 1%, 2%, 3%, 4%, 100% |
| **Sanitizers** | Hydrogen Peroxide ($H_2O_2$) | 1%, 2%, 3%, 4% |

*Diffusion Disk Method:* To evaluate the sensitivity of the fungal strains, fungal spores were evenly spread over the agar surface using a sterile spreader, Tween 20 to a 0.05% concentration was used to recollect the spores from a single colony. Sterile paper discs soaked with each concentration, detailed in **Table 1**, were carefully positioned over the inoculated PDA. Plates were incubated at 25°C to ensure optimal temperature for fungal growth. After three days of incubation, inhibition zones were measured, inhibition halo indicated antimicrobial effectiveness.

*Incorporating chemicals into fungal growth media:* Essential oils were carefully and thoroughly incorporated with PDA agar before pouring into plates. Two approaches were

evaluated: the number of recovery of cells and effect in growth rate. To evaluate the first case, standardized fungal spore suspensions are carefully spread onto the solidified agar surface, regarding the second one a small piece of agar was placed in the center of the plate. All essays were incubated, and plate count was performed to assess the number of cells. And growth monitoring of fungal colonies was observed and measured over 96 hours. Growth on essential oil-infused plates was compared to the results obtained in the normal growth rates essays for efficacy assessment.

*Spore Suspension Exposure:* From a 7-day culture a spore suspension was prepared by following flooding plate technique, with 0.01% Tween 20. The spore suspension was adjusted to a concentration of ten to six using a hemocytometer to count the cells. The evaluated antimicrobials were adjusted to a final volume of 900 microliters and 100 microliters of the spore suspension was added. After one minute of exploration, the samples were centrifuge, double washed and resuspended in 100 microliters of sterile distilled water. The suspension was then inoculated in PDA incubated and after 72 days, plate count was applied to evaluate cells recovery.

**Characterizing UV-C doses on *P. expansum* spore inactivation:**
A bench-top, batch type UV-C (254 nm) chamber was used for lab-scale UV treatment. A liquid suspension (8 mL) of *P. expansum* spores was UV-C treated. The times of exposure and corresponding doses of the treatments are displayed in **Table 2**. Treated spores were plated on PDA and incubated at 25 °C before colony enumeration.

**Table 2. Time of exposure and intensity of the treatment evaluated.**

| Exposure Time (SEC) | Dose |
|---|---|
| 10 | 0.45 kJ/$m^2$ |
| 20 | 0.9 kJ/$m^2$ |
| 30 | 1.35 kJ/$m^2$ |
| 40 | 1.8 kJ/$m^2$ |

**Decay evaluation in apples**
Apples cultivar Gala was inoculated in the surface, stem and calyx and treated with 3% hydrogen peroxide, 30 seconds of UV-C light, or a combined treatment. To evaluate the combined treatment, two more tests were performed. First, to evaluate the order in which they should be applied, inoculated apples were first exposed to hydrogen peroxide, air dried and then exposed to UV-C treatment. Then the opposite was performed. To determine the implications of the way that the antimicrobial solution was applied, submerge in and rinsing were the two options analyzed. In the first scenario, apples were fully submerged in a 3% hydrogen peroxide solution and let air dry. In the second scenario, apples were rinsed with 3% hydrogen peroxide. Apples were then stored at room temperature (for the purpose of accelerating decay process) and observed for fungal infection severity.

**PART II: Studies on Foodborne Pathogens (*Listeria Monocytogenes*)**

**Preparation of *Listeria* cultures:**
*L. monocytogenes* strains 360, 043, 027, 041, and 021 used in this study were isolated from various fresh produce. Non-pathogenic strain, *L. innocua*, was tested as a surrogate for *L. monocytogenes*

cocktails. All work was conducted in a biosafety cabinet unless otherwise specified. Frozen stock of each culture was streak plated onto Tryptic Soy Agar with Yeast Extract (TSAYE) to produce isolated representative colonies. Representative colonies of each strain were transferred to TSBYE and incubated at 37 °C for 24h. The incubated broth was then spread plated onto TSAYE and incubated under the same conditions. Bacterial lawns were harvested using 7 mL of deionized water. Deionized water was chosen as a medium as to not leave any residues post drying onto the surface of apple, as saline residue could contribute to UV-C shielding of the bacteria.

**Antimicrobial screening against**
A disc diffusion assay was performed on TSAYE inoculated with 300 µL of *L. innocua* broth. After incubating at 37 °C for 24h results were observed. Hydrogen peroxide, citric acid, acetic acid, and thyme oil were determined to have an inhibitory effect on *L. innocua* at 3%, 0.5M, 3%, and 100%, respectively. Tea tree oil was determined to have insignificant inhibitory effect. A broth dilution assay was performed with effective antimicrobials from which minimum inhibitory and bactericidal concentrations were determined. Minimum antimicrobial concentration with no growth was determined to be the Minimal Inhibitory Concentration (MIC) for the tested species. For tubes with no growth after 24 hours, 100 µL of broth was transferred into a 10 µL tube of TSAYE and incubated for 48 hours. Growth was assayed and the minimum antimicrobial concentration that led to no growth after incubation was determined to be the Minimal Bactericidal Concentration (MBC).

**Evaluate UV-C efficacy on apples:**
The same UV-C chamber described above for fungi treatment was used for *L. monocytogenes* study. Whole apples (Gala) were prepared by submerging in 70% ethanol for 1 min and drying for an hour and thirty min. 300 µL of cocktail was spot plated onto the equatorial region of the apple in an area of $\approx 2.5$ cm$^2$. Inoculated apples were dried (<1h), individually transferred to the UV-C box, and exposed to UV under selected time. Six replicate apples were used for each treatment time, and three apples were used as untreated controls, from which the average recovery enumeration was used to determine the treatment reduction from the enumeration of treated apples. Control apples were enumerated at the beginning, middle, and end of experiment. During treatment it was ensured that the inoculated area was facing the UV-C emitters. For enumeration, apples were transferred to a sterile U-line bag and submerged in saline solution (100 mL, 85%). Apples were hand massaged for 30 s in bag to detach bacteria from the surface of apples. Resulting solutions were plated on TSAYE and Neogen Harlequin (selective media for *L. monocytogenes*). Enumerations of surviving bacteria were conducted after 48 h of incubation.

## RESULTS AND DISCUSSION

### PART I: Studies on Decay-Causing Fungi (*Penicillium Expansum*)

**Antimicrobial screening against *P. expansum*:**
Results of antimicrobial screening using diffusion disk method (**Figure 2**) showed that hydrogen peroxide (1-3%) significantly inhibited the growth of *P. expansum* on the growing media, while other tested chemicals (thyme oil, lemon oil, tea tree oil, lactic acid, acetic acid, had no inhibitory effect regardless of concentration. Essential oils directly incorporated into the fungal growing media also did not show any inhibition at the tested concentrations. When spores were suspended in the tested chemicals, hydrogen peroxide also demonstrated the highest antifungal effect among all the samples evaluated, with an increased concentration (1 - 4%) leading to higher reductions of fungal spores (1.9 – 4.2 log CFU/mL). Therefore, hydrogen peroxide was selected for further evaluation with UV-C treatment in apples.

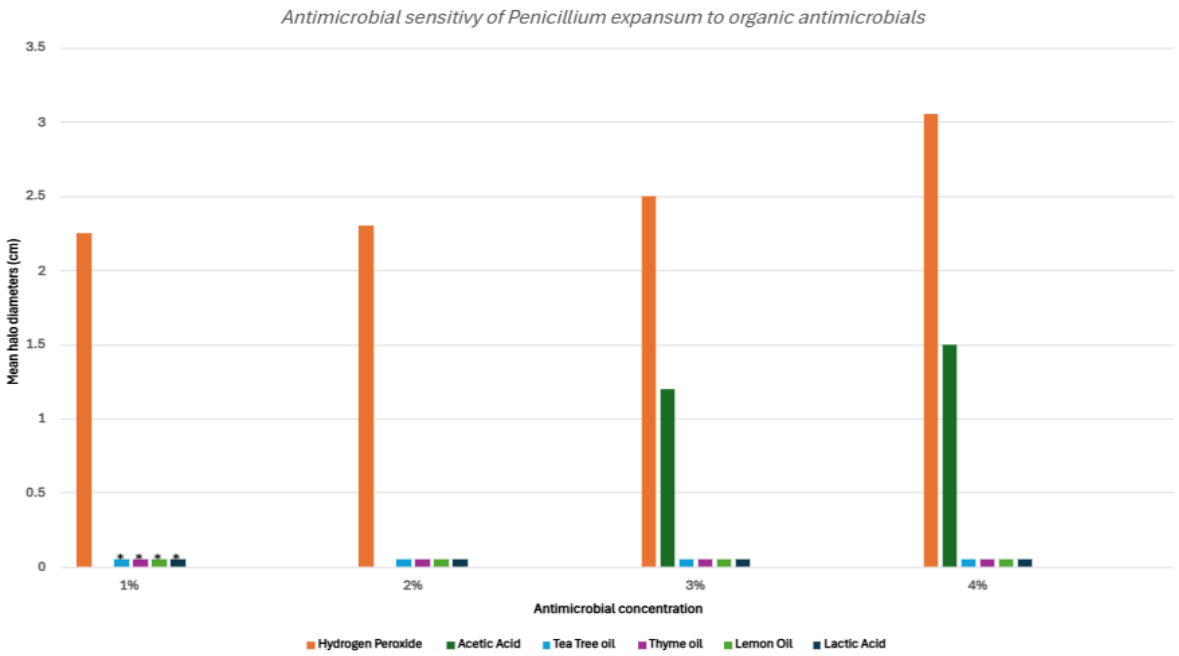


**Figure 2**: Antimicrobial screening using agar diffusion method. A larger halo diameter indicates a higher level of antimicrobial efficacy. Bars with * indicate no halo inhibition was observed for lactic acid, Tea tree oil, lemon oil or thyme oil.

**Characterizing UV-C doses on *P. expansum* spore inactivation:**
The number of reductions of *P. expansum* spores by UV-C was affected by the dose of light. While more replicates of experiments are needed for statistical analysis, it can be seen from the following data (**Figure 3**) that the selected doses inactivated fungal spores in the range of 0.9 – 2.7 log. Appropriate doses were selected for further testing with hydrogen peroxide in apples.

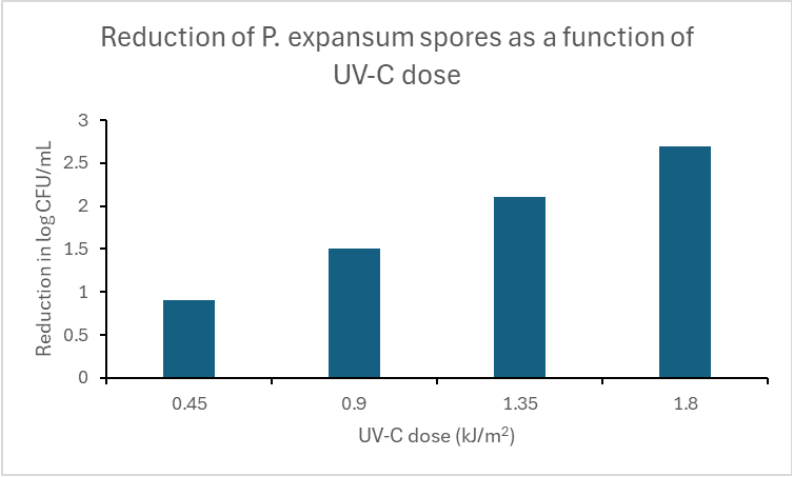


**Figure 3:** Reduction of *P. expansum* spores as a function of UV-C dose. Error bars are not shown as triplicate experiments have not been completed.

**Apple decay evaluation:**
UV-C treatment demonstrated significant effect in delaying the disease progress caused by *P. expansum* when the infection site is on apple surface. However, hydrogen peroxide did not perform as well in delaying apple decay when the infection sites were on apples surfaces. Both submerging and rinsing

seem to have the same results so far. **Figure 4** shows the photos of apple infection sites (surfaces) with no UV-C treatment (control), UV-C treatment for 30 s, 3% hydrogen peroxide, and 3% hydrogen peroxide followed by 30 s UV-C exposure. Photos were taken after 14 days of apple storage at room temperature.

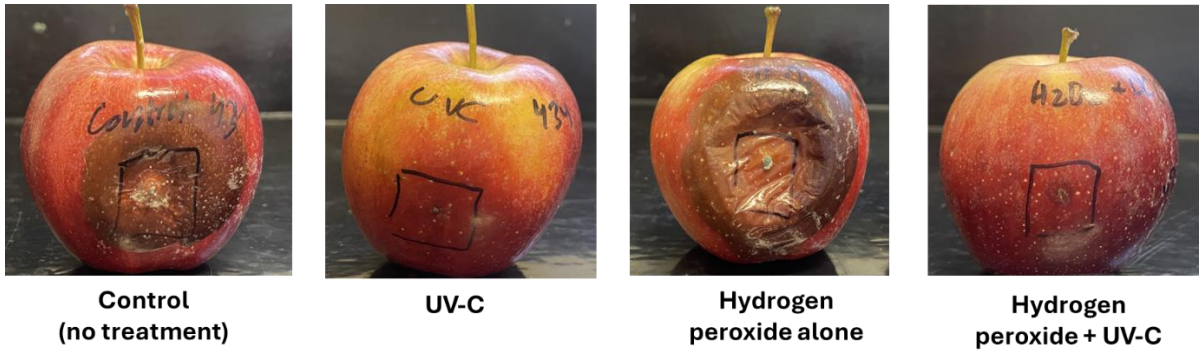


**Figure 4.** UV-C treatment on apple surface at 14 days

Nevertheless, hydrogen peroxide significantly reduced disease development when the infection zone was located on the calyx or the stem of apples (**Figure 5**). On the contrary, UV-C alone did not behave well when the infection zone is on the calyx or the stem, probably because the light could not fully reach target sites.

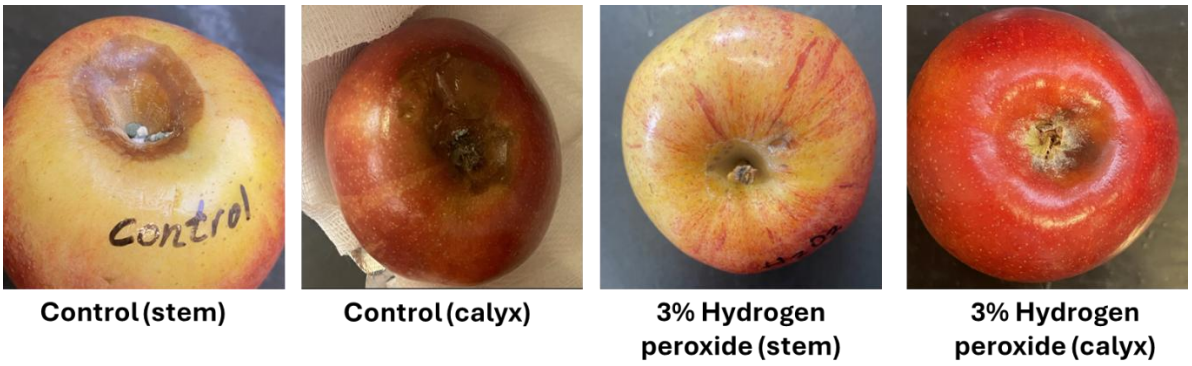


**Figure 5**. Hydrogen peroxide (3%) on apple stem and calyx at 14 days

When combining UV-C and hydrogen peroxide, better results are achieved probably due to the complementary effects of the two treatments (**Figure 6**). On one side, hydrogen peroxide can access the stem and calyx regions where UV-C cannot. UVC, on the other hand, helps inactivate spores when the wound is on the surface of the apple. Regarding the order of treatment, there seems to be no visual differences in the results after seven days in storage at room temperature**.**

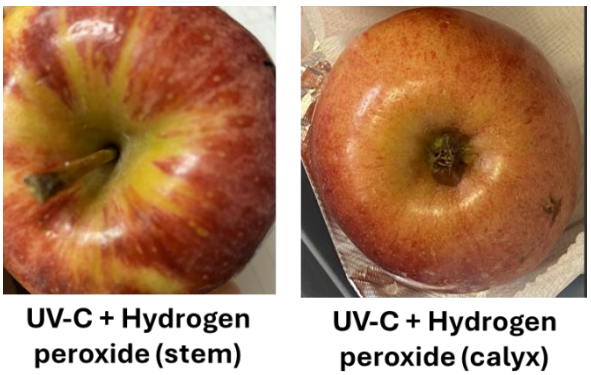


**Figure 6.** Combine treatment of UVC + hydrogen peroxide on apple stem and calyx at 14 days

**PART II: Studies on Foodborne Pathogens (*Listeria Monocytogenes*)**

**UV-C inactivation kinetics of *L. innocua* (nonpathogenic surrogate of *L. monocytogenes*):**
An inactivation kinetic curve for *L. innocua* by UV-C treatment was created from time kill data in planktonic trials. A reduction of 1.1 log per second treatment was determined. A steep shouldering effect is observed in apple treatments which limits the reduction that is achievable by UV-C alone, even at higher treatment doses. This is probably due to the waxy coating and microstructure that is on the surface of apples, along with the relatively robust genetic repair mechanisms that *Listeria* species possesses. This result allows for the UV-C shielding effect by apple microstructure and other surface characteristics to be quantified for in situ trials.

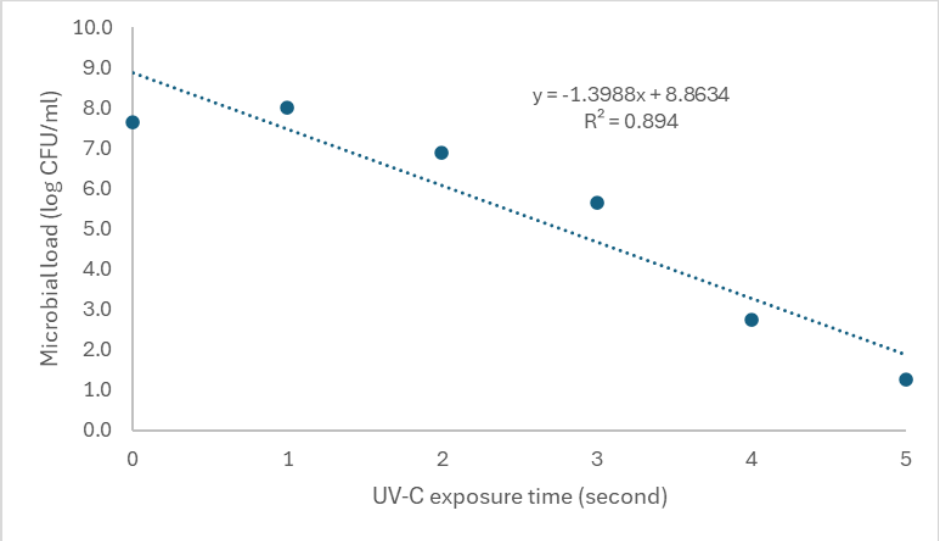


**Figure 7**. UV-C inactivation kinetics of *L. innocua* in saline solution.

**UV-C effectiveness on apple surfaces**:
UV-C of different treatment time (corresponding to different doses) were assessed for effectiveness against *L. monocytogenes* cocktail and *L. innocua* inoculated on apple surfaces (**Figures 8-10**). No statistical significance was found between *L. monocytogenes* cocktail and *L. innocua* for both high dosage group (**Figure 8**) and low dosage group (**Figure 9**), suggesting nonpathogenic *L. innocua* can be used as a surrogate for pathogenic *L. monocytogenes* cocktail. This information provides valuable insight for the Year 3 pilot-scale validation. Results also suggested that there was no statistical

difference in UV-C effectiveness on apples within the high dosage group or the low dosage group. As we refine our methods to better quantify lower-level recovery from apple surface, we hope to be able to achieve a kinetic kill curve for treatment dosage on the surface of apple with statistical difference between treatment points. From this kinetic kill curve, we can show the "shouldering" effect where low dosages display a log reduction that is not continuous into higher doses, leading to low level survivability regardless of intense treatment doses. This information will be important for determining the optimum UV-C dose that is effective and energy-saving for apple treatment.

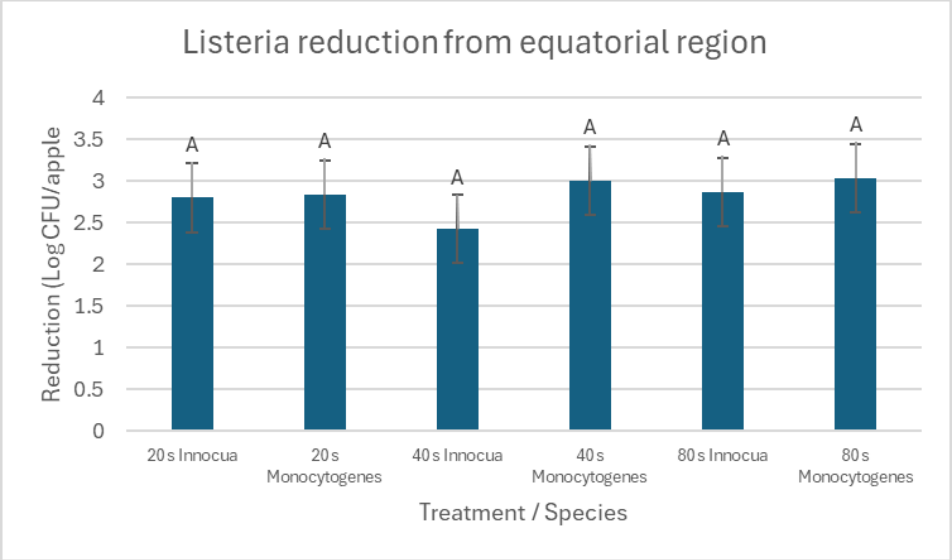


**Figure 8**. *L. innocua* and *L. monocytogenes* reduction in population from apple equatorial region by UV-C treatment of 20, 40, and 80 s. Same letters indicate a lack of significant difference at $P = 0.05$.

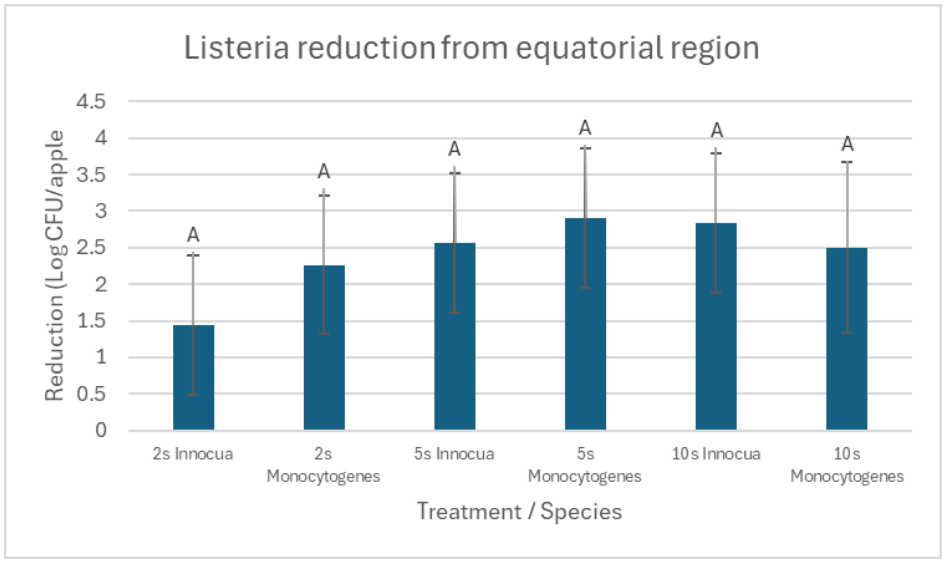


**Figure 9**. *L. innocua* and *L. monocytogenes* reduction in population from apple equatorial region by UV-C treatment of 2, 5 and 10 s. Same letters indicate a lack of significant difference at $P = 0.05$.

**Antimicrobial screening:**

Plate assay data (**Figure 10**) and bacterial MIC and MBC results (**Table 3**) indicated that hydrogen peroxide and citric acid were appropriate for future trials. While an inhibitory effect was observed at high concentrations of thyme oil and acetic acid, this was determined to not be feasible for larger-scale

trials. AOAC method of sanitizer verification is planned for all antimicrobial chemical species that will be investigated in the future UV-C + antimicrobial experimentation. The current data will help quantify and optimize the effectiveness of combined treatment on bacterial reduction.

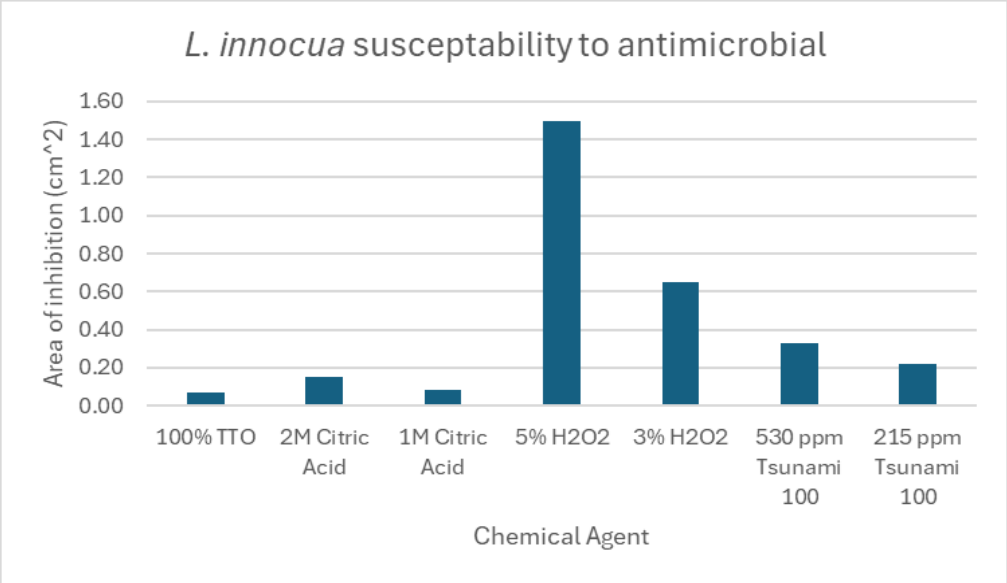


**Figure 10.** Area of inhibition of *L. innocua* inoculated on TSAYE incubated at 37 ˚C for 24 h.

**Table 3.** Minimum Inhibitory Concentration (MIC) for *L. innocua* and *L. monocytogenes* and Minimum Bactericidal Concentration (MBC) of *L. monocytogenes*

| Antimicrobial | *L. innocua* MIC | *L. monocytogenes* MIC | *L. monocytogenes* MBC |
|---|---|---|---|
| $H_2O_2$ | 300 ppm | 240 ppm | 240 ppm |
| Thyme Oil | 10% | >10% | >10% |
| Citric Acid | 1920 ppm | 4800 ppm | 38400 ppm |
| Acetic Acid | 3000 ppm | 150 ppm | 1200 ppm |

**Project/Proposal Title:** Interaction of microbiome and *Listeria* on apples during cold storage

**Report Type:** Final Report

**WTFRC Project Number:** AP-20-100A

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**Cooperators**: Stemilt Growers LLC.; Allan Brothers Inc., Hansen Fruit

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $84,704
**Total Project Request for Year 2 Funding:** $86,853
**Total Project Request for Year 3 Funding:** $88,076

**WTFRC Collaborative Costs:**

| Item | 2020-2021 | 2021-2022 | 2022-2023 |
|---|---|---|---|
| Salaries | | | |
| Benefits | | | |
| Wages | 2,509 | 2,548 | 2,588 |
| Benefits | 1,280 | 1,272 | 1,284 |
| RCA Room Rental | 6,750 | 6,952 | 7,160 |
| Shipping | | | |
| Supplies | 500 | 525 | 550 |
| Travel | 650 | 700 | 725 |
| Plot Fees | | | |
| Miscellaneous | | | |
| Total | 11,689 | 11,997 | 12,307 |

**Footnotes:**

**Budget 1**
**Primary PI:** Meijun Zhu
**Organization Name:** Washington State University
**Contract Administrator:** Anastasia Kailyn Mondy
**Telephone:**
**Contract administrator email address:** arcgrants@wsu.edu

| Item | 2020-2021 | 2021-2022 | 2022-2023 |
|---|---|---|---|
| Salaries | 31,500 | 32,760 | 34,070 |
| Benefits | 11,010 | 11,451 | 11,909 |
| Wages | 3,200 | 3,328 | 3,461 |
| Benefits | 305 | 317 | 329 |
| Equipment | | | |
| Supplies | 16,000 | 16,000 | 15,000 |
| Travel | 2,000 | 2,000 | 2,000 |
| Miscellaneous | 9,000 | 9,000 | 9,000 |
| Plot Fees | | | |
| Total | 73,015 | 74,856 | 75,769 |

**Footnotes:**

## OBJECTIVES

1. Examine survival of resident microbiota on apple surfaces stored under RA and CA.
2. Characterize the dynamic change of dominant and differential bacterial and fungal populations in the microbiome of fresh apples in the co-occurrence of *Listeria* under RA or CA storage.

## SIGNIFICANT FINDINGS

1. The *L. innocua* count was decreased by 1.5 $\log_{10}$ CFU/apple on Fuji apples under RA or CA storage.
2. *Enterobacteriaceae* had higher counts on apples contaminated with *L. innocua* than uninoculated control Fuji apples at RA or CA storage.
3. The number of *Pseudomonas* decreased during 36 weeks of cold storage. Introducing *L. innocua* on apples increased the reduction of *Pseudomonas* on apples, especially under CA storage.
4. Lactic acid bacteria count on apples slightly increased after 36 weeks of cold storage regardless of storage condition.
5. Populations of native bacteria and yeast and molds, particularly *Penicillium*, were increased on apples with or without *L. innocua* inoculation after 36 weeks of RA or CA storage.
6. A 9-month CA or RA storage had a great influence on fungal community structures; these significant differences were found on the phylum level, family level, genus level, and species level.
7. Inoculation with *L. innocua* significantly impacts the fungal community on Fuji apples before commercial storage or after 9 months of CA or RA storage.
8. *Basidiomycota* followed by *Ascomycota* are the dominant fungal phyla in Fuji apples, regardless of *L. innocua* inoculation and storage condition.
9. The relative abundance of *Basidiomycota* in uninoculated Fuji apples decreased after 9 months of CA or RA storage while *Ascomycota* increased.
10. The relative abundance of *Basidiomycota* and *Ascomycota* in inoculated apples remained stable after 9 months of RA storage. However, the relative abundance of *Basidiomycota* of inoculated apples decreased, while *Ascomycota* increased after 9 months of CA storage.
11. *Bulleribasidiaceae* is the dominant family in uninoculated apples followed by *Filobasidiaceae*. In inoculated apples, *Filobasidiaceae* is the dominant family, followed by *Bulleribasidiaceae* and *Pleosporaceae*. The abundances of these families changed after 9 months of CA or RA storage.
12. *Vishniacozyma* and *Filobasidium* are the dominant genera in uninoculate apples, accounting for 52.7% and 25.6% of total fungal genera. In inoculated apples, *Filobasidium, Vishniacozyma*, and *Alternaria* are dominant genera with relative abundances of 27.8%, 22.6%, and 21.6%, respectively. These genera changed after 9 months of CA or RA storage.
13. *Vishniacozyma victoriae and Filobasidium magnum* were the main species detected in both uninoculated and inoculated apples. The relative level of *Filobasidium magnum* decreased after 9 months of CA and RA storage regardless of inoculation.
14. *Tausonia pullulans* level were low in Fuji apples regardless of the inoculation but was extremely elevated in the inoculated apples after 9 months of RA storage (Fig. 5), increasing from 0.1% to 26.2%.
15. The introduction *of L. innocua* led to a significant decrease in bacterial diversities and richness on Fuji apples.
16. Over 36 weeks of CA storage, alpha diversity of bacterial communities gradually increased in *L. innocua*-contaminated Fuji apples, while it decreased on uninoculated Fuji apples.
17. Dynamic alterations were evident in bacterial communities at the phylum, family, genus, and species levels after the introduction of *L. innocua*.
18. *Deinococcus*, *Hymenobacter*, *Rhodococcus* and *Pantoea* were positively correlated with *L.*

*innocua*, while *Massilia* and *Pseudomonas* were negatively correlated with *L. innocua* on apples.

19. *Proteobacteria* dominated apple phyla with unique profiles across varieties, comprising 60-70% in Fuji and Granny Smith (GS) apples, and ~40% in Crispps Pink (CP) and WA 38 (WA) apples.
20. Each variety exhibited unique microbiome profiles at the family, genus, and species levels:
    - At the family level, CP was dominant by *Microbacteriaceae* and *Xanthobacteraceae*, GS by *Pseudomonadaceae* and *Enterobacteriaceae*, and WA by *Rhodobacteraceae* and *Cytophagaceae*.
    - At the genus level, CP was dominated by *Xanthobacter* and*Mesorhizobium*, GS by *Pseudomonas*, and WA by *Paracoccus* and *Rhodococcus*.
    - At the species level: *Acinetobacter johnsonii* was dominant in CP, *Pseudomonas veronii* in GS, and *Rhodococcus corynebacterioides* in WA.
21. WA apples displayed a unique microbiome compared to CP and GS apples, both at harvest and throughout storage.
22. Cold storage significantly altered the microbiome composition and diversity of each apples, with storage duration having a stronger effect than the storage environment (RA vs. CA).
23. The Chao1 index decreased over time, indicating reduced microbial richness during storage, with CP apples experiencing the largest decline and WA apples the smallest.

## METHODS

*1. Apple cultivar selection*

Fuji, Granny Smith (GS), WA38 (WA), and Cripps Pink (CP) apples were used in this study.

*2. Strain selection and inoculum preparation*

*L. innocua* isolates from Bidart apple facility and other processing plants were used to prepare a 3-strain cocktail of *L. innocua* inoculum per our well-established method (Sheng et al., 2018).

*3. Inoculation*

Washed and unwaxed apples of selected varieties were inoculated to establish $1\times10^6$ CFU/apple using a 3-strain cocktail of *L. innocua* per our well-established method (Sheng et al., 2018).

*4. Cold storage treatments*

Unwaxed and uninoculated or inoculated apples of selected varieties were randomly divided and subjected to well-controlled RA or CA storage for 9 months. 1% $CO_2$ and 1.2% $O_2$ were used in CA storage following the practices of commercial packing facilities for the selected varieties. A storage temperature of 33 ºF (1 ºC) was chosen for the selected apples. All fruits were subjected to 1-methyl cyclopropane (1-MCP, a maturation inhibitor) treatment once they are put in their respective storage rooms.

*5. Sampling during cold storage*

Fruits were sampled right before storage, at 3, 6, 12, 18, 24, 30, and 36 weeks of storage. Four replicates of 10 fruits each will be used on each sampling day at each storage condition.

*6. Surviving Listeria analysis*

On each sampling day, four sets of 10 apple fruits under the respective storage conditions were sampled and transported to the Food Microbiology Lab on the Pullman campus of Washington State University for microbial analyses. Upon arrival, *Listeria* survival of apple surfaces was analyzed immediately or within 24h per our well-established method (Sheng et al., 2018). If survival of *Listeria* on apple fruits was below the detection limit, the suspension was enumerated for Presence/Absence after 48h enrichment in Buffered *Listeria* Enrichment Broth (BLEB) and streaking onto a selective *Listeria* agar plate. Presumptive positive colonies were further confirmed by PCR (FDA, 2015).

*7. Resident microbiota enumeration*

To enumerate *Enterobacteriaceae*, the detached microbiota suspension was plated on TSAYE overlaid with Violet Red Bile Glucose agar and incubated at 35°C for an additional 24 h.

To enumerate *Pseudomonas*, the detached microbiota suspension was plated on TSAYE plates overlaid with *Pseudomonas* selective agar, and then incubated at 28°C for 5 days.

Total native bacteria were enumerated on TSAYE plates and incubated at 30°C for 3 days. Lactic acid bacteria were enumerated by pour plate method using de Man, Rogosa and Sharpe (MRS) agar and incubated at 35 ºC for 48 h.

Yeast and mold were plated on potato dextrose agar (PDA) and incubated at room temperature (~22°C) for 5 days. Colonies were classified into yeasts, molds, and *Penicillium* regarding morphological characteristics.

*8. Next-generation sequencing analysis of microbiome on apple surfaces*

1) Microbial detachment from apple surface

At each sampling day, 4 composite replications containing 16 uninoculated and/or inoculated apple fruits were collected. Microbial suspension detached from 16 apples was pooled together and used for DNA extraction as described in the following.

2) DNA extraction and purification

Genomic DNA was extracted from microbial samples collected above using commercial DNA extraction and purification kit from Qiagen (Valencia, CA) per our established method (Kang, Yang, Zhang, Ross, & Zhu, 2018). The concentration and quality of DNA will be measured using Nanodrop spectrometry (Thermo Scientific), while the quality of DNA will be monitored by DNA agarose gel.

3) Next-generation DNA sequencing

16S rRNA sequencing of the microbiome of Fuji apples targeted the V4 region was performed by the Initiative for Bioinformatics and Evolutionary Studies (IBEST) Genomics Resources Core at the University of Idaho using Illumina MiSeq dual-barcoded two-step PCR amplicon sequencing. To produce amplicons for sequencing, the V4 region of the bacterial 16S rRNA gene was amplified using universal primers (515F: GTGCCAGCMGCCGCGGTAA, 806R: GGACTACHVG GGTWTCTAAT) with flanking regions ACACTGACGACATGGTTCTACA or TACGGTAGCA GAGACTTGGTCT at F515 or R806, respectively.

16S rRNA sequencing of the microbiome of CP, GS, and WA apples was conducted at Omega Bioservices (GA, US), targeting the V3–V4 region using primers (IlluminaF: CCTACGGGNGGCWGCAG; IlluminaR: GACTACHVGGGTATCTAATCC).

For the fungal community, the internal transcribed spacer region (ITS1) of the fungal ribosomal RNA gene was amplified using the prepared microbial DNA and universal primers of ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2: 5'- GCTGCGTTCTTCATCGATGC-3' with flank regions ACACTGACGACATGG TTCTACA and TACGGTAGCAGAGACTTGGTCT at ITS1F and TIS2, respectively, for the first PCR (Schlatter, Yin, Hulbert, & Paulitz, 2020; Schoch et al., 2012) (Schlatter et al., 2020; Schoch et al., 2012).

Libraries were prepared with Kapa HiFi PCR kit, and sequencing was performed on an Illumina MiSeq platform using a 2 × 300 paired end per established methods.

*9. Bioinformatics analysis of apple microbiome under storage*

Raw DNA sequence reads from the Illumina MiSeq will be demultiplexed and classified using the established method by bioinformaticist at each sequencing facility.

*10. Statistical analysis.*

Data were analyzed using the analysis of variance (ANOVA), followed by Turkey multiple comparison with SAS software. *P* values less than 0.05 were considered statistically significant. Data were presented as mean ± SEM, averaged from four composite replicates, each with 16 fruits. Microbiome analyses were performed using R Statistical Software (v 4.4.2).

## RESULTS AND DISCUSSION

1. *Counts of L. innocua and resident bacteria on apples inoculated with or without L. innocua.*

After 36 weeks of storage, *L. innocua* were decreased by 1.5 $\log_{10}$ CFU/apple on apples under RA or CA storage (Fig. 1). Total plate count (TPC) was not influenced by the introduction of *L. innocua*, where gradually increased by 1.6 $\log_{10}$ CFU/apple after 36 weeks of RA or CA storage (Fig. 2A). *Enterobacteriaceae* were slightly higher on *L. innocua* inoculated apples compared with uninoculated apples after 24 weeks of RA or CA storage. A higher reduction of *Enterobacteriaceae* was found in CA storage compared with RA storage regardless of *L. innocua* inoculation (Fig. 2B). A higher reduction of *Pseudomonas* was found on *L. innocua* inoculated apples compared with uninoculated apples, especially under CA storage (Fig. 2C). This might be due to the competitive effects *Pseudomonas* with *Listeria*, *Pseudomonas* species such as *Pseudomonas fluorescens* was found to have competitive relationship with *L. monocytogenes* in liquid culture after 14 days incubation at 4°C (Buchanan & Bagi, 1999). *Pseudomonas* decreased on apples regardless of storage treatment and *L. innocua* inoculation. CA storage caused higher reduction of *Pseudomonas* on inoculated and uninoculated apples compared with RA storage (Fig. 2C). Lactic acid bacteria slightly increased on uninoculated apples after 36 weeks of CA or RA storage (Fig. 2D).

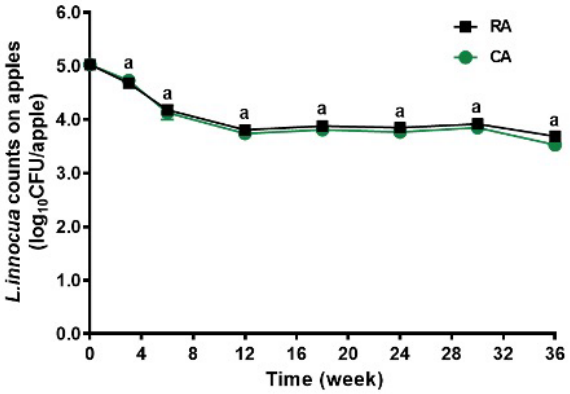


**Fig. 1**. *L. innocua* counts on Fuij apples during 36 weeks of cold storage. [a]Mean at each sampling point without comm letter differ significantly ($P$<0.05). Mean ± SEM, n = 64.

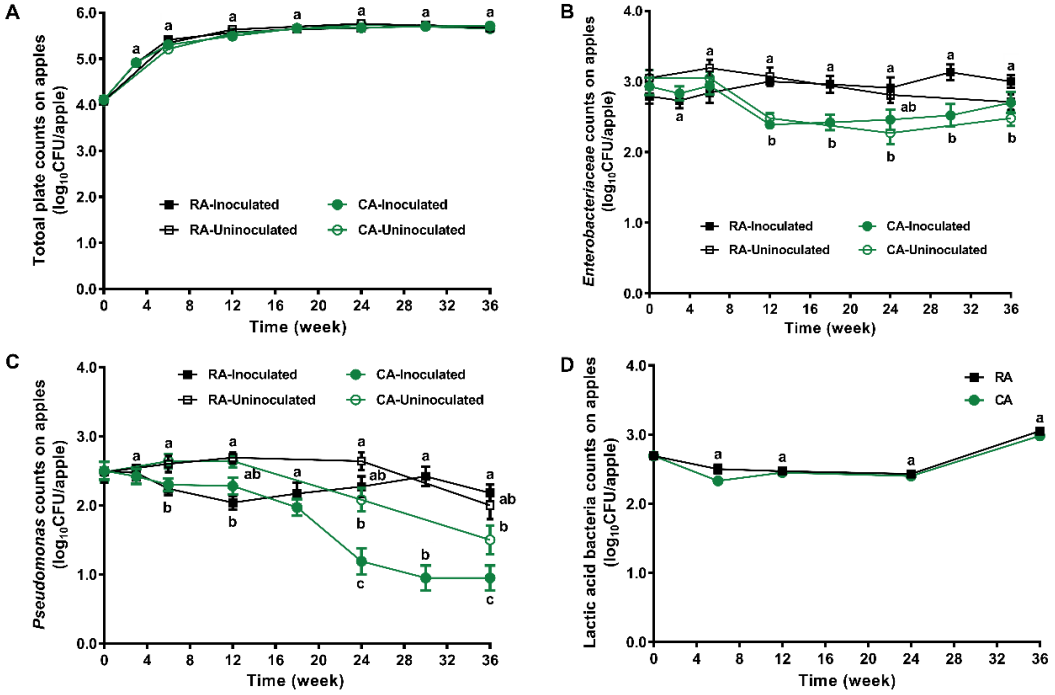


**Fig. 2**. The count of resident bacteria on Fuji apples with or without *L innocua* over 36 weeks RA and CA storage. A. Total plate count; B. *Enterobacteria*; C. *Pseudomonas*; D. Lactic acid bacteria. [a-c]Mean at each sampling point without comm letter differ significantly ($P$<0.05). Mean ± SEM, n = 64.

2. *Yeast and mold count on apples inoculated with or without L innocua*

Yeast and mold counts increased by 0.5 $\log_{10}$ CFU/apple after 36 weeks of RA or CA storage. Introducing *L. innocua* on apples did not impact the behavior of yeast and mold on apples. *Penicillium* increased on apples regardless of storage condition during 36 weeks of storage.

*3. Overall comparison of bacterial composition of apples before and after inoculation.*

Fig. 3 depicts the microbiome distribution and diversity of Fuji apples before and immediately after inoculation with *L. innocua*. Prior to the introduction of *L. innocua*, 15 bacteria phyla were identified, with *Proteobacteria*, *Deinococcus-Thermus*, *Actinobacteria*, *Cyanobacteria*, *Bacteroidetes,* and *Firmicutes* being the most prevalent phyla (Fig. 4A). Following inoculation, there is a notable reduction in bacterial phyla diversity (Fig. 3B-D), with only 5 phyla, including *Proteobacteria*, *Firmicutes, Bacteroidetes, Cyanobacteria* and *Actinobacteria* being identified (Fig. 4A).

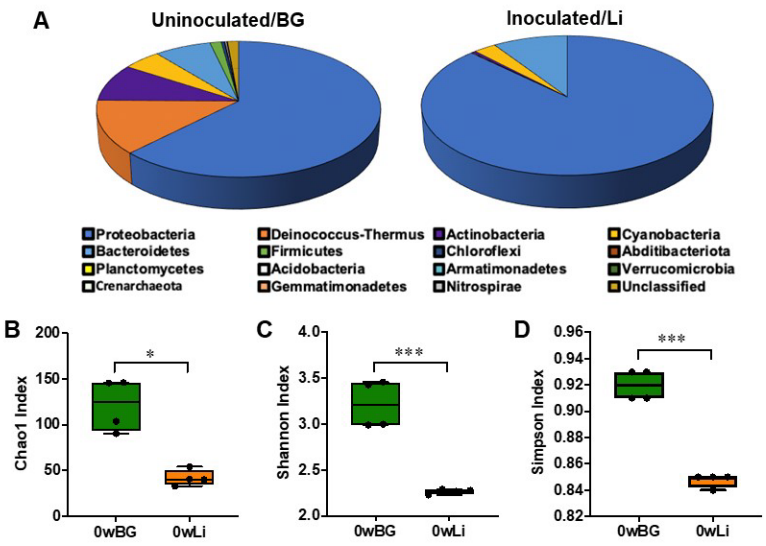


**Fig. 3. The microbiome distribution and diversity on Fuji apples with or without *L. innocua.*** A. Bacterial phyla. B-D. Alpha diversity. Li: Apple inoculated with *L. innocua*; BG: apples without inoculation. Mean ± SEM, n = 4, each with 16 apples. * and *** indicated statistically significant difference at $P < 0.05$ and 0.001.

*2. Overall comparison of bacterial composition of Fuji apples during 36 weeks of CA storage*

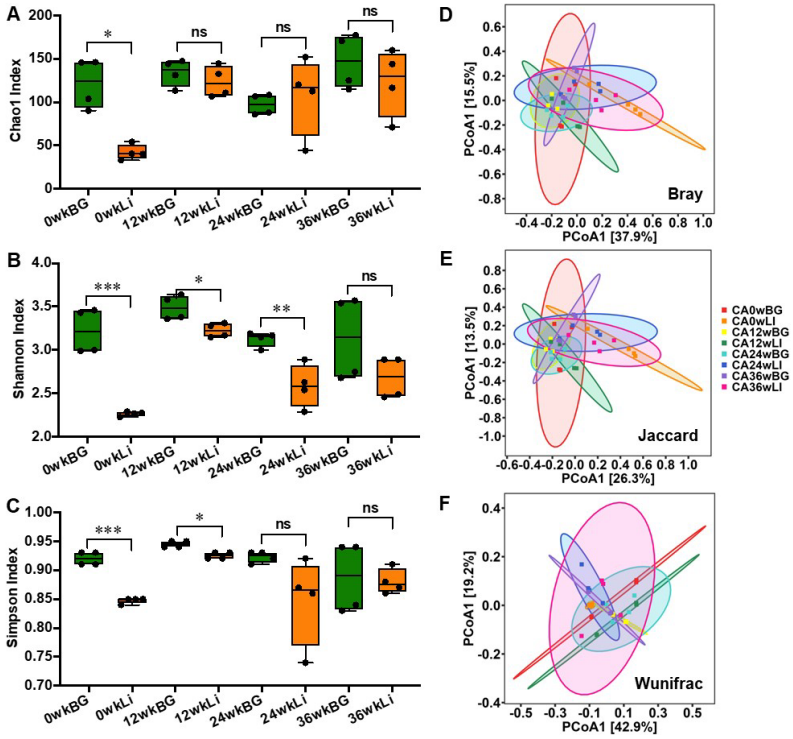


**Fig. 4. Microbiota diversity of Fuji apples with or without *L. innocua* over 36 weeks of CA storage.** A-C. Alpha diversity. D-F. Beta diversities. Li: Apple inoculated with *L. innocua*; BG: apples without inoculation. Mean ± SEM, averaged from 4 replicate, each with 16 apples. * and *** indicated statistically difference at $P < 0.05$ and 0.001.

Over 36 weeks of CA storage, alpha diversities, reflecting the numbers of species (richness) within individual samples, showed a gradual increase in *L. innocua*-inoculated apples, while a decline was observed in uninoculated apples (Fig. 4A-C). Beta diversities, differences in bacterial community compositions, demonstrated distinct composition profiles between *L. innocua*-inoculated and uninoculated Fuji apples throughout the 36 weeks of CA storage (Fig. 4D-E).

*3. Bacterial composition of Fuji apples with or without L. innocua at the phylum level over CA storage*

Fig. 5 presents a Chord diagram, illustrating the relative abundances of bacterial phyla of apples with and without inoculation over a

36-week CA storage period. During this duration of CA storage, there were dynamic changes in bacterial phyla on both *L. innocua*-inoculated and uninoculated Fuji apples (Fig. 5). Specifically, over the 36 weeks of CA storage, *Proteobacteria* decreased by 24.48% and 45.48% on uninoculated apples and inoculated apples, respectively. *Deinococcus-Thermus*, initially absent on *Listeria*-inoculated apples, increased to ~11% by the end of the storage period (Fig. 5). In contrast, it remained relatively stable on uninoculated apples over 36 weeks of CA storage. *Actinobacteria* increased by 24.38% on *Listeria*-inoculated apples and 26.38% on uninoculated apples after 36 weeks of CA storage (Fig. 5). *Cyanobacteria* increased on *Listeria*-inoculated apples while remaining unchanged on uninoculated apples over the entire CA storage (Fig. 5). *Bacteroidetes* decreased by ~5% on uninoculated Fuji apples but showed no change on inoculated apples after 36 weeks of CA storage (Fig. 5).

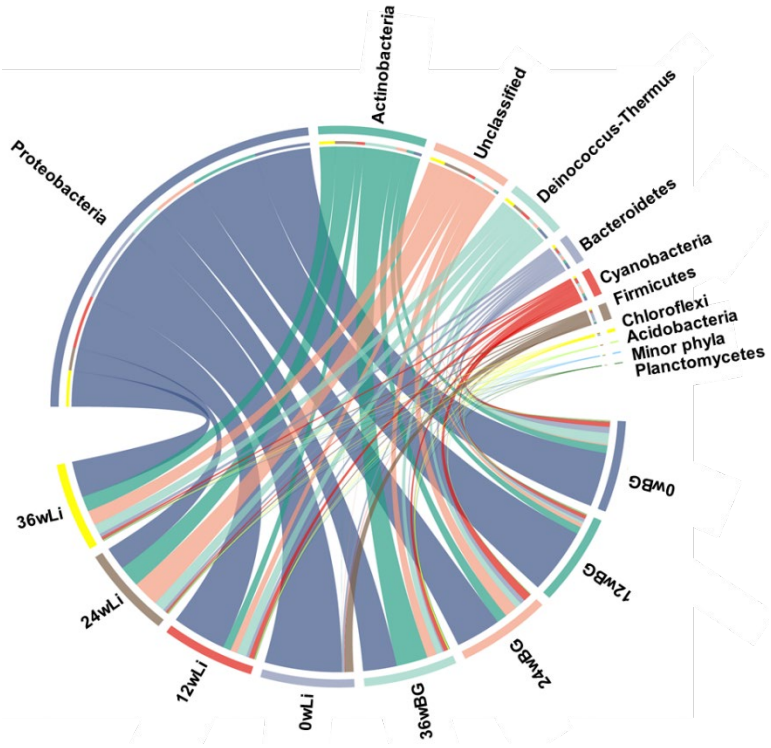


**Fig. 5. Chord diagram depicting the relative abundances of bacterial phyla on Fuji apples with or without *L. innocua* during 36 weeks of CA storage.** Minor phyla: average relative abundance less than 0.1%. Li: apples contaminated with *L. innocua*. BG: apples without *L. innocua* contamination. The relative abundance was averaged from four replicates, each with 16 apples.

*4. Bacterial families of Fuji apples with or without L. innocua over 36 weeks of CA storage*

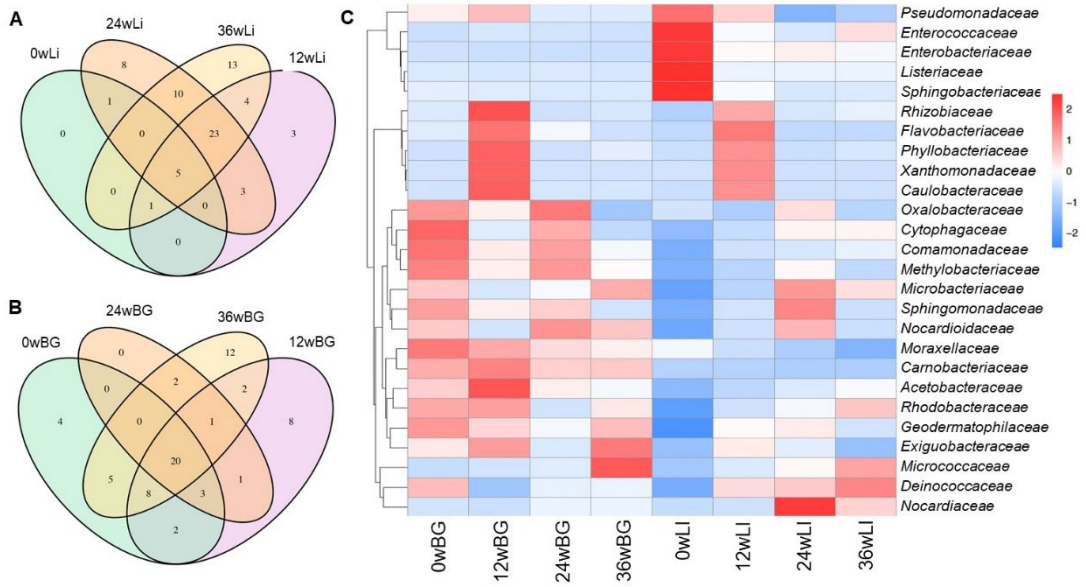


**Fig. 6. Bacterial families on Fuji apples with or without *L. innocua* contamination during 36 weeks of CA storage.** A. Venn diagram for inoculated apples. B. Venn diagram for uninoculated apples. C. Heatmap. Li: apples inoculated with *L. innocua*. BG: apples without *L. innocua* inoculation. The relative abundance in each treatment was averaged from four replicates, each with 16 apples.

Fig. 6 employs Venn diagrams to illustrate shared and unique bacterial families and a heatmap to showcase abundance and clustering patterns of these families on apples with and without *L. innocua* contamination during a 36-week CA storage period. Throughout this storage period, uninoculated apples had a higher number of overlapping bacterial families compared to *Listeria*-inoculated apples (Figure 6A-B). Specifically, *Pseudomonadaceae* and *Exiguobacteraceae* were significantly more abundance on uninoculated apples than on *L. innocua*-inoculated apples under CA storage (Fig. 6C). The introduction of *L. innocua* on apples significantly increased the relative abundance of *Pseudomonadaceae* but decreased the relative abundance of other bacterial families under 36 weeks of CA storage (Fig. 6C). *Cytophagaceae* was significantly higher on *L. innocua*-inoculated apples compared to uninoculated apples after 36 weeks of CA storage. As anticipated, *Listeriaceae* was exclusively found on *L. innocua*-inoculated apples and gradually decreased over storage (Fig. 6C).

5. *Bacterial genera of Fuji apples with or without L. innocua over 36 weeks of CA storage.*

The star diagram (Fig. 7) depicts the relative abundances of bacterial genera on Fuji apples with and without *L. innocua* contamination throughout a 36-week CA storage period, offering a comprehensive visual overview of the distribution and dynamics of these genera over time. Notably, *Pseudomonas* was significantly more abundance on *L. innocua*-inoculated apples compared to uninoculated apples before storage (Fig. 7). Over 36 weeks of CA storage, *Pseudomonas* decreased on the inoculated apples while increasing on uninoculated apples (Fig. 7). By the end of CA storage, *Deinococcus* and *Hymenobacter* on *L. innocua*-inoculated apples were significantly higher than that on uninoculated apples (Fig. 7). *Rhodococcus* and *Pantoea* remained significantly higher on *L. innocua*-inoculated apples compared to uninoculated apples throughout the 36 weeks CA storage (Fig. 7).

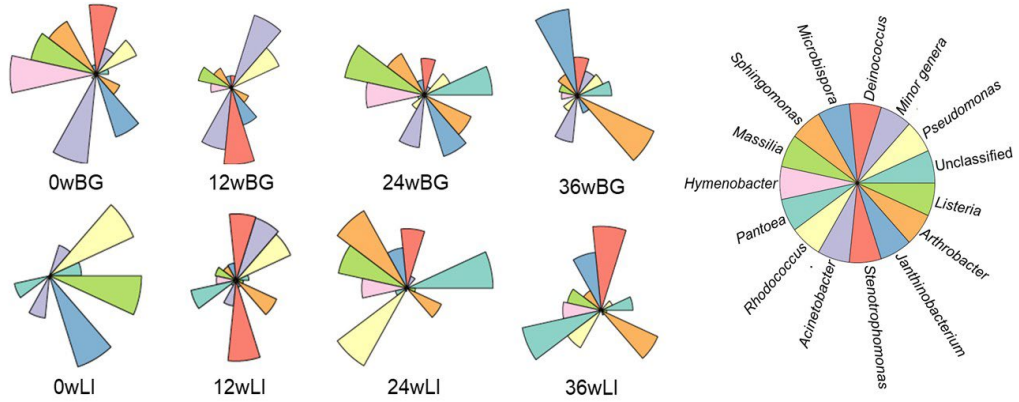


**Fig. 7. Star diagram showing the relative abundances of bacterial genera on Fuji apples with or without *L. innocua* contamination over 36 weeks of CA storage**. Minor genera are defined as those with an average relative abundance of less than 2.0%. Each sector represents the relative abundance of the respective genus.

*6. Bacterial species of Fuji apples with or without L. innocua over the CA storage.*

At species level, after 36 weeks of CA storage, *Pseudomonas umsongensis*, *Pseudomonas veronii*, *Comamonas terrigena*, and *Acinetobacter lwoffii* exhibited higher abundance on uninoculated apples compared to *L. innocua*-inoculated apples (Fig. 8). In contrast, *Rhodococcus fascians* were

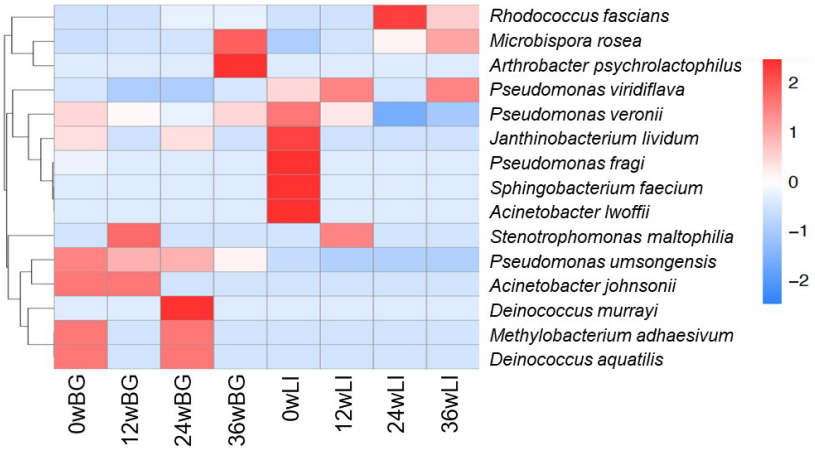


**Fig. 8. Heatmap of the relative abundance of bacterial species on Fuji apples with or without *L. innocua* over 36 weeks CA storage.**

significantly higher on *L. innocua*-inoculated apples than on uncontaminated apples at the end of 36 weeks of CA storage (Fig. 8). *Candidatus nitrososphaera* and *Aeromonas sharmana* showed significantly higher abundances on uninoculated apples compared to *L. innocua*-contaminated apples within 36 weeks of CA storage (Fig. 8). In summary, the introduction *of L. innocua* led to a significant decrease in bacterial diversities and richness on apples. Over the 36 weeks of CA storage, alpha diversities were gradually increased in *L. innocua*-contaminated apples while decreasing on uninoculated apples. Dynamic alterations in bacterial communities were observed at the phylum, family, genus, and species levels. Fig. 9 highlights the key taxa that were differentially enriched in apples with or without *L. innocua* inoculation, as identified through LEfSe analysis based on Linear discriminant analysis.

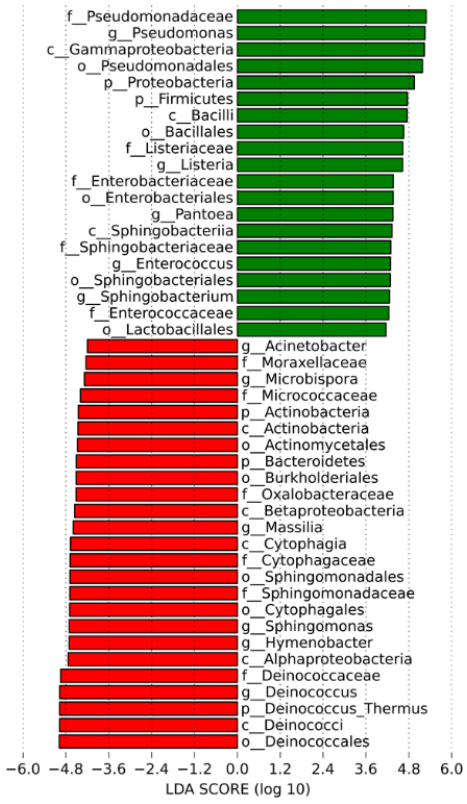


**Fig. 9. Main bacterial taxa that differ between *L. innocua* inoculated Fuji apples (LI, green) and uninoculated Fuji apples (BG, red)**.

*7. Fungal composition of apples inoculated with or without L. innocua at the phylum level*

At phylum level, the fungal population of Fuji apples, regardless of inoculation and storage condition, was dominated by *Basidiomycota* followed by *Ascomycota* (Fig.10). In uninoculated apples, the relative abundance of *Basidiomycota* decreased, while *Ascomycota* increased after 9 months CA or RA storage ($P< 0.05$) (Fig. 10BC). For inoculated apples, the relative abundance of *Basidiomycota* and *Ascomycota* remained stable for 9 months of RA storage, while *asidiomycota* decreased and *Ascomycota* increased after 9 months of CA storage ($P< 0.05$).

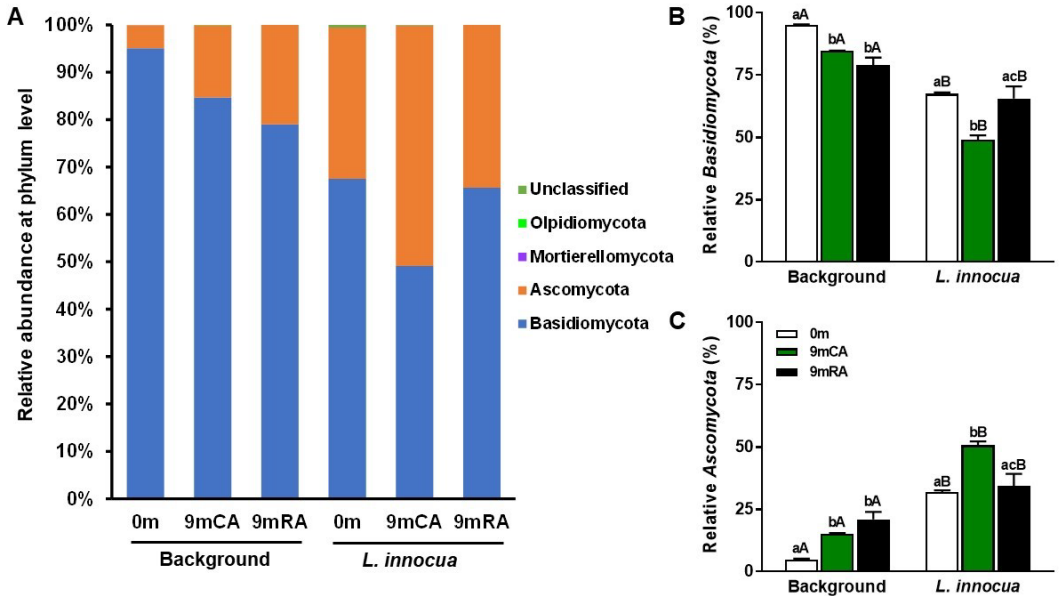


**Fig. 10**. **Relative abundance of fungal phyla detected in Fuji apples before and after 9 months of CA and RA storage.** [a-b]Mean without a common letter differ significantly ($P < 0.05$) for the inoculated apples (*L. innocua*) or non-inoculated apples (Background). [AB]Mean at each sampling point without a common letter differs significantly between the inoculated apples (*L. innocua*) and non-inoculated apples (Background) ($P < 0.05$).

*8. Fungal families of Fuji apples with or without L. innocua before and after 36 weeks of storage*

For non-inoculated apples, *Bulleribasidiaceae* is the dominant fungal family, which accounts for

54.6%, followed by *Filobasidiaceae*, accounting for 30.1% of the fungal families (Fig. 11). The relative abundances of *Bulleribasidiaceae* increased from 54.6% to 63-64% after 9 months of CA and RA storage, while the relative abundance of *Filobasidiaceae* was decreased to 8.4% and 2.4% after 9 months of CA and RA storage, respectively (Fig. 11). In *L. innocua* inoculated apples, *Filobasidiaceae* is the dominant family, accounting for 30.1% abundance, which is similar to that in non-inoculated apples, followed by *Bulleribasidiaceae* and *Pleosporaceae*, which accounting for 22.6% and 22.1%, respectively (Fig. 11). The population of *Bulleribasidiaceae* in the inoculated apples was lower than that in non-inoculated apples ($P$ <0.05), while the counts of *Pleosporaceae* was significantly higher compared to the non-inoculated apples ($P$ <0.05). The relative abundance of *Bulleribasidiaceae* in the inoculated apples remained stable after 9 months of CA and RA storage. The level of *Filobasidiaceae* and *Pleosporaceae* in the inoculated apples decreased significantly after 9 months of CA or RA storage ($P$ <0.05).

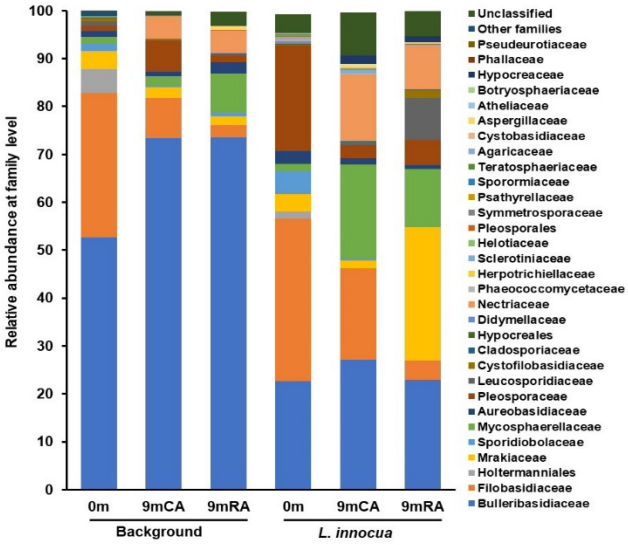


**Fig. 11. Relative abundance of fungal family detected in Fuji apple with or without *L. innocua* before and after 9 months of RA and CA storage.** Four replicates, with each replicate containing 16 apples.

*9. Fungal genera and species of Fuji apples with or without L. innocua before and after 9 months of CA or RA storage.*

*Vishniacozyma* is a dominant genus detected in non-inoculate apples, accounting for 52.6% abundance, followed by *Filobasidium*, accounting for 25.6% of total fungal genera (Fig. 12). Relative abundance of *Vishniacozyma* increased to 63-64% after 9 months of CA or RA storage ($P$ <0.05), while *Filobasidium* count decreased to 6.4% and 1.8%, respectively, after 9 months of CA or RA storage ($P$ <0.05) (Fig. 12). In *L. innocua*-inoculated apples, *Filobasidium, Vishniacozyma*, and *Alternaria* are dominated genera, which accounts for 26.8%, 22.6%, and 21.6% abundance, respectively (Fig. 12). Relative *Filobasidium* and *Alternaria* detected in the inoculated apples significantly decreased after 9 months of CA and RA storage ($P$ <0.05), while *Vishniacozyma* remained stable across storage (Fig. 12). Relative abundance of *Penicillium* family increased after 9 months of RA storage ($P$ <0.05), regardless of *L. innocua* inoculation. Relative abundance of Rhodotorula and Holtermanniella decreased after 9 months of CA or RA storage in both inoculated and non-inoculated apples ($P$ <0.05). Gibberella had a low abundance and remained low in the inoculated apples after 9 months of CA and RA storage but significantly increased in non-inoculated after 9 months of CA storage. More differential genera refer to Fig. 12.

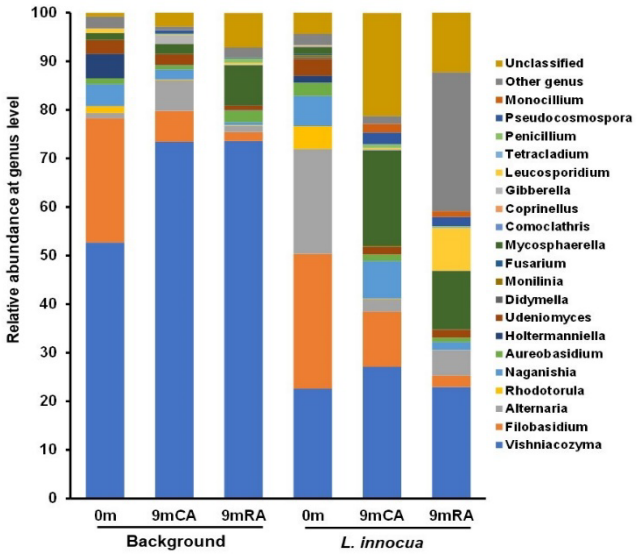


**Fig. 12. Fungal genera in Fuji apple with or without *Listeria innocua* before and after 9 months of CA and RA storage.** Four replicates, with each containing 16 apples.

At species level, *Vishniacozyma victoriae* was the main species on non-inoculated apples, accounting for 48.2% of total species, followed by *Filobasidium magnum* with 25.1% relative abundance. *Vishniacozyma victoriae* increased after 9 months of CA and RA storage, while *Filobasidium magnum* content decreased after 9 months of CA and RA storage ($P < 0.05$) (Fig. 13). In inoculated apples, both *Vishniacozyma victoriae* and *Filobasidium magnum* were the main species detected with 18.8% and 26.2% relative abundance, respectively (Fig. 13). *Vishniacozyma victoriae* level in the inoculated apples were similar before and after storage. The relative *Filobasidium magnum* content in the inoculated apples decreased after 9 months of CA and RA storage ($P < 0.05$) as observed in the non-inoculated apples (Fig. 13). *Holtermanniella takashimae* level was higher in non-inoculated apples than in inoculated apples and decreased after 9 months of CA or RA storage, regardless of the inoculation. Relative abundance of *Mycosphaerella tassiana* significantly increased after 9 months of RA storage (both inoculated and non-inoculated) and CA (inoculated apples) ($P < 0.05$) (Fig. 13). Relative abundance of *Tausonia pullulans* in apples was low regardless of the inoculation but was extremely elevated in the inoculated apples after 9 months of RA storage (Fig. 13), increasing from 0.1% to 26.2%. In conclusion, significant differences in the fungal community structure were found between apple samples taken from different sampling times for both inoculated and non-inoculated apples, and between apples inoculated with or without *L. innocua*.

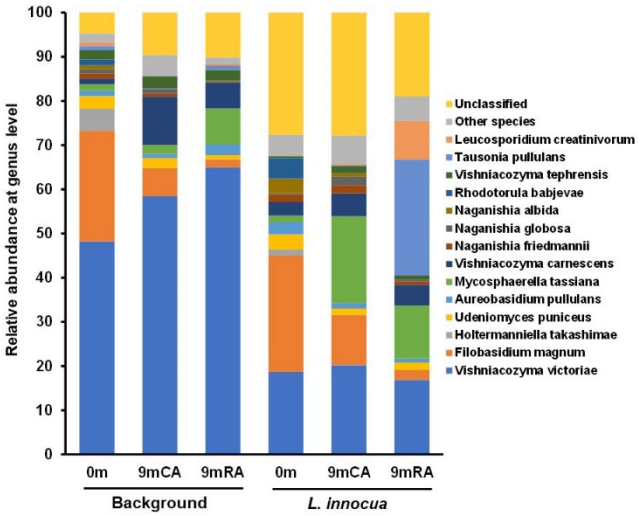


**Fig. 13. Relative abundance of fungal species of Fuji apple with or without *L. innocua* before and after 9 months of storage at commercial RA and CA room.** Four replicates with each replicate contains 16 apples.

*8. Overview of bacterial community among Cripps Pink, Granny Smith, and WA38 apples*

Similar to Fuji apples, *Proteobacteria* dominated the bacterial phyla in all varieties, comprising 40-70% of the community, followed by *Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria,* and *Planctomycetes*. These phyla collectively account for 91-98% of the total phyla (Fig. 14A). A heatmap visualization revealed significant differences in the abundance across varieties (Fig. 14B). Notably, GS had high level of *Proteobacteria*, while showing lower levels of *Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria,* and *Planctomycetes* ($P < 0.05$, Fig. 14B). Figs. 15 and 16 show bacterial families and genera associated with three apple varieties. Each characterized by a unique microbial composition. For CP, the dominant families include *Microbacteriaceae* (9.58%), *Xanthobacteraceae* (9.56%), *Micrococcaceae* (7.72%), *Phyllobacteri-aceae* (7.33%), *Sphingomonadaceae* (5.75%), *Cytophagaceae* (5.69%), *Bacillaceae* (4.16 %), and *Pseudomonadaceae* (4.10%). GS is dominated by *Pseudomonadaceae* (33.96%), *Enterobacteriaceae* (9.13%), *Xanthobacteraceae* (6.25%), *Moraxellaceae* (5.33%), *Micrococcaceae* (4.96%), *Phyllo-bacteriaceae* (4.42%), *Sphingomonadaceae* (2.93%), *Oxalobacteraceae* (2.78%). WA is enriched with *Rhodobacteraceae* (15.11%), *Cytophagaceae* (11.05%), *Nocardiaceae* (11.03%), *Micrococcaceae* (8.06%), *Deinococcaceae* (6.94%), *Sphingomonadaceae* (6.91%), *Acetobacteraceae* (5.33%), and *Comamonadaceae* (4.83%) (Fig. 15). At genus level, the key genera in CP include *Xanthobacter* (8.98%), *Mesorhizobium* (6.93%), *Microbacterium* (5.52%), *Hymenobacter* (4.87%), *Sphingomonas* (4.58%), and *Pseudomonas* (3.89%) (Fig. 16). GS is dominated by *Pseudomonas* (35.21%), followed by *Xanthobacter* (6.16%), *Acinetobacter* (5.35%), *Mesorhizobium* (4.39%), and *Sphingomonas* (2.33%). WA is enriched with *Paracoccus* (11.59%), *Rhodococcus* (10.61%), *Deinococcus* (6.78%), *Hymenobacter* (6.05%), *Kocuria* (5.69%), and *Sphingomonas* (5.22%) (Fig. 16). Each variety also exhibits unique dominant species: *Acinetobacter johnsonii* (2.38%) is dominant in CP, *Pseudomonas veronii* (22.33%) in GS, and *Rhodococcus corynebacterioides* (9.39%) in WA.

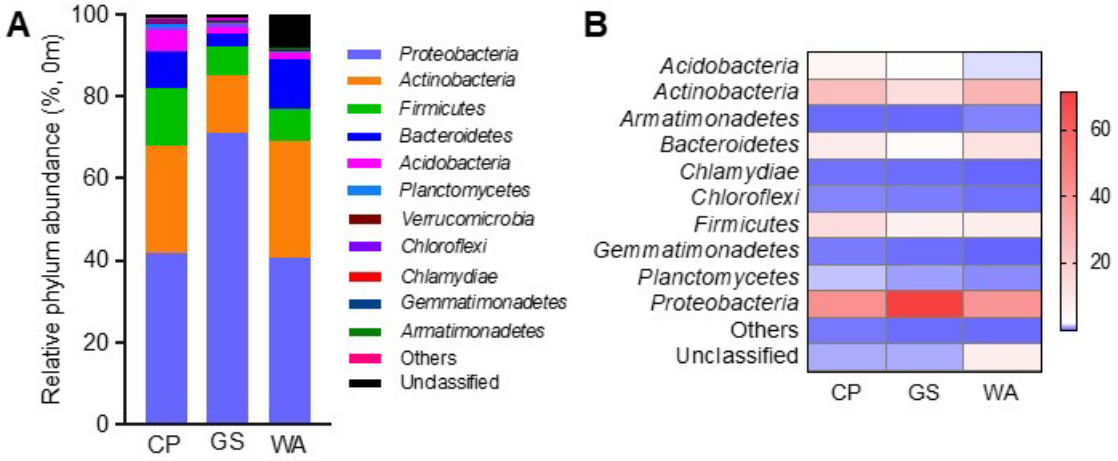


**Fig. 14**. **Bacterial phyla in Cripps Pink (CP), Granny Smith (GS), and WA38 (WA) apples.** A. Relative abundance. C. Heatmap. Color intensity represents relative abundance. Four replicates with each replicate containing 16 apples.

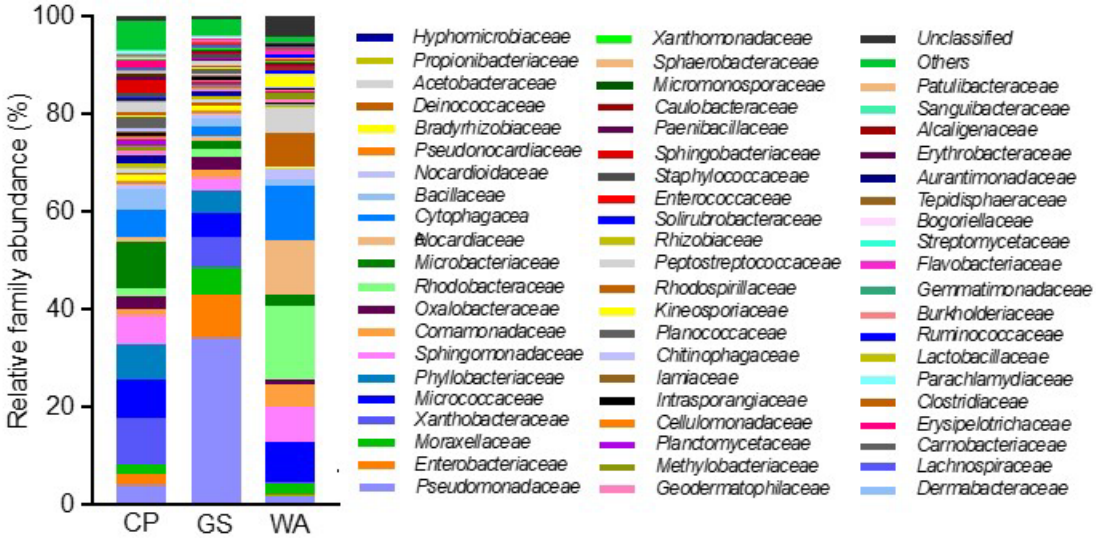


**Fig. 15 Relative abundance of bacterial family in Cripps Pink (CP), Granny Smith (GS), and WA38 (WA) apples.** Colored segment within each bar corresponds to different bacterial families. Four replicates with each replicate containing 16 apples.

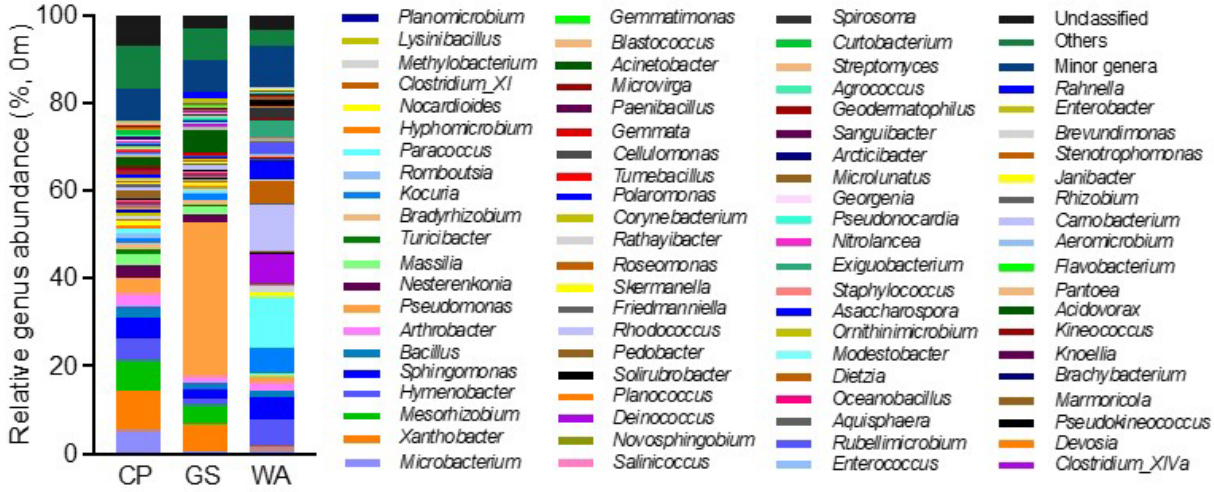


**Fig. 16 Relative abundance of bacterial genera in Cripps Pink (CP), Granny Smith (GS), and WA38 apples (WA).** Colored segment within each bar corresponds to different bacterial genera. Four replicates, with each replicate containing 16 apples.

*9. Overall comparison of bacterial composition among apple varieties during 36 weeks of storage*

Beta diversity analysis, which assesses overall compositional differences in the microbial communities, revealed significant differences in the bacterial community composition among the apple varieties at harvest. WA (purple) displayed a more distinct microbiome profile compared to CP (red) and GS (green) apples (Fig. 17). Fig. 18 illustrates the beta diversity of the apple microbiome across three varieties over 36 weeks of storage. Within each variety, beta-diversity shows a dynamic shift over the 9 months of RA or CA storage, indicating that cold storage drastically impacted microbiome composition. The storage environment, (RA or CA) had a minor influence on microbiome composition (Fig. 18). The alpha diversity-Chao1 index, an estimate of the total species richness in a microbiome sample, varied among the apple varieties and across the storage time points (Fig. 19). Before storage, WA (149.25 ± 9.03) had a significantly lower Chao1 index than CP (302.11 ± 11.57) and GS (290.39 ± 24.17). In general, the Chao1 index tended to decrease over the storage, indicating a potential decrease in microbial richness. For CP, the Chao1 index decreased gradually over time, with the lowest values observed at 9 months of storage (Fig. 19A). In WA, the Chao1 index also declined, but to a lesser extent than CP and GS (Fig. 19).

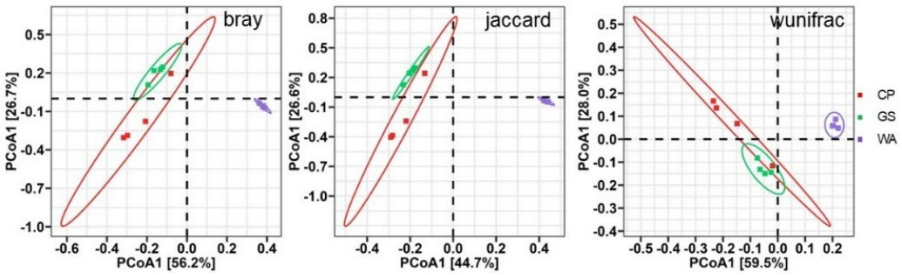


**Fig. 17. Beta diversity of Cripps Pink (CP), Granny Smith (GS), and WA38 (WA) apples before storage.** Four replicates, each with 16 apples.

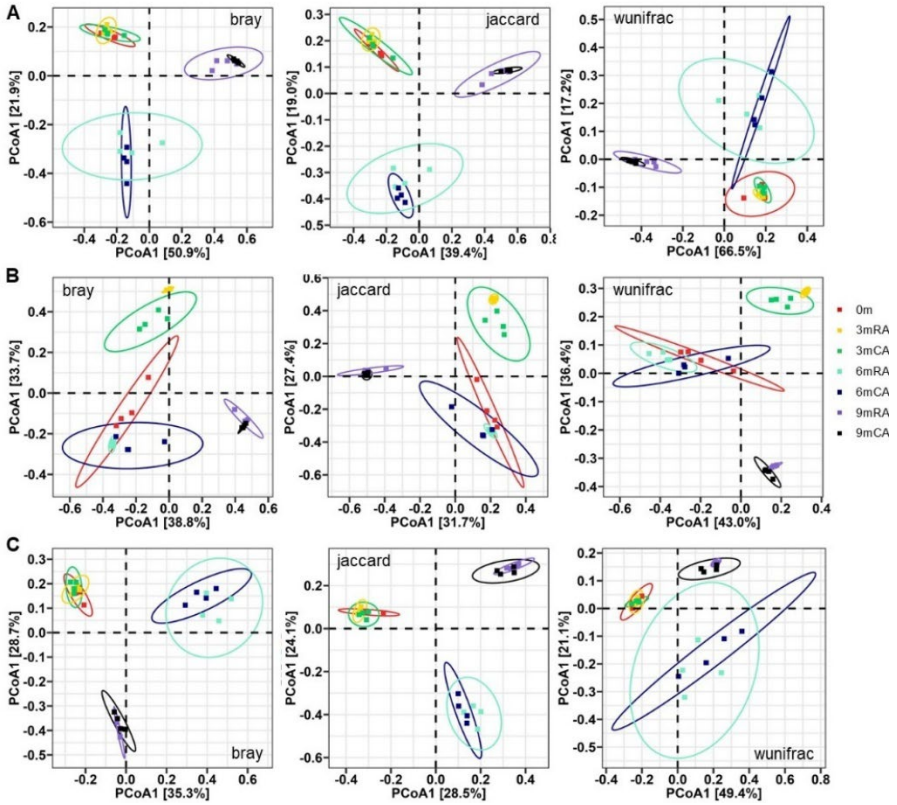


**Fig. 18. Beta diversity of Cripps Pink (CP, A), Granny Smith (GS, B), and WA38 (WA, C) apples over 36 weeks of storage.** Four replicates, each with 16 apples.

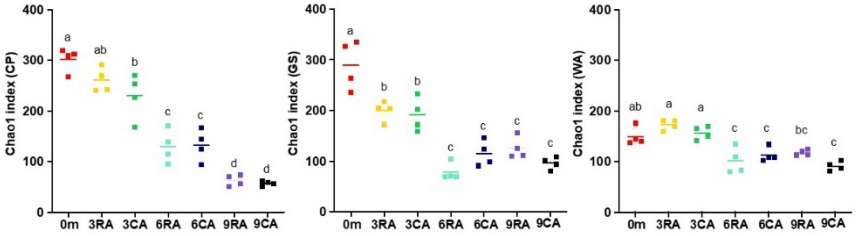


**Fig. 19. Alpha diversity Chao1 indext of Cripps Pink (CP), Granny Smith (GS), and WA38 (WA) apples before storage.** [a-c]Mean at each sampling point without comm letter differ significantly ($P$<0.05). Four replicates, each with 16 apples.

**References**


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## EXECUTIVE SUMMARY

**Project Title:** Interaction of microbiome and *Listeria* on apples during cold storage

**Keywords:** *Listeria innocua*; apples; commercial storage; microbiome; microbial diversity; varietal difference; fungal community.

### Abstract

The population of *L. innocua* declined during storage under both RA and CA storage. Introduction of *L. innocua* led to an increase in *Enterobacteriaceae* and a decrease in *Pseudomonas* counts compared to uncontaminated apples. Yeast and mold populations, particularly *Penicillium*, increased after 36 weeks of storage. *L. innocua* inoculation significantly reduced bacterial diversity and richness on apples. The microbiome composition differed at all taxonomic levels (phylum, family, genus, species) between inoculated and uninoculated apples over the storage periods. Uninoculated apples exhibited higher bacterial diversity and richness than inoculated apples at selected sampling points. Furthermore, distinct fungal community structures were observed between apples, influenced by storage conditions, duration, and inoculation status. Fungal diversity appeared higher at lower taxonomic levels. Varietal comparison revealed that *Proteobacteria* was a dominate phylum across all apple varieties, comprising 40-70% of the microbial phyla, with significant differences between varieties. Each apple variety exhibited unique profiles at the family, genus, and species levels. For CP, dominant families included *Microbacteriaceae* and *Xanthobacteraceae*, while GS was characterized by *Pseudomonadaceae* and *Enterobacteriaceae*, WA was enriched with *Rhodobacteraceae* and *Cytophagaceae*. At the genus level, CP is dominated by *Xanthobacter* and *Mesorhizobium*, GS by *Pseudomonas*, and WA by *Paracoccus* and *Rhodococcus*. Each variety had unique dominant species: *Acinetobacter johnsonii* in CP, *Pseudomonas veronii* in GS, and WA by *Rhodococcus corynebacterioides*. Cold storage significantly impacts the microbiome composition and richness of apples, with storage duration having a stronger influence than the storage environment (RA vs. CA). WA apples exhibited a distinct microbiome profile compared to CP and GS apples at harvest and throughout storage. Microbial richness, measured by the Chao1 index, declined over time across all varieties, with CP showing the greatest reduction and WA the least, indicating varietal differences in microbiome. These findings suggest that the apple microbiomes are dynamic, influenced by variety, storage, and inoculation status. *L. innocua* inoculation alters both bacterial and fungal community on apples, presenting potential opportunities for the development of novel biological control strategies to mitigate *Listeria* contamination and decay in apples.

**Project Title:** Reducing $CO_2$-related disorders during Honeycrisp rapid CA treatment

**Report Type:** Continuing Project Report-No Cost Extension

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**Cooperators**: Emmi Klarer, Sarah Gabel, Stemilt Growers LLC.

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $22,000
**Total Project Request for Year 2 Funding:** $85,000
**Total Project Request for Year 3 Funding:** $85,000

**Other related/associated funding sources:** Awarded
**Funding Duration:** 2022 - 2025
**Amount:** $115,317/3 yrs.
**Agency Name:** USDA-ARS, In-house project
**Notes:** In-house project with complimentary objectives. Funds for storage maintenance and costs ($8000/yr), supplies and materials ($3000/yr), travel ($1000/yr), and 0.1 FTE (PI) and 0.1 FTE (technical).

**Other related/associated funding sources:** Awarded
**Funding Duration:** 2024 - 2028
**Amount:** $555,828/4 yrs.
**Agency Name:** USDA-NIFA
**Notes:** Project Title: "Benefits of and barriers to dynamic controlled atmosphere (DCA) storage: Analyses needed for technology uptake by the U.S. apple industry". Lead institution: Cornell University (PD: Chris Watkins). Project was funded through the SCRI program beginning in FY24.

**Budget 1**
**Primary PI:** David Rudell
**Organization Name:** USDA-ARS
**Contract Administrator:** Sharon Blanchard
**Telephone:** 509-664-2280 (SB)
**Contract administrator email address:** Sharon.Blanchard@usda.gov

| Item | 2022 | 2023 | 2024 |
|---|---|---|---|
| Salaries (GS-7)* | | 43,311 | 45,764 |
| Benefits (40%) | | 18,124 | 18,305 |
| Wages | 5,000 | 5,000 | 5,000 |
| Benefits | | | |
| Equipment | | | |
| Supplies | 5,000 | 4,565 | 3,931 |
| Travel | | | |
| Miscellaneous** | 12,000 | 12,000 | 12,000 |
| Plot Fees | | | |
| Total | 22,000 | 85,000 | 85,000 |

**Footnotes: *Estimated 3% salary increase; **22% of instrument service contract**

**Objectives**:
1. Determine influence of $CO_2$ levels on disorder development during rapid CA treatment.
2. Determine impact of initial fruit temperature and delay of CA establishment during conditioning on disorder development.
3. Monitor flesh chemistry to indicate which treatment conditions may elevate risk of developing soft scald/soggy breakdown or $CO_2$-related/other disorders.

## SIGNIFICANT FINDINGS

1. Rapid CA conditioning eliminated soft scald/soggy breakdown.
2. Rapid CA conditioning does not compromise quality (6 months) regardless of how long it was delayed.
3. $CO_2$-related symptoms developed as a result of elevated $CO_2$ during rapid CA in the second season.
4. Apples held for a shorter period in rapid CA under elevated $CO_2$ had reduced internal browning.
5. Internal browning associated with elevated $CO_2$ during rapid CA develops after transfer to long-term CA (low $CO_2$) storage.
6. $CO_2$ sensitivity was orchard specific.

## METHODS

As the light crop load, hail, and lack of disorders impacted results from our first year of the project, we repeated year 1 experiments in year 2. In year 2, we also included air stored treatments without conditioning as a storage regime not likely to control bitter pit and soft scald/soggy breakdown to establish susceptibility of each lot. Year 2 activities are outlined by objective below.

Objective 1: Determine influence of $CO_2$ levels on disorder development during rapid CA treatment.

Honeycrisp apples were harvested approximately 1 week prior to commercial harvest, at commercial harvest, and 1 week after commercial harvest from the same block in Quincy, WA. Harvest maturity (starch index, internal ethylene concentration, firmness, titratable acidity, and soluble solids) and external/internal appearance were evaluated, and fruit were imaged using a digital camera. Apples were treated with 1-MCP (1 ppm), then stored in 2.5% $O_2$ and (0.5, 1, 2, 3, or 5%) $CO_2$ for 7 days at 50 °F. To distinguish soft scald from $CO_2$-related internal browning, 2 trays of DPA (2000 ppm drench) treated apples and 2 trays of untreated (no 1-MCP or DPA) apples from the last harvest were stored in 2.5% $O_2$ and 5% $CO_2$ for 7 days at 50 °F in separate CA chambers as controls. Following conditioning, apples were stored for 6 months in 2.5% $O_2$ and 0.5% $CO_2$ at 37 °F upon which external and internal disorders, firmness, titratable acidity, soluble solids, and defect incidence were evaluated. This year an air storage control was added, with 2 trays of untreated apples from each harvest timepoint stored at 37 °F in ambient air for the duration of the experiment.

Objective 2: Determine impact of initial fruit temperature and delay of CA establishment during conditioning.

To determine the impact of delayed CA establishment during conditioning, Honeycrisp apples were harvested at commercial harvest from 3 different orchards: 2 near Quincy, WA and 1 near Mattawa, WA. Harvest maturity (starch index, internal ethylene concentration, firmness, titratable acidity, and soluble solids) and external/internal defects were evaluated, and fruit were imaged using a digital camera. Apples were treated with 1-MCP (1 ppm) then conditioned at 50 °F in CA (atmosphere established at 0, 4, or 8 days) in 2.5% $O_2$ and (2.5 or 5%) $CO_2$. Once in CA, apples were conditioned at 50 °F until day 10 after harvest. Following conditioning, apples were stored in 2.5% $O_2$ and 0.5%

$CO_2$ at 37 °F for 6 months upon which external and internal disorders, firmness, titratable acidity, soluble solids, and defect incidence were evaluated.

To determine the impacts of conditioning temperature during rapid CA, Honeycrisp apples were harvested approximately one week after commercial harvest from an orchard in Quincy, WA. Harvest maturity (starch index, internal ethylene concentration, firmness, titratable acidity, and soluble solids) and external/internal defects were evaluated, and fruit were imaged using a digital camera. Apples were treated with 1-MCP (1 ppm) and immediately placed in CA in 0.5% $O_2$ and 2.5% $CO_2$ at (37, 46, or 50 °F) for 7 days. Following conditioning, apples were stored in 2.5% $O_2$ and 0.5% $CO_2$ at 37 °F for 6 months upon which external and internal disorders, firmness, titratable acidity, and soluble solids were evaluated.

Objective 3: Monitor flesh chemistry to indicate which treatment conditions may elevate risk of developing soft scald/soggy breakdown or $CO_2$-related browning.

Honeycrisp apples were picked at three timepoints (from Objective 1; 1 week prior to commercial harvest, at commercial harvest, and 1 week after commercial harvest), treated with 1-MCP, and immediately pulled down to 2.5% $O_2$ and 0.5, 1, 2, 3, or 5% $CO_2$ and conditioned for 1 week at 50 °F (as described in objective 1). One tray of each $CO_2$ treatment was sampled at 0-, 2-, 4- and 7-days during conditioning to determine if markers of $CO_2$ sensitivity increase with disorder risk and/or symptom development. The cortex samples were flash frozen in liquid nitrogen and cryo-preserved for chemical analysis. Data processing targeted chemistries associated with $CO_2$-sensitivity and internal browning discovered in our previous project (Rudell and Mattheis, 2023).

Current (Year 3) season:

Year 2 results were also impacted by low disorder incidence. To attempt to account for this in Year 3, we determined further activities would include harvests from additional locations. Honeycrisp were harvested from 9 different orchards in Mattawa, Royal City, Quincy, and Bridgeport, WA, approximately at commercial harvest during fall 2024. As in the previous experiments, harvest maturity (starch index, internal ethylene concentration, firmness, titratable acidity, and soluble solids) and external/internal appearance were evaluated, and fruit were imaged using a digital camera. Apples were treated with 1-MCP (1 ppm) at harvest, then stored in 2.5% $O_2$, and 0.5, 1, 2, 3, or 5% $CO_2$ for 7 days at 50 °F. One tray of each $CO_2$ treatment was sampled at 0- and 7-days postharvest. The cortex samples were flash frozen in liquid nitrogen and cryo-preserved for chemical analyses. Two trays of DPA (2000 ppm) treated apples and two trays of untreated (no 1-MCP or DPA) apples from the last harvest were stored in 2.5% $O_2$ and 5% $CO_2$ for 7 days at 50 °F in separate CA chambers as controls. Following conditioning, apples were placed into CA storage for 6 months in 2.5% $O_2$ and 0.5% $CO_2$ at 37 °F. External and internal disorders, firmness, titratable acidity, and soluble solids will be evaluated after long term storage.

The conditioning temperature experiment from year 1, objective 2 was also repeated, but with fruit picked from an orchard in Mattawa with a recent history (Year 2) of $CO_2$ sensitivity. Apples were harvested approximately one week after commercial harvest. Harvest maturity (starch index, internal ethylene concentration, firmness, titratable acidity, and soluble solids) and external/internal appearance were evaluated, and fruit were imaged with a digital camera. Apples were treated with 1-MCP (1 ppm) and immediately placed in CA in 0.5% $O_2$ and 2.5% $CO_2$ at 37, 46, or 50 °F for 7 days. Following conditioning, apples were stored in 2.5% $O_2$ and 0.5% $CO_2$ at 37 °F for 6 months. External and internal disorders, firmness, titratable acidity, and soluble solids will be evaluated after long term storage.

**RESULTS AND DISCUSSION**

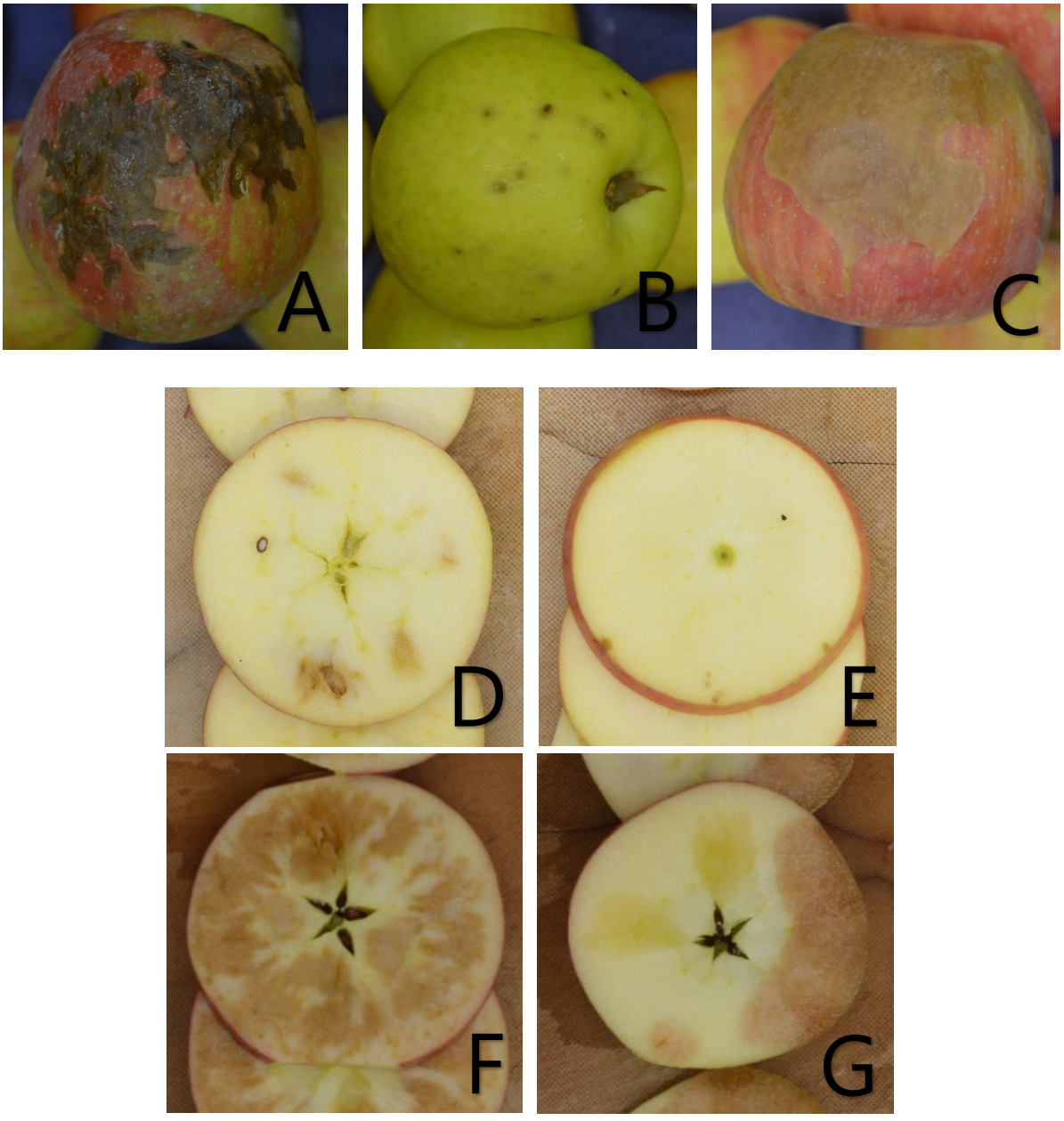


Figure 1. Examples of observed symptoms. (A) Leather Blotch (B) Bitter Pit (C) Soft Scald (D) Lens-shaped Cavities (E) Corky Spots (F) $CO_2$ related internal Browning and (G) Soggy Breakdown. D and E are typical symptoms of $CO_2$ sensitivity.

*Impact of harvest maturity and $CO_2$ level during rapid CA on fruit quality and disorder outcome*

Honeycrisp apples were harvested from orchard 2 (see Objective 2) 3 times at 1-week intervals around commercial harvest to account for differences in susceptibility to bitter pit, soft scald, soggy breakdown, and $CO_2$ sensitivity related to maturity. Average starch index values were 4, 7, and 7 (1-8

scale) for harvests 1, 2, and 3, respectively. Fruit quality was not consistently impacted by storage atmospheric composition (Table 1). As in the previous season, neither flesh firmness nor titratable acidity (tartness) were not impacted by the rapid CA conditioning protocol nor $CO_2$ level. Bitter pit, flesh cavities, and soft scald/soggy breakdown did not develop in or on apples stored in CA (Table 2). With rapid CA temperature conditioning followed by CA storage, disorder incidence was low. Significant soft scald/soggy breakdown incidence only developed on or in untreated, air stored fruit. This indicates apples from this block at all 3 maturities were prone to develop the disorder, but not bitter pit or $CO_2$ related browning. It also indicates that rapid CA conditioning was an effective disorder control.

Table 1. Honeycrisp quality was not consistently impacted following 6 months CA storage by conditioned in varying levels of $CO_2$, regardless of harvest maturity. Data were analyzed comparing $CO_2$ conditioning treatments within a single harvest using SAS Proc ANOVA (Tukey's, $p<0.05$). Letters indicate significant groups within a harvest timepoint.

| **Harvest** | **Conditioning $CO_2$** | **Firmness (lb)** | **TA (g/L)** | **°Brix** |
|---|---|---|---|---|
| Early | 0.50% | 13.59 a | 3.96 ab | 12.52 a |
| | 1% | 13.21 a | 3.72 ac | 12.32 a |
| | 2% | 13.26 a | 3.89 ab | 12.26 a |
| | 3% | 13.34 a | 4.05 b | 12.35 a |
| | 5% | 13.43 a | 3.95 ab | 12.42 a |
| | Air | 13.21 a | 3.51 c | 11.83 b |
| Commercial | 0.50% | 13.09 a | 3.27 a | 12.74 ab |
| | 1% | 13.13 a | 3.20 a | 12.86 b |
| | 2% | 13.08 a | 3.56 a | 12.84 b |
| | 3% | 13.30 a | 3.32 a | 13.03 b |
| | 5% | 13.54 a | 3.24 a | 13.08 b |
| | Air | 14.44 b | 3.30 a | 12.32 a |
| Late | 0.50% | 12.49 ab | 3.36 ab | 13.33 a |
| | 1% | 12.61 ab | 3.49 ab | 13.15 a |
| | 2% | 12.36 a | 3.42 ab | 13.59 a |
| | 3% | 12.42 ab | 3.38 ab | 13.42 a |
| | 5% | 12.35 a | 3.54 b | 13.69 a |
| | Air | 12.96 b | 2.29 c | 13.82 a |
| | No 1-MCP | 12.25 a | 3.24 a | 13.36 a |
| | DPA | 12.14 a | 3.39 ab | 13.23 a |

Table 2. Honeycrisp apples were prone to soft scald/soggy breakdown but conditioning eliminated the disorder. $CO_2$-related internal browning after 6 months was insignificant regardless $CO_2$ levels during conditioning. Data were analyzed comparing $CO_2$ conditioning treatments within a single harvest using a pooled z-test (SAS proc freq $p<0.05$). Letters indicate significant groups within a harvest timepoint.

| Harvest | $CO_2$ Treatment | Bitter Pit/ Leather Blotch | Cavities | Soft Scald | Soggy Breakdown |
|---|---|---|---|---|---|
| Early | 0.50% | 0 a | 0 a | 0 a | 0 a |
| | 1% | 0 a | 0 a | 0 a | 0 a |
| | 2% | 0 a | 0 a | 0 a | 0 a |
| | 3% | 0 a | 0 a | 0 a | 0 a |
| | 5% | 0 a | 0 a | 0 a | 0 a |
| | Air | 0 a | 0 a | 16.66 b | 16.66 b |
| Commercial | 0.50% | 0 a | 0 a | 0 a | 0 a |
| | 1% | 0 a | 0 a | 0 a | 0 a |
| | 2% | 0 a | 0 a | 0 a | 0 a |
| | 3% | 0 a | 0 a | 0 a | 0 a |
| | 5% | 0 a | 0 a | 0 a | 0 a |
| | Air | 2.77 a | 2.77 a | 19.44 b | 25.00 b |
| Late | 0.50% | 0 a | 0 a | 0 a | 0 a |
| | 1% | 0 a | 0 a | 0 a | 0 a |
| | 2% | 0 a | 0 a | 0 a | 0 a |
| | 3% | 0 a | 0 a | 0 a | 0 a |
| | 5% | 0 a | 2.77 a | 0 a | 0 a |
| | Air | 0 a | 3.03 a | 24.24 b | 30.30 b |
| | No 1-MCP | 0 a | 0 a | 0 a | 0 a |
| | DPA | 0 a | 0 a | 3.22 a | 3.22 a |

*$CO_2$-related internal browning and cavities develop in CA after rapid CA conditioning*

Honeycrisp apples were harvested from 3 different orchards approximately at commercial harvest. CA conditions were established following different periods during conditioning to determine whether a delay in CA establishment would reduce $CO_2$-sensitivity, while still reducing bitter pit and soft scald and/or soggy breakdown. Across locations, fruit quality was not consistently impacted by delaying CA establishment or $CO_2$ levels during rapid CA conditioning (not shown). Any differences were primarily attributable to relative harvest maturity. Starch index values for Orchards 1-3 were 5.7, 7.4, 7.6 (1-8 scale), respectively. This indicates Orchard 1 (Mattawa) was the least mature. Elevated bitter pit incidence was only evident in fruit from Orchard 1 if rapid CA (5% $CO_2$) was delayed until day 8 of the conditioning period (Table 3). Orchard 1 developed severe internal browning and cavities, both of which are typically associate with $CO_2$-sensitivity. More severe browning symptoms, surprisingly, developed in apples conditioned under 2.5% compared with 5% $CO_2$ which developed more severe cavities than browning. Cavities also developed in apples harvested from Orchard 3 and conditioned under 5% $CO_2$. Incidence of both of these symptoms in

both orchards was less with delay of CA establishment during conditioning regardless of $CO_2$ level during the CA portion of the conditioning period. While symptoms developed at sometime during CA storage (2.5% $O_2$: 0.5% $CO_2$) following conditioning, reducing time exposed to elevated $CO_2$ during conditioning resulted in lower incidence. $CO_2$-related symptoms resulting from elevated $CO_2$ during conditioning developing during subsequent long term CA storage under low $CO_2$ percentages is consistent with limited $CO_2$ symptoms found during Year 1. While further seasons with more substantial $CO_2$-related symptom incidence are required to support these findings, the results indicate that Honeycrisp exposed to high $CO_2$ during conditioning may be destined to develop symptoms regardless of the subsequent storage atmosphere. Another remaining question that has not been addressed using this protocol is when or how long symptoms take to develop following transfer into the long-term CA conditions.

Table 3. Incidence of $CO_2$-related internal browning of Honeycrisp at 6 months CA storage was orchard specific and increased with duration of elevated $CO_2$ exposure during conditioning. Data were analyzed comparing conditioning treatments ($CO_2$ and CA) within an orchard using a pooled z-test (n=36, $p<0.05$). Letters indicate significant groups within an orchard.

| Orchard | CA Start Day | Conditioning $CO_2$ | Bitter Pit/ Leather Blotch | Internal Browning ($CO_2$) | Cavities |
|---|---|---|---|---|---|
| Orchard 1 | 0 | 2.50% | 2.86 ab | 37.14 ac | 20.00 ab |
| | | 5% | 0 a | 11.76 b | 38.24 a |
| | 4 | 2.50% | 0 a | 50.00 a | 25.00 a |
| | | 5% | 0 a | 3.03 bd | 24.24 a |
| | 8 | 2.50% | 0 a | 19.44 bc | 25.00 a |
| | | 5% | 11.11 b | 0 d | 5.56 b |
| Orchard 2 | 0 | 2.50% | 0 a | 0 a | 0 a |
| | | 5% | 0 a | 0 a | 2.86 a |
| | 4 | 2.50% | 0 a | 0 a | 0 a |
| | | 5% | 0 a | 0 a | 2.78 a |
| | 8 | 2.50% | 0 a | 0 a | 0 a |
| | | 5% | 0 a | 0 a | 0 a |
| Orchard 3 | 0 | 2.50% | 0 a | 0 a | 0 a |
| | | 5% | 0 a | 0 a | 11.11 b |
| | 4 | 2.50% | 0 a | 2.78 a | 0 a |
| | | 5% | 0 a | 0 a | 5.56 ab |
| | 8 | 2.50% | 0 a | 2.86 a | 0 a |
| | | 5% | 0 a | 2.78 a | 2.78 ab |

*Impact of temperature during rapid CA conditioning on fruit quality and disorder outcome*

Honeycrisp apples were harvested from Orchard 2 one week after commercial harvest. Fruit was conditioned for 10 days using rapid CA conditions with elevated $CO_2$ (2.5%) at different temperatures and then transferred to long term CA storage. Conditioning temperature did not consistently impact fruit quality (not shown). $CO_2$-related disorders did not develop regardless of rapid CA conditions.

This is consistent with results from other activities using fruit from this orchard in Year 2. Also, as with earlier activities using apples from Orchard 2, soft scald/soggy breakdown developed on air stored apples confirming that this orchard was prone to developing these disorders (Table 4). As in the earlier activities, apples from orchard 2 were also not sensitive to elevated $CO_2$ during conditions.

Table 4. No disorders developed on Honeycrisp from Orchard 2 conditioned using rapid CA. Soft scald/soggy breakdown developed on unconditioned, air stored apples. Data were analyzed comparing conditioning temperature using a pooled z-test (n=36, $p<0.05$).

| **Conditioning Temperature (°F)** | **Internal Browning** | **Cavities** | **Soft Scald** | **Soggy Breakdown** |
|---|---|---|---|---|
| 37 | 0 a | 0 a | 0 a | 0 a |
| 46 | 0 a | 0 a | 0 a | 0 a |
| 50 | 0 a | 0 a | 0 a | 0 a |
| Air | 0 a | 3.03 a | 24.24 b | 30.30 b |
| No 1-MCP | 0 a | 0 a | 0 a | 0 a |
| DPA | 0 a | 0 a | 3.22 a | 3.22 a |

*Changes of specific flesh chemicals are linked with internal browning and $CO_2$ levels associated with disorder risk.*

Although flesh tissue was sampled at 0, 2, 4, and 7 days during rapid CA conditioning different $CO_2$ setpoints, $CO_2$-related internal browning symptoms did not develop regardless of harvest maturity (from Orchard 2). Flesh chemistry related to $CO_2$-sensitivity and disorder risk was not analyzed as in Year 1. This aspect was the primary reason for requesting a no-cost extension as determining whether levels of these chemicals. Determining whether chemical changes associated with risk in our earlier work (Rudell and Mattheis, 2023; Fig. 2) can be monitored to indicate disorder risk during rapid CA conditioning was one of our key objectives. However, the fruit used for this objective in both seasons failed to develop the disorder, regardless of $CO_2$ levels during conditioning. Consequently, in Year 3, we have harvested fruit from 9 orchards at different maturities (as in activities outlined under Objective 1) as part of this effort. Given that $CO_2$ sensitivity during rapid CA seems to be orchard specific in Year 2, it is expected that 1 or more orchards will prove disorder-prone and provide evidence for this risk assessment protocol.

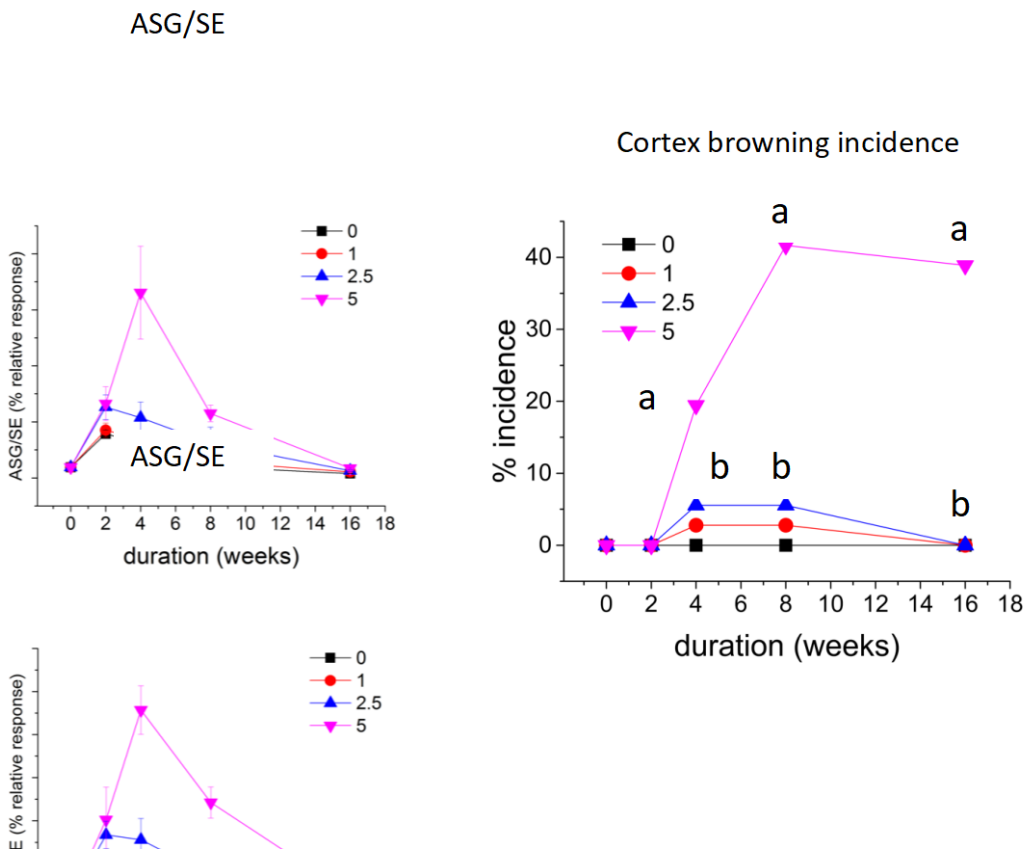


Figure 2. Two ratios of natural chemicals found in apple flesh associated with risk of developing internal $CO_2$-related browning (left) and internal browning development in Fuji stored at 2.5 % $O_2$ and 0.5, 1, 2.5, and 5 % $CO_2$ (right). This experiment was the outcome of an objective of our previous project (Rudell and Mattheis, 2023) where natural peel chemicals associated with risk of $CO_2$-related disorders were found. Apples stored under the highest levels of $CO_2$ developed browning and this was reflected by the ratio of ASG/SE before symptoms developed.

**Project/Proposal Title:** Fate of *Listeria* on fresh apples as affected by commercial apple waxes

**WTFRC Project Number:** AP-20-104A

**Report Type:** Final Project Report

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**Cooperators**: Stemilt Growers LLC.; Hansen Fruit; Allan Brothers; Pace International LLC.; Jones-Hamilton Co.

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $ 83,842
**Total Project Request for Year 2 Funding:** $ 85,841
**Total Project Request for Year 3 Funding:** $ 86,419

**WTFRC Collaborative Costs:**

| Item | 2020-2021 | 2021-2022 | 2022-2023 |
|---|---|---|---|
| Salaries | | | |
| Benefits | | | |
| Wages | 2,104 | 2,135 | 2,168 |
| Benefits | 1,089 | 1,093 | 1,110 |
| RCA Room Rental | 2,250 | 2,316 | 2,385 |
| Shipping | | 275 | 275 |
| Supplies | 250 | 275 | 275 |
| Travel | 350 | 375 | 400 |
| Plot Fees | | | |
| Miscellaneous | | | |
| Total | 6,043 | 6,469 | 6,613 |

**Footnotes:**

**Budget 1**
**Primary PI:** Meijun Zhu
**Organization Name:** Washington State University
**Contract Administrator:** Anastasia Kailyn Mondy
**Telephone:**
**Contract administrator email address:** arcgrants@wsu.edu

| Item | 2020-2021 | 2021-2022 | 2022-2023 |
|---|---|---|---|
| Salaries | 32,667 | 33,974 | 35,333 |
| Benefits | 6,632 | 6,898 | 7,173 |
| Wages | | | |
| Benefits | | | |
| Equipment | | | |
| Supplies | 28,500 | 28,500 | 28,500 |
| Travel | 2,000 | 2,000 | 2,000 |
| Miscellaneous | 8,000 | 8,000 | 6,800 |
| Plot Fees | | | |
| Total | 77,799 | 79,372 | 79,806 |

**Footnotes:**

## OBJECTIVES

1. Examine the fates of *Listeria,* resident bacteria, and yeast/mold on apples applied with commercial apple wax under subsequent cold storage.
2. Evaluate the fates of *Listeria* on waxed apples contaminated during wax application under subsequent cold storage.
3. Investigate the serotype-specific survival of *Listeria* on waxed apples and the killing effects of residual sanitizers on the fates of *Listeria* and resident microbes on waxed apples during subsequent cold storage

## SIGNIFICANT FINDINGS

1. The dry temperature, whether at 22 °C (72 °F), 45 °C (113 °F), or 60 °C (140 °F), had no discernible impact on the survival of *Listeria innocua* on wax-coated apples from the selected varieties.
2. The population of *L. innocua* on unwaxed apples decreased by 1.9 $\log_{10}$ CFU/apple over the course of 18 weeks of refrigerated air storage.
3. *L. monocytogenes* reduced by 1.8-2.0 $\log_{10}$ CFU/apple on waxed apples during 12-week cold storage, regardless of the type of wax coating.
4. The fate of *Listeria* on wax-coated apples was similar to that on unwaxed apples.
5. The fate of *L. innocua* on Granny Smith apples exhibited comparable trends to those observed on Fuji apples, irrespective of the specific type of wax coating.
6. A significant risk of cross-contamination of *L. monocytogenes* occurred during the wax coating application process, from inoculated apples to waxing brushes, and from contaminated brushes to uninoculated apples, highlighting the importance of waxing station sanitation.
7. The die-off rate of *L. monocytogenes* on wax-coated apples contaminated during wax coating process was not significantly different from those contaminated before wax coating.
8. Different *L. monocytogenes* serotypes, including 1/2a, 1/2b, and 4b, exhibited distinct survival profiles on Granny Smith apples.
9. Serotype 1/2a displayed the highest resilience, maintaining a high population on Granny Smith apples throughout storage. In contrast, serotype 4b, linked to the caramel apple outbreak, exhibited the lowest survivability, with a rapid decline observed within 48 h of attachment at 22 °C (72 °F).
10. A 30-sec treatment with peroxyacetic acid (PAA) at 80 ppm resulted in a ~1.4 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* on apples but had no residual killing effect on *L. monocytogenes* or resident microbes on wax-coated apples during the subsequent 16 weeks of cold storage. The count of *L. monocytogenes* recovered from wax-coated apples treated with 80 ppm PAA was significantly lower than that of control apples washed with tap water.
11. *L. monocytogenes* remained viable on waxing brushes during 12 weeks of ambient holding.
12. Including fungicides in the wax coating effectively reduced yeasts and molds on wax-coated apples; however, it did not impact *L. monocytogenes* survival.
13. The wax coating did not affect the survival of yeasts and molds on apples, irrespective of the apple cultivars; an increase of 0.4-0.5 $\log_{10}$ CFU/apple was observed after 18 weeks of cold storage, independent of the type of wax treatment applied.
14. Wax coating increased the glossiness of apples, regardless of wax treatment.

15. The firmness of apples decreased after 18 weeks of commercial storage, regardless of whether a wax coating was applied or the types of coating used. However, wax coating reduced the firmness loss in Granny Smith apples across all coating types.
16. Total soluble solids (TSS) were maintained in both unwaxed and wax-coated apples after 18 weeks of cold storage. Titratable acidity (TA) decreased in both unwaxed and wax-coated Fuji apples, while wax coating reduced TA loss in Granny Smith apples.
17. The application of the wax coating, irrespective of type, had no impact on interior and exterior disorders on Fuji and Granny Smith apples, but it significantly reduced internal browning in Granny Smith apples.

## METHODS

1. Strain selection

*L. monocytogenes* strains for BSL2 lab storage: A panel of *L. monocytogenes* serotypes consisting of serotypes 1/2a, 1/2b, and 4b was selected and used in this study (Table 1). To confirm the serotype-specific survivability of *L. monocytogenes* strains, we also used an additional set of strains covering these three serotypes, including NRRL B-57618, NRRL B-33466, and NRRL B-33053 (Table 1).

Table 1. Detailed information on *L. monocytogenes* strains used in this study.

| Strain No. | Serotype | Source | Antibiotics resistance |
|---|---|---|---|
| LS808 | 1/2a | Linked to a celery outbreak | Erythromycin at 2.5 mg/l |
| LS810 | 1/2b | Linked to a cantaloupe outbreak | Erythromycin at 2.5 mg/l, Rifampicin at 100 mg/l |
| LS1062 | 4b | Linked to apple outbreak | Streptomycin at 1000 mg/l |
| NRRL B-57618 | 1/2a | 2011 cantaloupe outbreak isolate | None |
| NRRL B-33466 | 1/2b | Processing plant environmental isolate | None |
| NRRL B-33053 | 4b | 1983 Coleslaw outbreak isolate | None |

*L. innocua* strains employed for commercial cold storage: *L. innocua*, a widely used surrogate for *L. monocytogenes,* was used to investigate the fates of *Listeria* during commercial cold storage. A 3-strain cocktail of *L. innocua* isolates, sourced from an apple packing facility and other fresh produce processing plants, was prepared using our established methodology.

2. Apple inoculation

Apples were contaminated with *Listeria* prior to the waxing application: Washed and unwaxed apples of the selected varieties without cuts or bruises were individually and separately inoculated to establish $1\times10^6$ CFU/apple of a 3-strain cocktail of *L. monocytogenes* or *L. innocua* per our well-established method. Additionally, to rule out confounding effects from potential antagonistic interactions among strains, each serotype from the LS808, LS810, and LS1062 set, as well as from the *L. monocytogenes* NRRL B-57618, B-33466, and B-33053 set, was used to prepare individual inocula. The resulting inoculum was used separately for apple inoculation. The inoculated apples were held at 22 °C (72 ºF) for 24 h before the wax coating was applied.

Apples were contaminated during waxing application: To test the potential of *L. monocytogenes* cross-contamination from apple-to-brush and brush-to-apple, one waxing brush was used to coat one *L. monocytogenes* inoculated apple; then, this contaminated brush was used to wax five uninoculated apples in a sequence (Fig. 1).

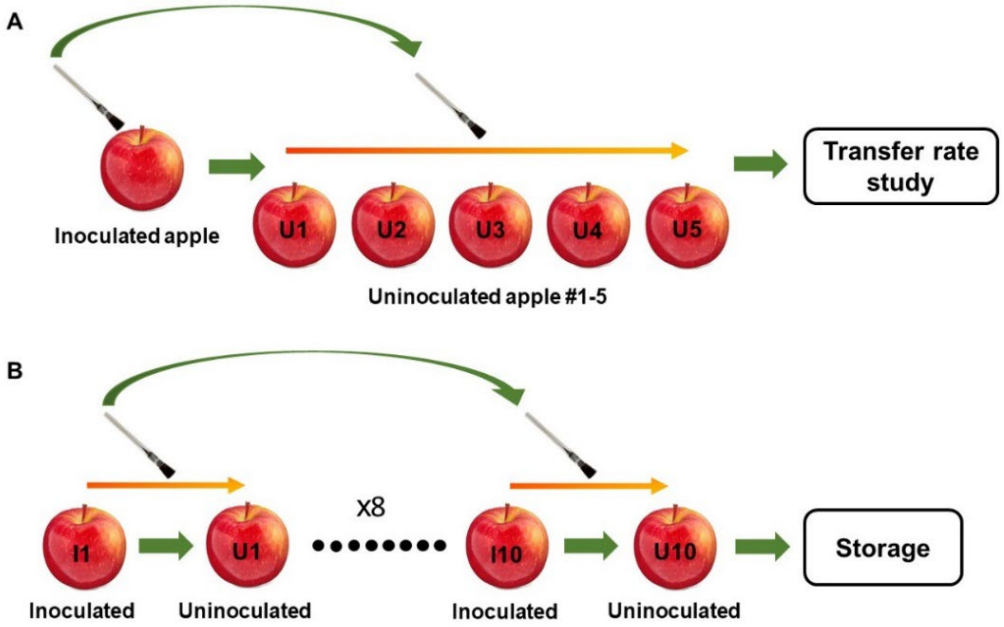


**Fig. 1**. **Illustration for the preparation of waxed apples contaminated with *Listeria monocytogenes* during wax coating.** A. Wax-coated apples for the apple-to-brush and brush-to-apple transfer rate study. B. Wax-coated apples for the storage study. I: inoculated apple; U: uninoculated apple.

3. Waxing application

Wax selection: Three commercial apple fruit waxes, namely Prima Fresh 360 HS (PF360), Prima Fresh 606 EU (PF606) or Shield Brite AP-450 (AP-450) were used in the proposed studies.

Waxing application: Each wax solution was manually applied evenly to both inoculated and uninoculated apple surfaces of the selected cultivars unless specified. To assess the fate of *Listeria* on waxed apples subjected to cross-contamination during the waxing process, brushes contaminated with *L. monocytogenes* were used for manual wax application. This enables the cross-contamination of *L. monocytogenes* to uninoculated apples (Fig. 1).

4. Wax coating drying

To evaluate the impacts of wax coating drying conditions/temperatures on the survival of *Listeria* on waxed apples, apples immediately following wax coating were subjected to different drying temperatures (~22 °C/72 °F, 45 °C/113 °F, or 60 °C/140 °F) for 2 min, followed by an additional 5 h drying at room temperature (~22 °C/72 °F) before being subjected to cold storage (Fig. 2).

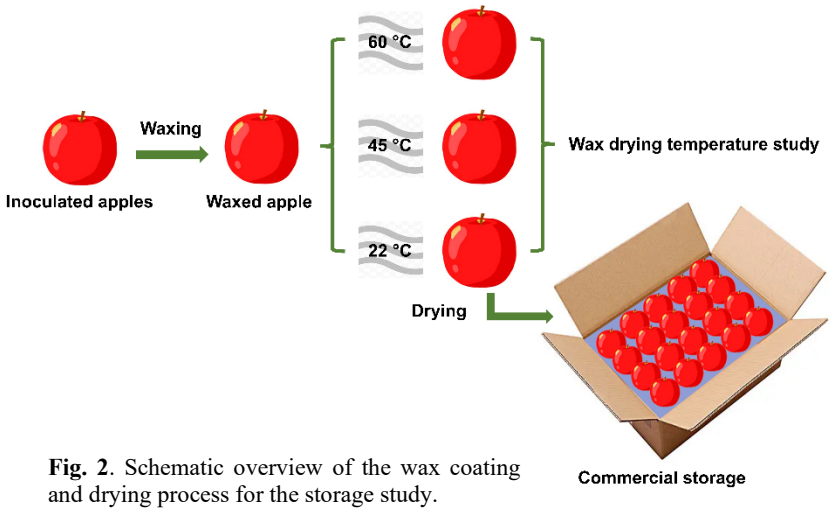


**Fig. 2**. Schematic overview of the wax coating and drying process for the storage study.

5. Cold storage treatments and sampling

BSL2 lab cold storage: Uninoculated or inoculated apples (with or without wax coating) from the selected cultivars were subjected to 1 °C (34 °F) or ambient temperature storage for up to 16 weeks. Samples were taken at designated intervals to enumerate *L. monocytogenes* or resident microbiota (background bacteria or yeast/mold). Two independent and sequential trials were conducted, each using a different lot of apples. In each independent trial, 20 apples per treatment were sampled on each sampling day.

Commercial facility storage: Uninoculated apples and apples inoculated with a 3-strain *L. innocua* cocktail and coated with different wax coatings were stored at 1 °C (34 °F) for up to 18 weeks in refrigerated air (RA) room within a commercial packing facility. Apples of each treatment combination were sampled during storage to enumerate the survival of *L. innocua* and yeast/mold. The study was conducted over two consecutive years. Four sets of 10 apples were used for each wax treatment on each sampling day.

6. Residual killing effects of antimicrobial interventions

Fresh, unwaxed Granny Smith apples of uniform size (220-240 g) were selected and dip-inoculated with a three-strain *L. monocytogenes* cocktail as previously described. The inoculated apples were treated with 80 ppm peroxyacetic acid (PAA), a commonly used sanitizer in spray bar interventions, for 30 seconds. After treatment, the apples were dried at room temperature for 3 h and then manually wax-coated with PrimaFresh 360 HS as outlined above. Apples inoculated and treated with tap water served as controls.

The wax-coated apples were subsequently dried at room temperature for 4 h before being stored at 1 °C (34 °F) and ~90 % relative humidity for up to 16 weeks. Temperature and humidity levels were monitored daily using a hygro-thermometer (Extech Instruments) throughout the storage period. A sample size of 40 apples per sampling time and treatment was used.

7. Survival microorganism analysis

*Listeria* enumeration: At each sampling day, *Listeria* survival on waxed apples under the respective storage (BSL2 or commercial facility) was detached and serially diluted. Appropriate dilutions were plated on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) plates overlaid with modified Oxford agar per our established method. For the serotype-specific survival profile analysis, the detached microbial suspensions were plated onto TSAYE plates with erythromycin, erythromycin and rifampicin, and streptomycin for the enumeration of serotype 1/2a serotype 1/2b, and serotype 4b, respectively.

All plates were incubated at 35 °C (95 °F) for 48 h and subsequently enumerated. If the survival of *Listeria* on apple fruit fell below the enumerative detection limit, the suspension was assessed for presence/absence after 48 h of enrichment in Buffered *Listeria* Enrichment Broth (BLEB) and streaked onto a selective *Listeria* agar plate. Presumptive positive colonies were further confirmed by PCR (FDA, 2015).

Resident microbiota: Microbial suspension at appropriate dilutions was also plated on duplicate Potato Dextrose Agar plates supplemented with 0.1 g/l chloramphenicol for yeast and mold counts. The PDA plates were incubated at room temperature (~22 °C/ 72 °F) for 5 days.

8. Fruit quality analysis

At harvest or 18-week storage, fruit quality parameters such as firmness, total soluble solids, and titratable acidity, as well as external and internal disorders, including superficial scald and lenticel decay, were assessed at the end of cold storage by the WTFRC quality lab using established methods (Shen et al., 2021). A sample size of 10 apples per replicate with 4 independent replicates per wax type was used for internal and external disorder assessment.

9. Glossiness measurement

The gloss index of apples was determined at 60° with a gloss meter (Novo-Curve, Rhopoint Instrumentation, East Sussex, UK). The gloss units (GU) were directly measured on the fruit surface with 10 randomly selected spots per fruit. A total of 10 apple fruits per treatment condition was used for gloss analysis.

10. Statistical analysis.

Data were analyzed with IBM SPSS 19.0 (Chicago, IL). Mean differences were assessed through a one-way analysis of variance (ANOVA), followed by a Tukey multiple comparison test. *P* values less than 0.05 were considered significant differences.

## RESULTS AND DISCUSSION

1. Survival of *L. monocytogenes* in wax coating solutions

*L. monocytogenes* in wax solutions were reduced by ~2 $\log_{10}$ CFU/ml in 1-h contact regardless of wax type (Fig. 3). AP-450 showed a superior antimicrobial efficacy against *L. monocytogenes*, followed by PF 606 and PF 360. *L. monocytogenes* in AP-450 wax solution dropped to below the limit of detection (1 CFU/ml) after 24-h incubation compared with 5.3 and 3.1 $\log_{10}$ CFU/ml reductions observed in PF 606 and PF 360 wax solutions (Fig. 3).

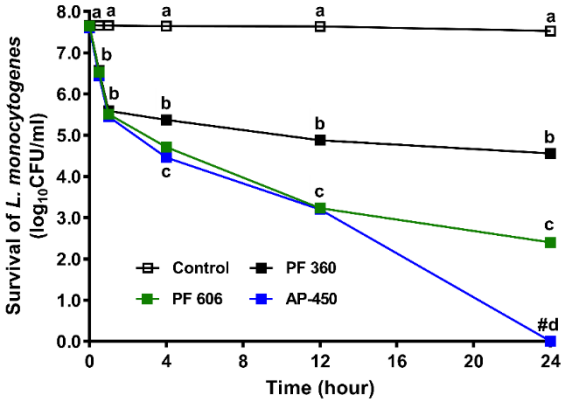


**Figure 3. Survival of *L. monocytogenes* in apple wax coating solutions at RT.** Control: water. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360; PF 606: PrimaFresh 606. Mean ± SEM, n = 9. [a-d] Mean at each sampling point without a common letter differ significantly ($P < 0.05$).

2. Effects of wax drying temperatures on survival of *L. innocua*, yeast and mold counts, and the glossiness of the waxed apples

During the commercial apple packing line process, wax coatings of apples are dried as they pass through a heated air dryer (42-45 °C/ 108-113°F). To simulate this commercial drying process in our study, we first evaluated the impact of wax coating drying temperatures of 22 °C (72 °F), 45 °C (113 °F), and 60 °C (140 °F) on the survival of *L. innocua* on apples and the glossiness of waxed apples in our BSL2 lab. Data in Fig. 4 indicated that the drying temperature did not affect the

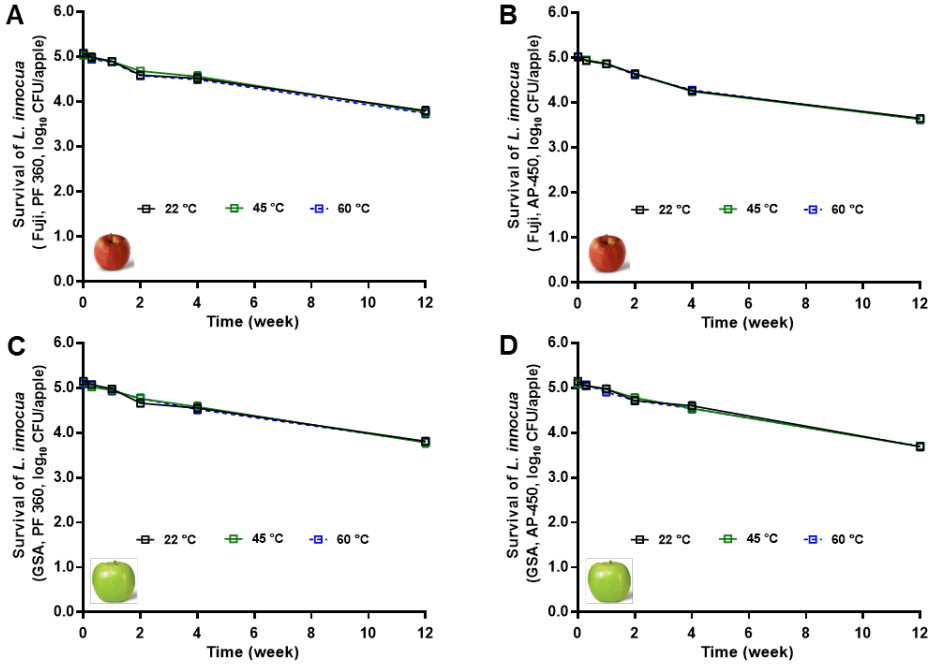


**Figure 4. Impact of wax drying temperature on *L. innocua* on waxed apples during 12 weeks of storage at 1 °C (33 °F).** A. Fuji apples coated with PrimaFresh 360 HS (PF 360); B. Fuji apples coated with Shield-Brite AP 450 (AP 450); C. Granny Smith apples coated with PF 360; D. Granny Smith apples coated with AP 450 Mean ± SEM, n = 12.

fate of *L. innocua* on apples during the 12 weeks of storage at 1 °C (34 °F). Populations of *L. innocua* on waxed apples gradually declined, resulting in 1.4-1.5 $\log_{10}$ CFU/apple reduction of *L. innocua* by the end of storage, regardless of apple varieties, drying temperature, and wax type (Fig. 4). Similarly, the application of a shellac-based wax at 25 °C (77 °F), 50 °C (122 °F), and 60 °C (140 °F), for 2 min caused comparable reductions of *E. coli* on the stem scar area of oranges (Pao, Davis, Kelsey, & Petracek, 1999). However, another study found that applying Shield-Brite AP-40 on apples at 55 °C (131 °F), drying caused additional 1.0 and 0.4 $\log_{10}$ CFU/apple reduction of *Escherichia coli* O157:H7 and *Salmonella*, respectively, compared to drying at 21 °C (70 °F), (Kenney & Beuchat, 2002). These different observations may be due to variations in pathogens and the wax coating formulations.

Similarly, yeast and mold count on PF 360- or AP 450-coated Fuji apples (Fig. 5AB) and GSA (Fig. 5CD) were not affected by drying temperature, showing 0.5-0.6 $\log_{10}$ CFU/apple increase during the initial 4 weeks of cold storage and remaining stable over subsequent 8 weeks of cold storage (Fig. 5).

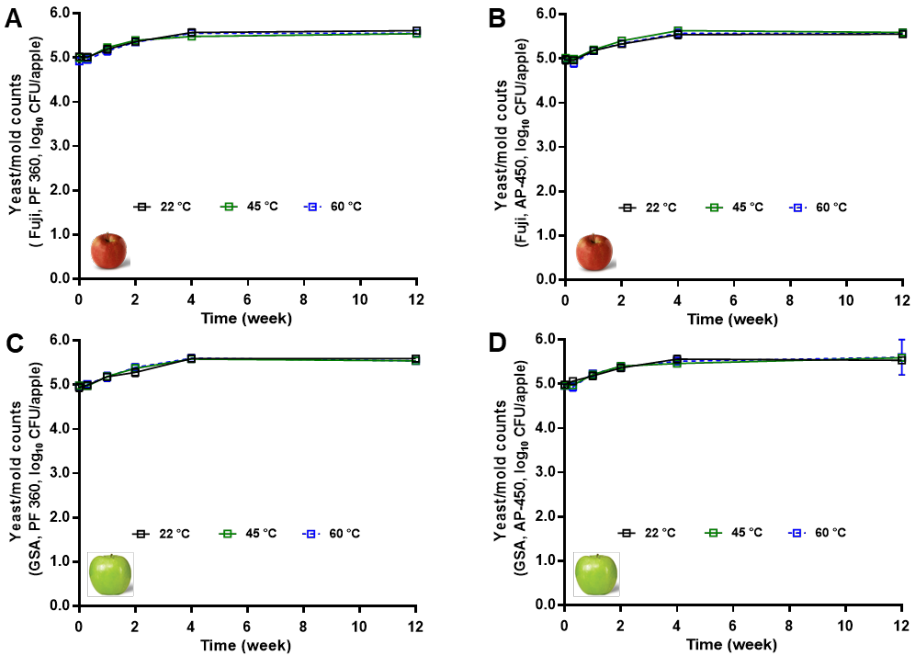


**Figure 5. Impact of wax drying temperature on yeast and mold counts on waxed apples during 12 weeks of storage at 1 °C (34 °F).** A. Fuji apples coated with PrimaFresh 360 HS (PF 360); B. Fuji apples coated with Shield-Brite AP-450 (AP-450); C. Granny Smith apples coated with PF 360; D. Granny Smith apples coated with AP-450. Mean ± SEM, n = 12.

The gloss indices of apples increased after coating with wax solutions, with an increase of 11.2-12.2 GU observed for apples coated with PF 360 and 18.7-20.0 GU for those coated with AP-450, compared to unwaxed apples (Su et al., 2023). The drying temperature had no impact on gloss indices, regardless of the wax type and apple varieties. Based on these findings, a drying temperature of 22 °C (72 °F) was selected for preparing wax-coated apples during the subsequent studies.

3. Transfer of *L. monocytogenes* from apple-to-brush and brush-to-apple during wax application

To test the potential of *L. monocytogenes* cross-contamination from apple-to-brush and brush-to-apple, one waxing brush was used to coat one *L. monocytogenes* inoculated apple; then, this contaminated brush was used to wax five uninoculated apples in a sequence (Fig. 1A). During PF 360 wax coating application, there were 3.7, 3.5, 3.3, 2.9, and 2.7 $\log_{10}$ CFU/apple of *L. monocytogenes* transferred from the inoculated apple (6.2 $\log_{10}$ CFU/apple) to uninoculated apple 1 to apple 5, respectively (Fig. 6A). After waxing the 5$^{th}$ uninoculated apple, 3.6 $\log_{10}$ CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 6B). Similarly, for apples with a higher contamination level (8.4 $\log_{10}$ CFU/apple), 5.8, 5.6, 5.0, 4.8 and 4.6 $\log_{10}$ CFU/apple of *L. monocytogenes* were transferred to uninoculated apple 1 to apple 5 during wax coating application (Fig. 6C). After waxing of the 5$^{th}$ uninoculated apple, 5.5 $\log_{10}$ CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 6D). A similar transfer rate of *L. monocytogenes* from the inoculated

apple to the waxing brush and uninoculated apples was found for AP-450, regardless of the initial contamination level (Fig. 6).

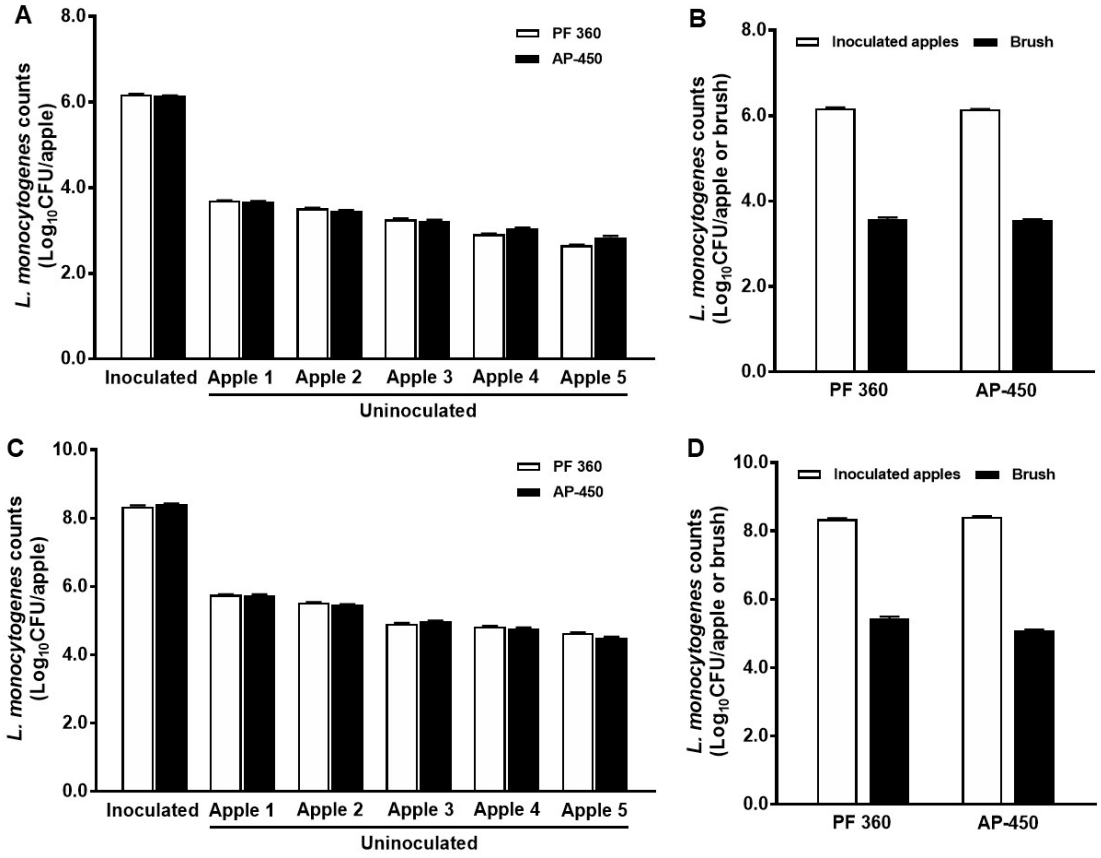


**Figure 6**. Transfer of *L. monocytogenes* from inoculated apples to uninoculated apples and waxing brushes during wax coating. A. Transfer from inoculated apples (~6 $\log_{10}$ CFU/apple) to uninoculated apples; B. Transfer from inoculated apples (~6 $\log_{10}$ CFU/apple) to waxing brushes. C. Transfer from high level inoculated apples (~8 $\log_{10}$ CFU/apple) to uninoculated apples; D. Transfer from high level inoculated apples (~8 $\log_{10}$ CFU/apple) to waxing brushes. Apple 1-5: *L. monocytogenes* on uninoculated apples transferred from contaminated waxing brushes. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360. Data were presented with mean ± SEM, n = 24.

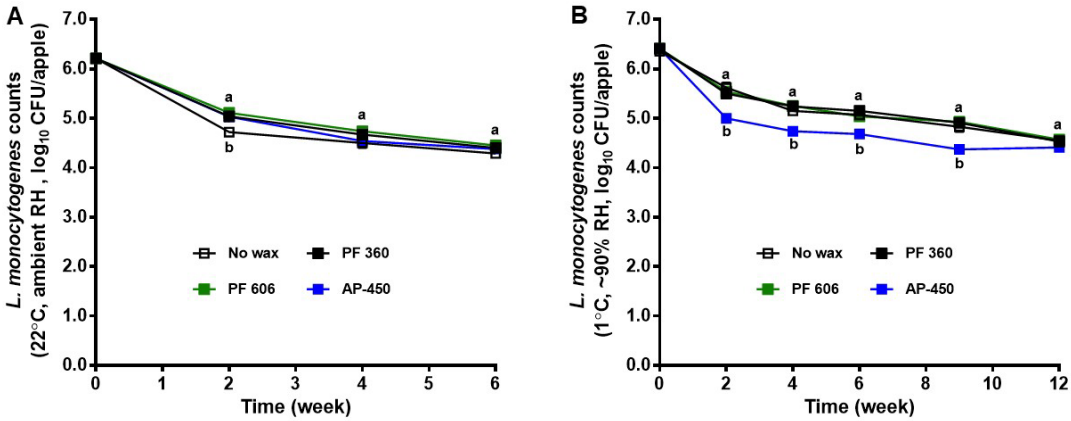


**Figure 7**. Fates of *L. monocytogenes* on wax-coated apples contaminated before wax coating application for up to 12 weeks of storage. A. ambient T and RH. B. 1 °C (34 ºF) and ~90 % RH; No wax: unwaxed control apples; PF 360: apple coated with PrimaFresh 360; PF 606: apple coated with PrimaFresh 606; AP-450: apple coated with Shield-Brite AP-450; RH: relative humidity. Mean ± SEM, n = 40. [a-b] Means at each sampling point without common letter differ significantly ($P < 0.05$).

3. Survival of *L. monocytogenes* on waxed apples contaminated during different waxing schemes

To represent wax applications at apple packinghouses, three commonly used fruit wax coatings, PF 360, PF 606, and AP-450 were applied to the inoculated fruits, followed by up to 12-week storage. *L. monocytogenes* showed a similar trend on waxed apples under cold storage; there were 1.8-2.0 $\log_{10}$ CFU/apple reductions of *L. monocytogenes* on apples during 12 weeks of cold storage regardless of wax coating type, though the reduction on AP-450 waxed apples was higher ($P < 0.05$) at 2-9 weeks of storage (Fig. 7B). The application of wax coating had a minor impact on the survival of *L. monocytogenes* on apples regardless of storage temperature (Fig. 7).

Given the prevalence of *Listeria* species in waxing areas (Ruiz-Llacsahuanga, Hamilton, Zaches, Hanrahan, & Critzer, 2021; Simonetti et al., 2021), it is likely that *L. monocytogenes* can be introduced to wax-coated apples during the wax-coating process. Therefore, we next examined the fate of *L. monocytogenes* on PF 360 coated apples introduced during wax coating with the same contamination level as pre-contaminated apples at ~6 $\log_{10}$ CFU/apple (Fig. 1B). *L. monocytogenes* was reduced by 1.8 $\log_{10}$ CFU/apple after 12 weeks of cold storage (Fig. 8), which had a similar trend as *L. monocytogenes* introduced to apples before waxing application whether apples had an initial population of ~6 $\log_{10}$ CFU/apple (Fig. 7B) or ~8 $\log_{10}$ CFU/apple (Fig. 8).

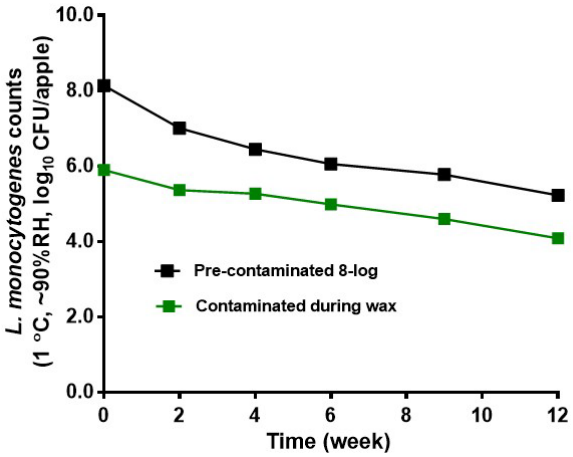


**Figure 8**. Fates of *L. monocytogenes* on wax-coated apples introduced during PrimaFresh 360 coating application for up to 12 weeks of storage. Source apples were inoculated with ~8 $\log_{10}$ CFU/apple of *L. monocytogenes* before wax coating (black line). Mean ± SEM, n = 40.

4. Impacts of fungicide application in PrimaFresh 360 coating on fates of *L. monocytogenes* and endogenous yeasts and molds on waxed apples during 12 weeks of cold storage

Fungicides can be incorporated into wax coating solutions under commercial apple waxing. To evaluate the potential impacts of fungicide applications during wax coating on the fate of *L. monocytogenes* on waxed apples, PF 360 wax coating was further applied in combination with two widely used fungicides, fludioxonil, and natamycin, followed by 12 weeks of cold storage. As shown in Fig. 9A, fludioxonil or natamycin in the fruit wax coating did not impact ($P > 0.05$) the behavior of *L. monocytogenes* on waxed fruits. Populations of *L. monocytogenes* decreased by 1.7-1.8 $\log_{10}$

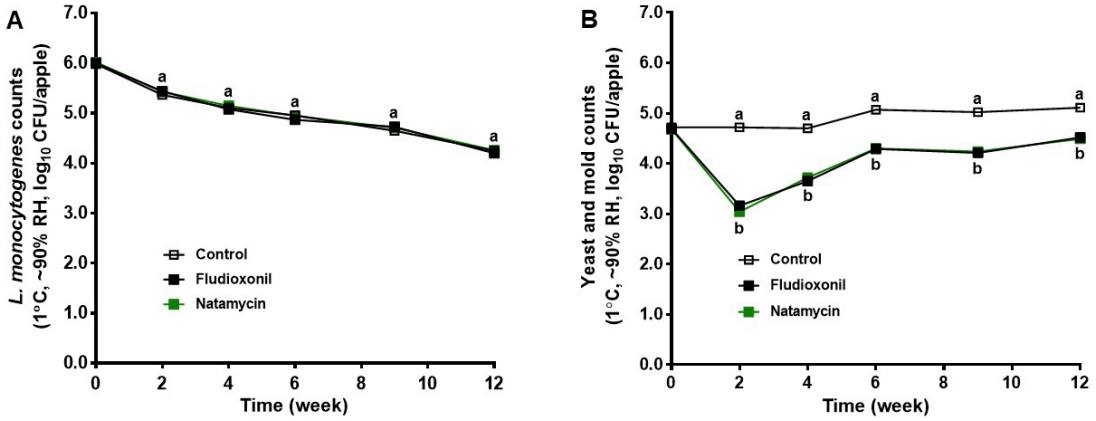


**Figure 9**. Impacts of fungicide application in PrimaFresh 360 coating on fates of *L. monocytogenes* (A) and endogenous yeasts and molds (B) on wax-coated apples during 12 weeks of cold storage. Mean ± SEM, n = 40. [a-b] Means at each sampling point without common letter differ significantly ($P < 0.05$).

CFU/apple on PF 360-coated apples regardless of fungicide application after 12 weeks of cold storage (Fig. 9A). Including fungicides in a wax solution reduced yeast and mold counts on waxed apples by 1.5-1.6 $\log_{10}$ CFU/apple at 2-week cold storage, but the counts then gradually increased to 4.5 $\log_{10}$ CFU/apple at 12-week cold storage (Fig. 9B). Fludioxonil and natamycin had similar effectiveness ($P > 0.05$) in controlling yeasts and molds on waxed apples.

5. Serotype-specific survival of Granny Smith apples during 48 h of attachment

The initial inoculation level of the *L. monocytogenes* 3-strain cocktail on apples was ~5.3 log CFU/apple, with ~4.9 $\log_{10}$ CFU/apple for each serotype (Fig. 10A). After 48 h of the establishment at RT, the counts of *L. monocytogenes* cocktail, LS808 (1/2a), and LS810 (1/2b) on GSA increased by 0.44-0.5 $\log_{10}$ CFU/apple (Fig. 10A-B, $P < 0.05$), while the culturable count of LS1062 (4b) significantly decreased by 1.36 $\log_{10}$ CFU/apple ($P < 0.05$) (Fig. 10A). To rule out the possibility that the reduction in the 4b strain was due to the potential antagonistic interactions among strains, each serotype was individually inoculated on apples, and the survival patterns of each serotype on apples mirrored those observed in the cocktail environments (Fig. 10). To further confirm serotype-specific survival and attachment, an additional set of *L. monocytogenes* strains linked to fresh produce outbreaks (Table 1) was examined individually on apples during 24 and 48 h of attachment. Their survival again mirrored the LS808, 810, and 1062 set (Data not shown).

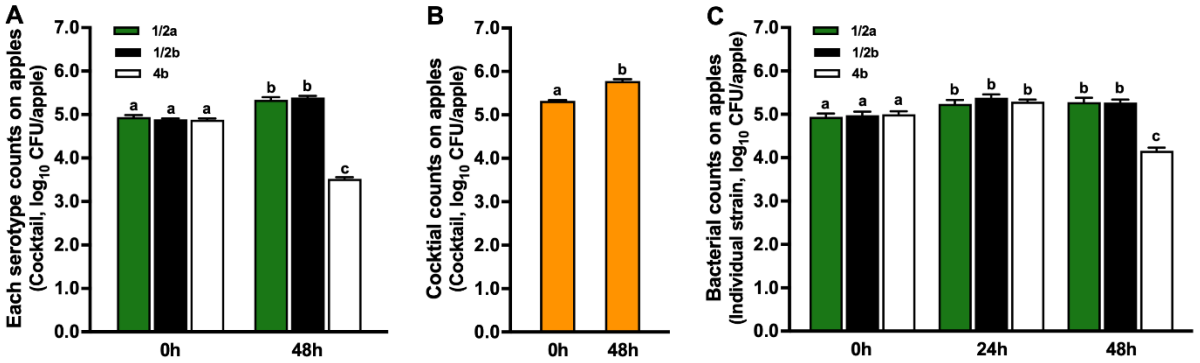


**Figure 10. Counts of *L. monocytogenes* on unwaxed Granny Smith apples during 48 h attachment at 22 °C.** A. Counts of each serotype in the cocktail inoculated on apples, immediately after inoculation (0 h) and 48 h post-inoculation (48 h). B. Counts of the *L. monocytogenes* cocktail inoculated on apples at 0 h and 48 h post-inoculation. C. Counts of each serotype individually inoculated on apples 0 h, 24 h, and 48 h post-inoculation. Mean ± SEM, n = 20. Different

Over 12 weeks of 1 °C (34 °F) or RT storage, the total counts of the 3-strain *L. monocytogenes* recovered from waxed GSA, regardless of wax coating application or the type of wax coating, remained relatively stable (Figs. 11-12). Each serotype exhibited a unique survival profile during 1 °C (34 °F) storage. After a sharp decrease post 48 h ($P < 0.05$), serotype 4b remained relatively low (3.5 $\log_{10}$ CFU/apple) but stable counts on GSA over the subsequent 12 weeks of 1 °C (34 °F) storage, irrespective

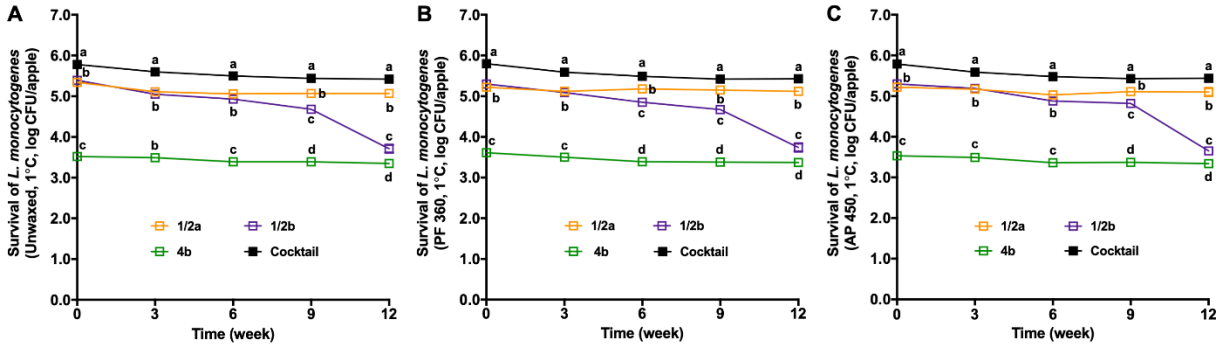


**Figure 11. Survival of *L. monocytogenes* serotypes on Granny Smith apples during 12 weeks of storage at 1 °C (33 °F).** A. Unwaxed apples; B. PrimaFresh 360 (PF 360) coated apples; C. Shield-Brite AP-450 (AP-450) coated apples. Mean ± SEM, $n = 40$. Different letters (a-d) indicate significant differences at each sampling point ($P < 0.05$).

of wax coating or the type of wax (Fig. 11). Among the serotypes tested, serotype 1/2a was the most resilient, maintaining a high population level (5.3 $\log_{10}$ CFU/apple) throughout the entire storage duration, regardless of the type of wax coating. Serotype 1/2b counts on GSA remained relatively stable during the first 9 weeks of storage but exhibited a drastic reduction (1.7 $\log_{10}$ CFU/apple) ($P < 0.05$) by the end of the 12-week storage. The fate of each serotype strain was comparable to unwaxed GSA (Fig. 11), indicating that the wax coating had minimal impact on the survival of distinct serotypes ($P > 0.05$).

The fate of each *L. monocytogenes* serotype as well as the 3-strain cocktail, on waxed GSA during ambient temperature storage showed similar trends to those observed under cold storage conditions (Fig. 12). Serotype 4b exhibited a swift decline, while serotype 1/2a became the dominant serotype on the surface of GSA.

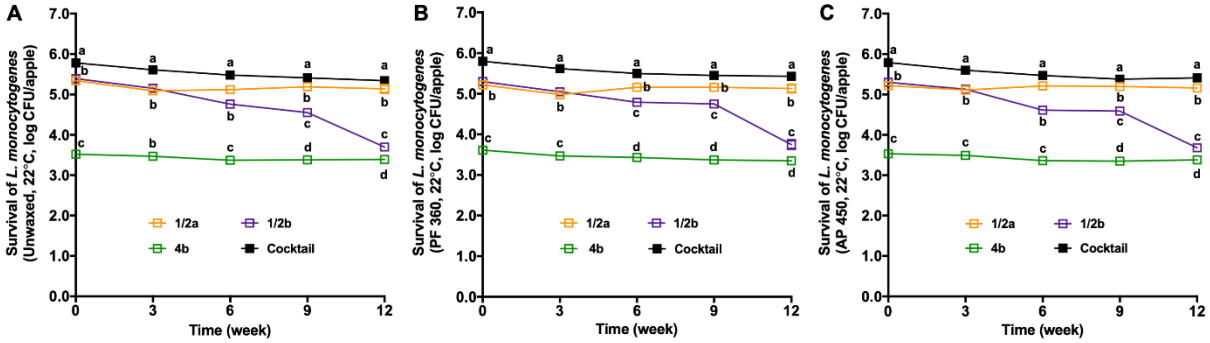


**Figure 12. Survival of *L. monocytogenes* serotypes on Granny Smith apples during 12 weeks of storage at 22 °C (72 °F).** A. Unwaxed apples; B. PrimaFresh 360 (PF 360) coated apples; C. Shield-Brite AP 450 (AP 450) coated apples. Mean ± SEM, $n = 40$. Different letters (a-d) indicate significant differences at each sampling point ($P < 0.05$).

6. Residual killing effects of antimicrobial interventions

PAA is one of the most frequently used sanitizers in the Washington apple industry and can be applied at 80 ppm without the need for further rinsing (FDA, 2017). A 30-sec treatment with PAA at 80 ppm resulted in a ~1.4 $\log_{10}$ CFU/apple reduction of *L. monocytogenes* on apples before wax coating. However, PAA did not exhibit a residual killing effect on *L. monocytogenes* (Fig. 13A) or endogenous yeast and mold counts (Fig. 13B) on wax-coated apples during the subsequent 16 weeks of cold storage. The count of *L. monocytogenes* (Fig. 13A) and yeasts and molds (Fig. 13B) recovered from wax-coated apples treated with 80 ppm PAA was significantly lower than those on control apples washed with tap water.

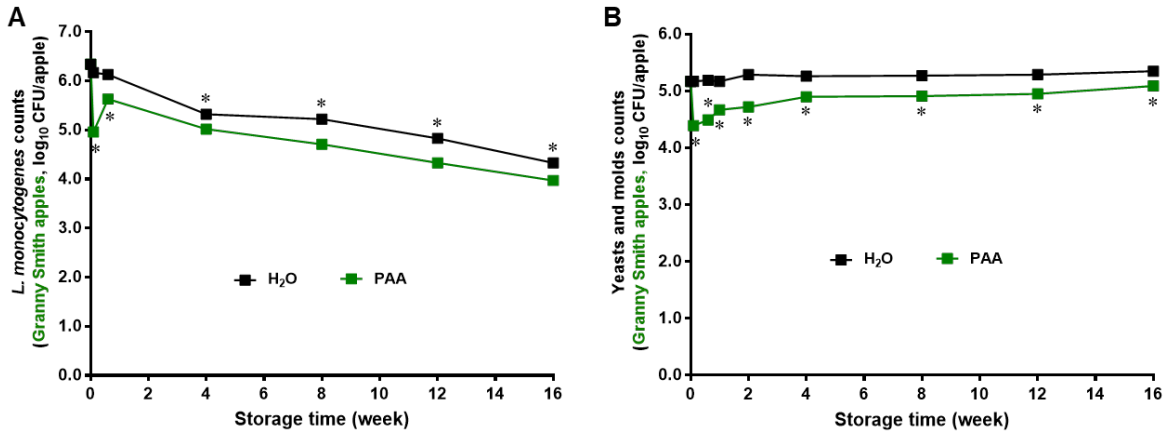


**Figure 13. The population of *L. monocytogenes* (A) and yeasts and molds (B) on PrimaFresh 360 (PF 360) coated Granny Smith apples washed with or without 80 ppm peroxyacetic acid (PAA) during 16 weeks of simulated cold storage at 1 °C (33 °F).** Mean ± SEM, $n = 40$. *: indicate significant differences at each sampling point ($P < 0.05$).

7. Persistence of *L. monocytogenes* on wax coating brushes

*L. monocytogenes* remained relatively stable on waxing brushes during 2 weeks of holding at 22 ºC (72 ºF). A 0.5 and 1.3 $\log_{10}$ CFU/brush reduction was observed on waxing brushes with initial contamination levels of 3.6 and 5.5 $\log_{10}$ CFU/brush, respectively (Fig. 14A). A similar contamination level and die-off rate ($P > 0.05$) of *L. monocytogenes* were found on waxing brushes used to apply AP-450 wax coating to apples using the same method (Fig. 14A). To evaluate the fate of *L. monocytogenes* on waxing brushes under long-term holding, the waxing brushes used to prepare PF 360-coated apples for the storage study were subjected to 12 weeks of ambient holding. After two weeks, a 1.2 $\log_{10}$ CFU/brush reduction in *L. monocytogenes* was observed, with populations remaining relatively stable at ~3 $\log_{10}$ CFU/brush during the subsequent ten weeks of holding (Fig. 14B).

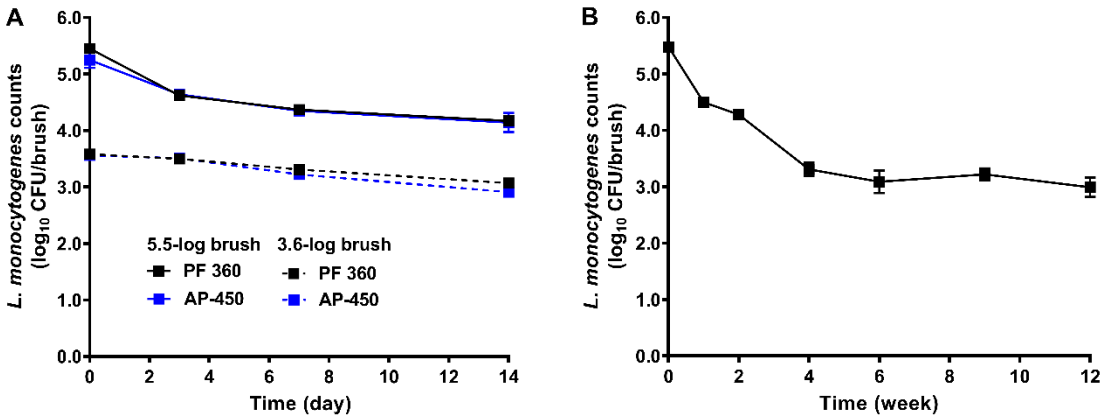


**Figure 14. Persistence of *L. monocytogenes* on waxing brushes during holding at ambient temperature**. (A) 14-day ambient holding of waxing brushes contaminated at low (3.6 $\log_{10}$ CFU/brush) and high (5.5 $\log_{10}$ CFU/brush) levels. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360. (B) 12-week ambient holding of waxing brushes coated with PF 360. Data are presented as mean ± SEM, n = 4-6. Brushes were contaminated with *L. monocytogenes* during the wax

8. Survival of *L. innocua* on different wax-coated apples during commercial cold storages.

The initial *L. innocua* counts on waxed and unwaxed Fuji (Fig. 15A) and Granny Smith (Fig. 15B) apples before storage was 5.6 $\log_{10}$ CFU/apple. During the first 6 weeks of cold storage, *L. innocua* counts decreased by 0.7-0.8 $\log_{10}$ CFU/apple on both unwaxed and wax-coated apples, regardless of the coating types and apple varieties (Fig. 15). Over the following 12 weeks of storage, *L. innocua* on

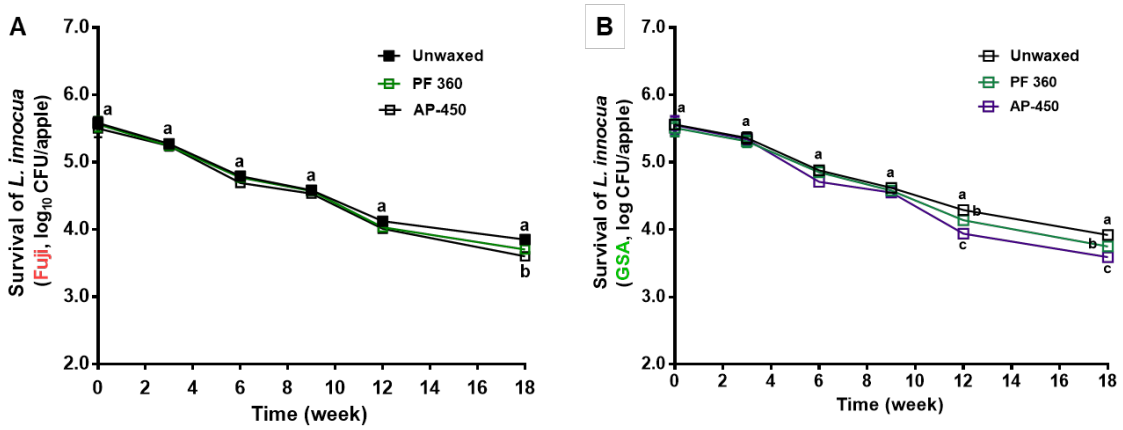


**Figure 15. Fates of *L. innocua* on Fuji (A) and Granny Smith (B) apples coated with or without wax during 18 weeks of commercial refrigerated air storage.** PF 360: PrimaFresh 360 HS; AP 450: Shield-Brite AP 450. Mean ± SEM, n = 40. Different letters (a-c) at each sampling point indicate significant differences ($P < 0.05$).

waxed apples further decreased by 1.8-2.0 $\log_{10}$ CFU/apple (Fig. 15). In summary, *L. innocua* survival was similar on unwaxed and waxed apples; however, wax coatings, regardless of type, slightly enhanced *Listeria* die-off during the 18 weeks of cold storage.

9. Yeast and mold count on wax-coated apples during commercial cold storage

The initial populations of yeasts and molds on Fuji (Fig. 16A) and Granny Smith (Fig. 16B) apples were 4.8-5.0 $\log_{10}$ CFU/apple (Fig. 16). Over 18 weeks of cold storage at 1°C (34°F), these populations gradually increased by 0.3-0.6 $\log_{10}$ CFU/apple (Fig. 16). The application of wax did not impact the survival of yeasts and molds during this period, with similar trends observed for both Fuji and Granny Smith apples. Similarly, a 0.5 $\log_{10}$ CFU/apple increase in yeasts and molds was noted on unwaxed Fuji apples after 24 weeks of storage at 1°C (34°F) (Sheng et al., 2018). Populations of yeast and mold on unwaxed Saltanat apples increased by 0.6-0.9 $\log_{10}$ CFU/cm$^2$ after 5 months of 2 °C (36 °F) storage (Juhneviča, Skudra, & Skudra, 2011).

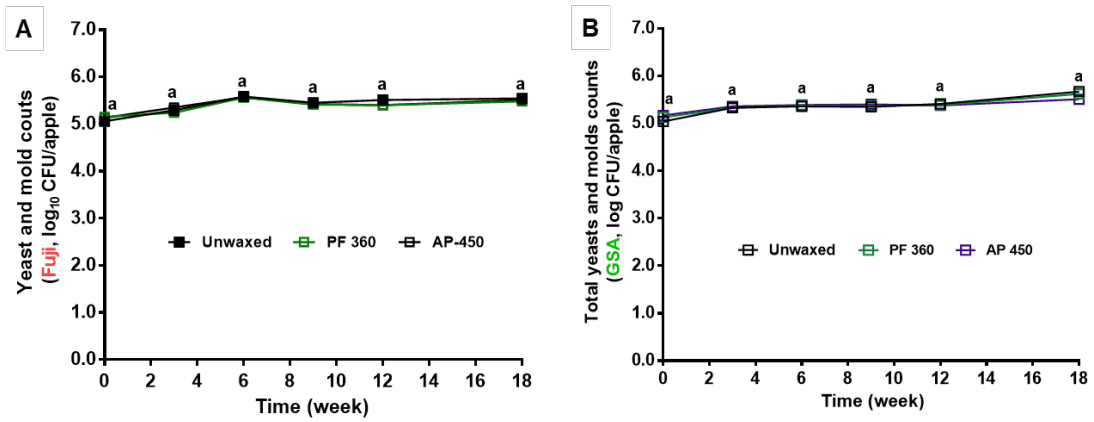


**Figure 16. Yeast and mold counts on Fuji (A) and Granny Smith (B) apples coated with or without wax during 18 weeks of commercial refrigerated air storage.** PF 360: PrimaFresh 360 HS; AP 450: Shield-Brite AP 450. Mean ± SEM, n = 40. Different letters (a-c) at each sampling point indicate significant differences ($P<0.05$).

10. Quality attributes of wax-coated apples during commercial cold storage

Wax coating significantly increased the glossiness of apples, regardless of the type of wax applied. After 18 weeks of commercial storage, the firmness of apples decreased in both waxed and unwaxed treatments, although wax coatings helped reduce the firmness loss in GSA across all coating types (Shen et al., 2025; Su et al., 2023). Total soluble solids (TSS) remained stable in both unwaxed and wax-coated apples during the 18-week cold storage period. Titratable acidity (TA) decreased in both unwaxed and wax-coated Fuji apples, but wax coating helped reduce TA loss in GSA apples (Su et al., 2023). The application of wax coatings, regardless of type, had no impact on interior and exterior disorders in Fuji and GSA, but it significantly reduced internal browning in GSA apples (Shen et al., 2025; Su et al., 2023). In agreement with our findings, 1% beeswax coating suppressed the increase of TSS in Generos, Starkrimson, Idared, and Jonagold apples during 4 months of cold storage at 2 °C (Anghel, 2011). TA decreased in both unwaxed and wax-coated GSA after 18 weeks of cold storage, consistent with our earlier finding on unwaxed Granny Smith apples over 24 weeks cold storage (Sheng et al., 2022).

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## EXECUTIVE SUMMARY

**Project Title:** Fate of *Listeria* on fresh apples as affected by commercial apple waxes

**Key words:** *Listeria monocytogenes*; serotype, apples; wax-coating; drying temperature waxing brush; cross-contamination; commercial storage.

### Abstract

This research examines the fate of *Listeria monocytogenes* on apples contaminated before and during wax coating, its persistence on contaminated waxing brushes, cross-contamination risks, and the serotype-specific survival of *L. monocytogenes* on wax-coated apples. The study further investigates the fate of *Listeria* and resident yeasts and molds on apples treated with commercial wax under cold storage, using *Listeria innocua* as a surrogate. Findings indicate that while wax coatings enhance apple glossiness, help maintain weight, and slightly reduce firmness loss, they offer limited antimicrobial effects against *Listeria*. Specifically, a reduction of about 1.9 $\log_{10}$ CFU/apple was observed over 18 weeks of commercial refrigerated air storage - comparable to that on unwaxed apples. The survival dynamics of *L. monocytogenes* also varied by serotype, with serotype 1/2a exhibiting higher resilience than others, such as serotype 4b, which declined more rapidly. There was also a notable cross-contamination risk among contaminated apples, uncontaminated apples, and waxing brushes during the simulated waxing process. *L. monocytogenes* remained viable on waxing brushes for up to 12 weeks at ambient temperature, with an initial 1.2 $\log_{10}$ CFU/brush reduction in the first two weeks, followed by stable populations at ~3 $\log_{10}$ CFU/brush during the subsequent ten weeks of holding. Additionally, the wax coating did not impact the survival of yeasts and molds on apples, with an increase of 0.4–0.5 $\log_{10}$ CFU/apple observed after 18 weeks of cold storage, regardless of wax type. Including fungicides in the wax coating effectively reduced yeasts and molds on wax-coated apples; however, it did not impact *L. monocytogenes* survival. Overall, this research underscores that while wax coatings offer some benefits for fruit quality, they are insufficient for controlling *Listeria* contamination risks during apple storage and pose a cross-contamination risk during the waxing process. The findings highlight the need for enhanced sanitation strategies and the development of wax coatings with antimicrobial properties.

**Proposal Title:** Ozone nanobubble to control *Listeria* and decay in organic apples

**Report Type:** Continuing Project Report

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**Cooperators:**

Stemilt Growers LLC.; Columbia Fruit Packers Inc.; En Solución technology; Pace International, LLC.

Cooperators from the apple industry will provide in-kind support in the forms of in-kind time commitment, technical input, and apple fruit donation for the proposed studies.

En Solución Technology: En Solución will provide a prototype nanobubbles unit to be integrated with the pilot apple processing facility for the proposed studies, and $10,000 cash donation per year.

Pace International LLC. Pace has helped with the pilot spray-bar brush bed system installation and will provide chemical sanitizers for the proposed studies as needed.

**Project Duration:** 3 Year

**Total Project Request for Year 1 Funding:** $80,990
**Total Project Request for Year 2 Funding:** $83,115
**Total Project Request for Year 3 Funding:** $87,759

**WTFRC Collaborative Costs:**

| Item | 2023-2024 | 2024-2025 | 2025-2026 |
|---|---|---|---|
| Salaries | | | |
| Benefits | | | |
| Wages | $1,983.00 | $2,054.00 | $4,368.00 |
| Benefits | $850.00 | $892.00 | $1,130.00 |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | $250.00 | $275.00 | $300.00 |
| Travel | $350.00 | $375.00 | $400.00 |
| Plot Fees | | | |
| Miscellaneous | | | |
| | | | |
| | | | |
| | | | |
| Total | $3,433.00 | $3,596.00 | $6,198.00 |

**Footnotes:**

**Budget 1**

**Primary PI:** Meijun Zhu
**Organization Name:** Washington State University
**Contract Administrator:** Anastasia Kailyn Mondy
**Telephone:** 509-335-2885
**Contract administrator email address:** arcgrants@wsu.edu

| Item | 2023-2024 | 2024-2025 | 2025-2026 |
|---|---|---|---|
| Salaries | $34,568.00 | $35,951.00 | $37,389.00 |
| Benefits | $8,321.00 | $8,653.00 | $9,000.00 |
| Wages | $6,024.00 | $6,265.00 | $6,516.00 |
| Benefits | $144.00 | $150.00 | $156.00 |
| RCA Room Rental | | | |
| Shipping | | | |
| Supplies | $24,000.00 | $24,000.00 | $24,000.00 |
| Travel | $1,500.00 | $1,500.00 | $1,500.00 |
| Plot Fees | | | |
| Miscellaneous | $3,000.00 | $3,000.00 | $3,000.00 |
| | | | |
| | | | |
| | | | |
| Total | $77,557.00 | $79,519.00 | $81,561.00 |

**Footnotes:**

## OBJECTIVES

1. Evaluate and optimize the efficacy of ozone nanobubbles against *L. monocytogenes* on fresh apples and water, which will be further compared with commonly used sanitizer PAA.
2. Evaluate the efficacy of ozone nanobubble interventions along with PAA against decay organisms on water and apples.
3. Verification of the effectiveness of the ozone nanobubble interventions against *L. monocytogenes* and decay organisms in a pilot-scale apple packing line.

## METHODS

### *1. Strain selection*

A panel of *L. monocytogenes* outbreak strains consisting of serotype 1/2a (an isolate from the 2011 Colorado cantaloupe outbreak, NRRL B-57618), 1/2b (an isolate from the 2011 Colorado cantaloupe outbreak, NRRL B-57623), 4b (an isolate from Bidart Bros. packing facility involved in 2014 California caramel apple outbreak, LS1016,) was used to prepare a 3-strain cocktail inoculum. These strains have been kept in a stock solution of trypticase soy broth supplemented with 0.6% (w/v) yeast extract (TSBYE) and 20% (v/v) glycerol at -80°C until used.

### *2. Apple cultivar selection*

Granny Smith and Gala apples were chosen for the proposed studies.

### *3. Inoculum preparation*

Before inoculation, each strain will be growth-phase synchronized twice in TSBYE broth by consecutively culturing at 37°C for 24h, then pelletized by centrifuging and re-suspended in 0.1% peptone water to achieve the target population density against a standard curve, depending on inoculation level. To prepare a 3-strain *L. monocytogenes* inoculum cocktail, each respective strain suspension will be mixed in equal proportions.

### *4. Apple inoculation*

Apples of the selected varieties without cuts or bruises and harvested at commercial maturity will be individually dip-inoculated to establish $1\times10^6$ CFU/apple of the 3-strain cocktail of *L. monocytogenes* per our well-established method (Shen et al., 2021; Sheng et al., 2018; Sheng et al., 2020b). The inoculated apples will be held at 22 °C for 48h before ozone nanobubble or PAA treatments.

### *5. Ozone nanobubbles solution preparations*

$O_3$-containing nanobubbles (ONB) were generated from an $O_3$ nanobubble generator (En Solucion, Austin, TX). The pH of ONB was adjusted to 3.0, 3.5, 3.7 or 4.0 using different acidulant including Nature's Shield 440-BF, lactic acid, malic acid, acetic acid, and hydrochloric acid (HCl). All solutions were used for intervention treatments immediately after preparation. The concentration of the ONB solutions was monitored using a dissolved $O_3$ sensor (Analytical Technology, Collegeville).

The pH and oxygen reduction potential (ORP, mV) of ONB solutions were recorded with Orion 8302BNUMD ROSS Ultra pH/ATC Triode and Orion 9678BNWP electrode, respectively, connected to a digital multi-parameter electrochemistry meter.

### *6. Ozone nanobubbles intervention and impact of the volume of washing system*

Inoculated apples (48h post-inoculation) were subjected to 3L ONB solutions for different contact times (30 -120 sec). Tap water and an 80 ppm peroxyacetic acid (PAA) wash served as negative and positive control, respectively. To evaluate the effect of wash solution volume on ONB efficacy, a 15 L

spray wash system was also included for comparison. Each experiment was conducted independently in triplicate.

### *7. Survival microorganism analysis*

At the end of each intervention, each apple will be individually transferred to a Whirl-Pak bag with 10 ml of neutralizing buffer and hand-rubbed for 80 s to detach *L. monocytogenes*. The detached microbial suspension of each apple post-sanitizer treatment will be serially diluted. Appropriate dilutions will be plated on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) plates overlaid with modified Oxford agar per our established method (Shen et al., 2019; Sheng et al., 2020a). All plates will be incubated at 35˚C for 48 h and enumerated. If survival of *Listeria* on apple fruit is below the enumerative detection limit, the suspension will be enumerated for Presence/Absence after 48h of enrichment in Buffered *Listeria* Enrichment Broth (BLEB) and streaked onto a selective *Listeria* agar plate. Presumptive positive colonies will be further confirmed by PCR (FDA, 2015).

## RESULTS AND DISCUSSIONS

1. The effectiveness of ONB against *L. monocytogenes* on apple surfaces

The initial inoculation level of *Listeria* spp. on apple surfaces was 6.0 - 6.4 $\log_{10}$ CFU/apple. The application of ONB at 4 ppm $O_3$ reduced *L. monocytogenes* on apple surface. However, the efficacy observed was relatively limited, a 2 min 4 ppm $O_3$ wash resulting in ~ 0.5 $\log_{10}$ CFU/apple reduction in 3-L dip-wash system, compared to 1.7 $\log_{10}$ CFU/apple resulted from 80 ppm PAA (Fig. 1). The concentrations of ONB solutions depleted by 4.0, 12.6, 28.0, 56.6 % after 2, 5, 10, and 30 min holding, respectively.

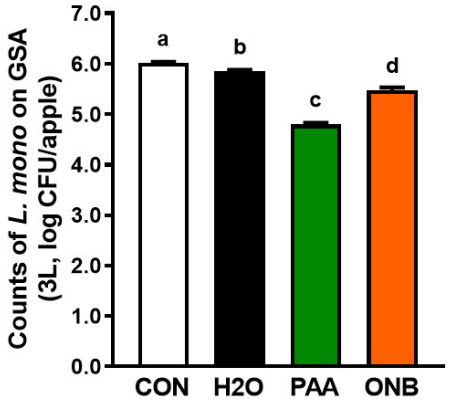


**Fig. 1.** Efficacy of 4 ppm ozone-containing nanobubbles (ONB) against *L. monocytogenes* on Granny Smith apples for 2-min contact in a 3L washing system. Mean ± SEM, n = 30. CON, inoculated apples without wash; H2O, tap water wash; PAA, 80 ppm peroxyacetic acid.

2. The incorporation of acidulants improved ONB efficacy against *L. monocytogenes*

To optimize and improve the efficacy of ONB solution, a commercial acidulant, NS440, was used in combination with ONB, which significantly improved the effectiveness of ONB on apples ($P < 0.05$, Fig. 2). A 2 min wash with 4 ppm ONB containing 15 - 30 and 60 - 200 ppm NS440, respectively, resulted in the reductions of *L. monocytogenes* by 0.8 - 1.2 and 1.5 - 1.6 $\log_{10}$ CFU/apple on apples (Fig. 2).

Similarly, applying ONB at 4 ppm with citric acid (CA), lactic acid (LA), malic acid (MA), acetic acid (AA), and HCl significantly enhanced its efficacy, reducing *L. monocytogenes* on apples by 1.3, 1.3, 1.4, 2.0, and 1.0 $\log_{10}$ CFU/apple, respectively (Fig. 3). Additionally, combining NS440, CA, and AA increased the ORP of ONB solutions, while the incorporation of LA and MA decreased due to the faster depletion of $O_3$.

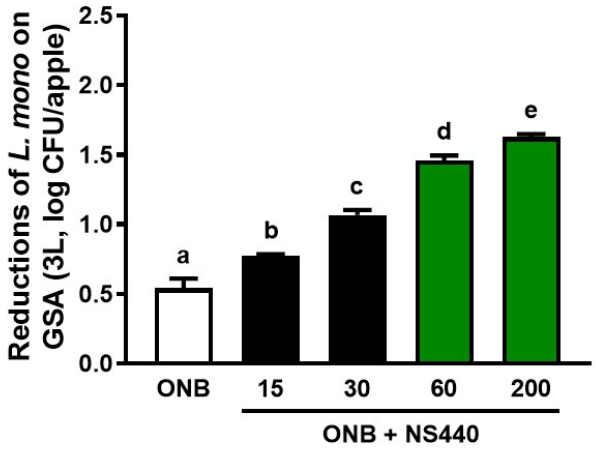


**Fig. 2.** Efficacy of 4 ppm ONB) in combination with 15-200 ppm Nature's Shield 440-BF (NS440) against L. monocytogenes on Granny Smith apples (GSA) for 2-min contact in a 3L washing system. Mean ± SEM, n = 30. ONB: 4ppm.

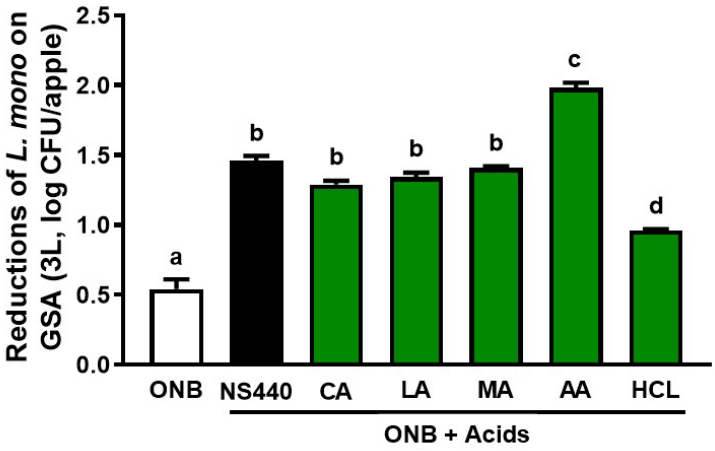


**Fig. 3. Efficacy of 4 ppm ONB with different acidulants against *L. monocytogenes* on Granny Smith apples (GSA).** NS440, Nature's Shield 440-BF; CA, citric acid; LA, lactic acid; MA, malic acid; AA, acetic acid, HCL, hydrochloric acid. All acidulants were applied to achieve an equivalent pH as NS440. Contact time: 2 min in 3L system. Mean ± SEM, n = 30.

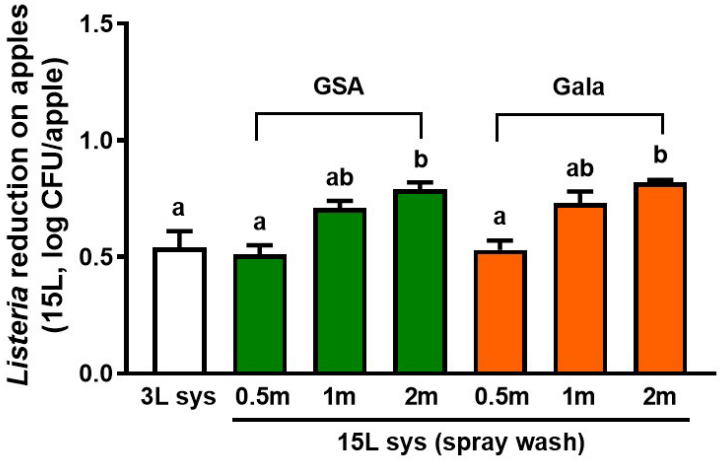


**Fig. 4. Effect of wash system volume on the efficacy of 4 ppm of ONB solution in reducing *L. innocua* populations on selected apple varieties. 0.5m-2m: contact time of 0.5 to 2 min.** Contact time for 3L system is 2 min. Mean ± SEM, n = 30.

3. Optimized efficacy of ONB with or without acidulants in spray-wash system

To optimize the ONB solution, the impact of wash system volume on its efficacy was evaluated. Increasing the system volume from 3L to 15L, combined with a spray-wash approach, significantly enhanced the efficacy of ONB on apples. A 2-min wash with ONB containing 4 ppm $O_3$ in the 15L reduced *L. innocua* by 0.8 $\log_{10}$ CFU/apple on Granny Smith apples, compared to 0.5 $\log_{10}$ CFU/apple reduction in 3L system. Similar reductions were also observed for Gala apples (Fig. 4). Reducing the contact time from 2 min to 1 min slightly decreased ONB's anti-*Listeria* effectiveness on apples, with a further reduction observed at 0.5-min contact (Fig. 4).

The effectiveness of ONB combined with selected organic acidulants was further tested in the 15L spray-wash system. The addition of CA and LA to a 4 ppm ONB solution significantly enhanced anti-*Listeria* efficacy compared to ONB alone 15 L system. A 2-min spray-wash in 15L system reduced *L. innocua* on Granny Smith apples by 2.1 $\log_{10}$ CFU/apple (Fig. 5). However, the efficacy diminished when the contact time was reduced from 2 min to 0.5 min (Fig. 5).

In summary, ONB alone demonstrates limited efficacy against *Listeria* on apples. Increasing the wash system volume and incorporating acidulants significantly enhanced its effectiveness. Further optimization efforts are ongoing.

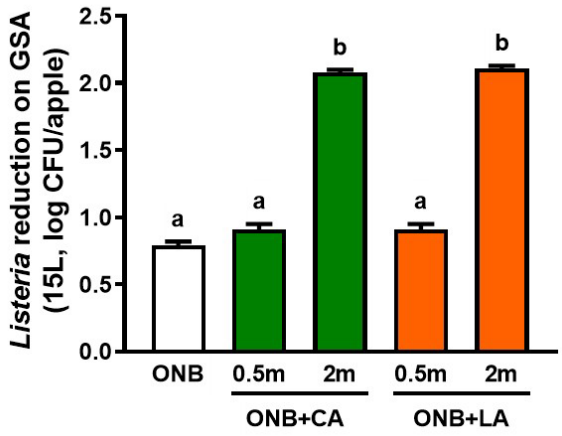


**Fig. 5. Efficacy of 4 ppm ozone-containing nanobubbles (ONB) against *L. innocua* on Granny Smith apples for 0.5- and 2-min contact in a 15L spray-wash system.** CA, citric acid; LA, lactic acid. Mean ± SEM, n = 30.

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Shen, X., Su, Y., Hua, Z., Sheng, L., Mendoza, M., He, Y., Green, T., Hanrahan, I., Blakey, R., Zhu, M.J., 2021. Effectiveness of low-dose continuous gaseous ozone in controlling *Listeria innocua* on Red Delicious apples during 9-month commercial cold storage. Frontiers in microbiology 12, 712757.

Sheng, L., Hanrahan, I., Sun, X., Taylor, M.H., Mendoza, M., Zhu, M.J., 2018. Survival of *Listeria innocua* on Fuji apples under commercial cold storage with or without low dose continuous ozone gaseous. Food microbiology 76, 21-28.

Sheng, L., Shen, X., Ulloa, O., Suslow, T., Hanrahan, I., Zhu, M.J., 2020a. Evaluation of JC9450 and neutral electrolyzed water in controlling *Listeria monocytogenes* on fresh apples and preventing cross-contamination. Frontiers in microbiology 10, 3128.

Sheng, L., Shen, X., Zhu, M.J., 2020b. Screening of non-pathogenic surrogates of *Listeria monocytogenes* applicable for chemical sanitizer interventions of fresh apples. Food Control 110, 106977.

**Project Title:** Finding peel surface properties linked with green spot of WA 38

**Report Type:** Final Project Report

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**Cooperators/Collaborators**: Bruno Carra (WSU)

**Project Duration:** 1-Year

**Total Project Request for Year 1 Funding:** $ 4,000

**Other related/associated funding sources: The endowment fund of the Musacchi program's supports this research**
**Funding Duration:** 2024

**Budget 1**

**Primary PI:** Stefano Musacchi
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**Contract administrator email address:** kevin.rimes@wsu.edu
**Station Manager/Supervisor:** Darla Ewald
**Station manager/supervisor email address:** dewald@wsu.edu

| Item | 2024 |
|---|---|
| Salaries | |
| Benefits | |
| Wages | |
| Benefits | |
| RCA Room Rental | |
| Shipping | |
| Supplies | $4,000.00 |
| Travel | |
| Plot Fees | |
| Miscellaneous | |
| | |
| | |
| | |
| Total | $4,000.00 |

**RE-CAP OBJECTIVES:**

**1. Cuticle deposition during development in WA 38 and the parent cultivars Honeycrisp and Enterprise. Fruit growth will be monitored.**

**2. Development of lenticels and their extent of opening. Cross-sections of the fruit's skin will be taken to monitor the development of epidermis and hypodermis in symptomatic and asymptomatic fruit.**

**SIGNIFICANT FINDINGS:**

1. ***Cuticle deposition during development in 'WA 38' and the parent cultivars 'Honeycrisp' and 'Enterprise'.***
   - A new stage of "WA 38" green spot development was identified during the sampling of the cuticle throughout the season. We named "green halo" first observed on 6/4/2024 = 47 DAFB.
   - Across 18 weeks, we followed the apple growth and noticed some differences among varieties.

***2. Development of lenticels and their extent of opening.***

- Fruit growth in the green spot region (GS) is inhibited, as indicated by slight depressions of the fruit surface in those regions where green spots form. This confirms the results of the previous project, and GS seems to be likely linked to the lenticel damage.

- Bags confirmed the results from previous studies. Early bagged fruit did not develop signs of "Severe" green spot, while the control showed 29.7 % of “Severe” symptoms (culled apples).
- No significant differences were found in the incidence of GS within each bearing wood.
- Lenticels in “bag” apples presented a higher AO infiltrated area than the "control" ones on 98 and 155 DAFB.

## RESULTS AND DISCUSSION

### 1. Cuticle deposition during development in ‘WA 38’ and the parent cultivars ‘Honeycrisp’ and ‘Enterprise’.

During season 2024, we focused on investigating the cuticle deposition throughout fruit development for 'WA 38' and the parents, 'Enterprise' and 'Honeycrisp'. On 5/20/2024 (32 days after full bloom =DAFB for 'WA 38' and 'Honeycrisp', and 37 DAFB for 'Enterprise'), we started the fruitlets sampling of all 3 cultivars; the selection criteria accounted for both east and west side of the trees, all bearing positions in the canopy, and both king and lateral types of fruit. For 'WA 38', the sampling was weekly from 5/20/2024 to 7/30/2024, then became biweekly (twice a month), while for 'Enterprise' and 'Honeycrisp', was done twice monthly.

Once in the laboratory, apples were photographed on graph paper, weighted and described for any misshapenness, blush, presence/absence of green spot for every timepoint. Epidermal peel disks (8 to 10 mm punch, depending on the stage of development) were excised from each apple in the equatorial area (2 disks/apples up to 7/2/24 = 75 DAFB, then 3 disks/apples until harvest on 9/25/2024 = 160 DAFB) and placed into scintillation vials with an enzyme solution containing both pectinase and cellulase, sodium azide and citric acid as described in Lai et al., 2016 and kept at room temperature until further analysis. The punctured apples were saved and subjected to allometric measurements (included maximum diameter) to define the growth of the different areas of the fruit along the season (Skene, 1966).

For the three cultivars, we built calibration curves utilizing the "detached" apples for the cuticle deposition sampling, where fruit weight resulted in a polynomial function of the apple diameter. The $R^2$ were 0.9871, 0.9812, and 0.9885 for 'Enterprise', 'Honeycrisp', and 'WA 38' (Figure 1), respectively.

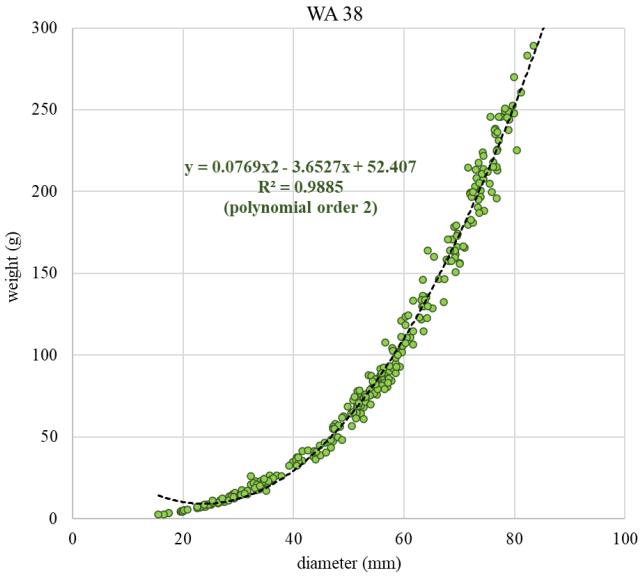


***Figure 1:*** *'WA 38' relationship between fruit weight and its diameter for data collected in 2024 at the Sunrise Farm (WSU).*

At the first measurement on 5/20/2024, when the fruit diameter range across the 3 cultivars was 15 to 26 mm, another set of 20 east-facing apples per cultivar were tagged "on-tree" (Figure 2) and photographed weekly with the same angle settings to track the fruit growth. Post-processing the fruit maximum diameter measured from the images (N=10 best apple captures) allowed us to quantify the apple growth and present it as millimeters per day (mm/day, Figure 3). The growth chart presents weekly fruit development as each cultivar's growth speed. This parameter was compared across varieties to assess the hypothesis that there could be a difference in 'WA 38' growth compared to the two parents.


***Figure 2:*** *'WA 38' and its parents pictured on 7/9/2024 in the weekly photographs to track the fruit dimensions and growth in 2024 at the Sunrise Farm (WSU).*

Across 18 weeks, the apple growth showed some differences that we are investigating (Figure 3). During the sampling for the cuticle development throughout the season, we were able to identify a new stage for 'WA 38' green spot development that we named "green halo" first observed on 6/4/2024 = 47 DAFB (avg. 31 mm max diameter) and characterized by a darker green ring around lenticels mainly in the shoulder and equatorial area of the apples (not shown).

Another experiment was carried out in 2024 to quantify the stress and strain relaxation by apple region and developmental stage for all three cultivars. It is well known in literature that, during their development, apples undergo a series of stress events that cause strain of their cuticle (Knoche and Lang, 2017, Knoche et al., 2018). The hypothesis behind this experiment was that the green spot (GS) onset could be related to a "skin strain event" during the apple development that causes irreversible damage to the cuticle, potentially impacting skin and cortex integrity and jeopardizing marketability. The reason for investigating by region relies upon the fact that GS is more frequent in the shoulder and equatorial areas of the apple than in the calyx. The first timepoint was at approximately 40 mm apple diameter (6/18/2024 = 61 DAFB); at that time, one 8-mm-disk of cuticle per each region was excised from each apple (blushed side) and placed into the enzyme solution described above; the three regions in the apple were "shoulder (S)", "equator (E)", and "calyx (C)". The total fruit per sampling point were 25. For the second time point, at approximately 55 mm fruit size (7/16/2024 = 89 DAFB), the number of regions of interest increased from 3 to 5. In fact, intermediate regions were included and named "shoulder-to-equator (S-E)" and "equator-to-calyx (E-C)". Sampling was completed "at harvest" for each cultivar: 9/10/24 for 'Honeycrisp' (145 DAFB) and 9/24/24 for 'WA 38' and 'Enterprise' (159 DAFB for 'WA 38'). Those ongoing biophysical analyses can eventually provide valuable insights into the cuticle properties of the three cultivars under study and their potential role in GS onset.

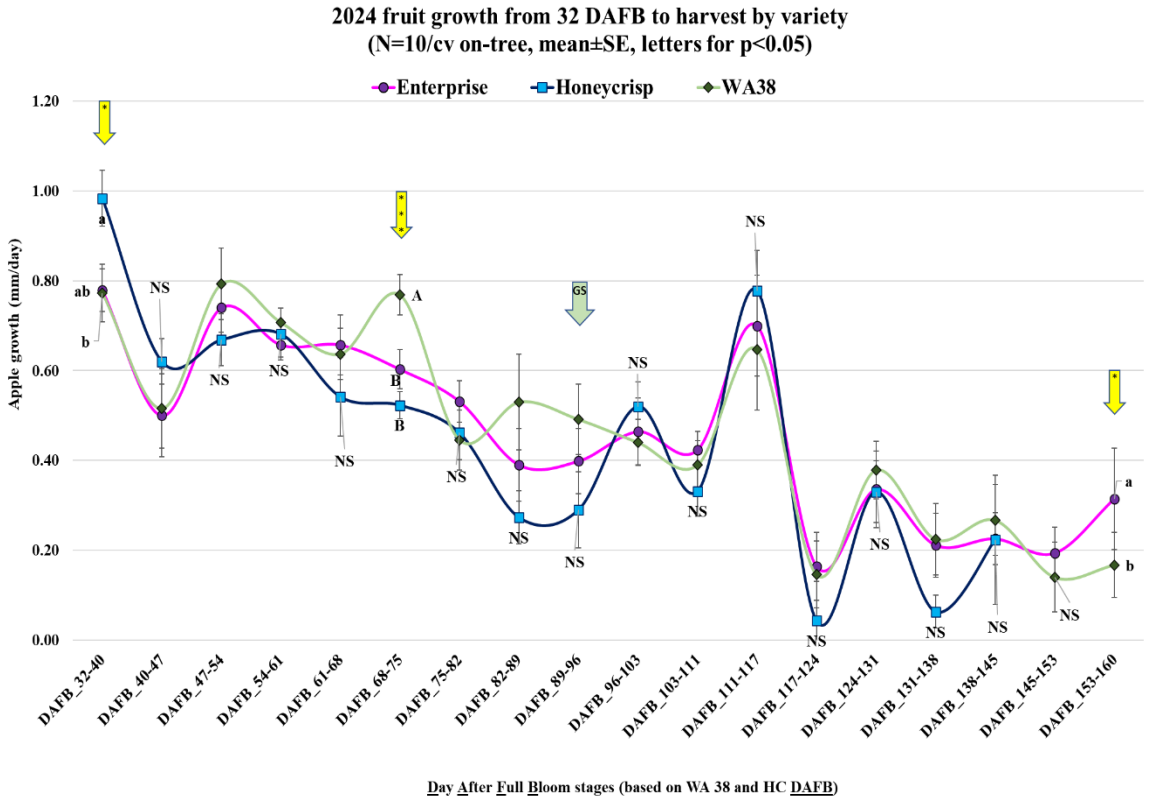


***Figure 3:*** *'WA 38', 'Enterprise', 'Honeycrisp' on trees apples photographed weekly from May to September 2024 at the Sunrise Farm (WSU). The maximum fruit diameter measured from the images (N=10 best apple captured) was used to calculate the apple growth (millimeter per day = mm/day). The three yellow arrows pointing down indicate the 3 weeks in the season when the apple growth between the three cultivars resulted significantly different. Significance: * <0.05, *** <0.001, NS = not significant; same letter associate to the means indicate no difference. The green arrow marked with "GS" indicated the onset of green spot on 'WA 38' in 2024 in the experimental site. Error bars represent the standard error of the means.*

**2. Development of lenticels and their extent of opening. Cross-sections of the fruit's skin will be taken to monitor the development of epidermis and hypodermis in symptomatic and asymptomatic fruit.**

Preliminary results on 'WA 38' symptomatic and asymptomatic apples in 2023 provided some leading clues that helped direct our research for 2024. Fruit growth in the green spot region (GS) is inhibited, as indexed by slight depressions of the fruit surface in those regions where green spots form. Preliminary data indicates that cuticle mass is higher within these green spot regions (22.4 ± 0.3 g $m^{-2}$) as compared to non-affected control areas (20.3 ± 0.2 g $m^{-2}$). This is primarily due to increased wax deposition (9.0 ± 0.2 within GS vs. 6.9 ± 0.2 g $m^{-2}$ control), while cutin deposition appears unaffected (13.4 ± 0.2 within GS vs. 13.4 ± 0.2 g $m^{-2}$ control). Non-affected 'WA 38' fruit skin does not show any irregularities in cutin or wax deposition compared to other cultivars. The mass of cuticle per unit area in these non-affected areas is within the range normally encountered in apple varieties (range 20.3 ± 0.3 to 36.2 ± 0.9 g $m^{-2}$; grand mean of 22 apple varieties 26.7 ± 0.3 g $m^{-2}$; Khanal et al., 2013). The

occurrence of cracks in severe green spot symptomatic peel is indicative of increased mechanical stress. This is consistent with increased strain release upon wax extraction in the green spot affected area. That wax inhibits strain relaxation, a typical strategy in apple to avoid surface defects.

In Sheick et al., 2022, we had already observed that 'WA 38' lenticels were frequently at the center of the green spot symptoms, but not always. Lenticels are reported to be "weak points for mechanical stress as fruit enlarges," as reported by Duríc et al., 2015. In this objective, we focused on the development of lenticel and its involvement in the evolution of GS. On 6/5/2024, twenty 'WA 38'/G41 trees trained to spindle were selected for a similar amount of fruit and vigor by visual estimation. Ten of those twenty trees (randomized across 5 orchard rows) were tagged as "bag" trees and each cluster was hand thinned down to the strongest fruitlets and then bagged as described in Sheick et al., 2022. For each "bag" tree, at least 5 apples were left outside bags to account for an "internal control" and the average of bags/tree was 61 ranging from 47 to 95 bag/tree. The other ten trees represented the "control" and were handled by hand thinning like the "bag" trees, leaving only the strongest fruitlet in each cluster. The average apples/tree at that time was 66, ranging from 38 to 103 apples/tree across the 10 trees. During the growing season, we utilized those trees as source of fruit for sampling at 3 key developmental timepoints (7/3/24 = 76 DAFB = 50-55 mm, 7/25/2024 = 98 DAFB = 58-60 mm, and 9/20/24 at harvest = 155 DAFB = >73 mm) to compare lenticels development and microcracking between "bag" apples and "control" apples after 10-min-staining in 0.1 % (w/v) aqueous Acridine Orange (AO) + 0.05% Silwett® L-77 solution. Ten-mm-stained disks were visualized at the Nikon SMZ18 stereomicroscope under visible light and under incident fluorescence light (using a P2-EFLC EGFP LP HC filter, emission 515-555 nm) and photographed to calculate the lenticel density and the AO infiltrated area as $mm^2$ per lenticel (Figure 4). This experiment can make us postulate that there is possibly less mechanical stress in the skin of bagged fruit (for unknown reasons) and less need for wax deposition (data not shown).

At harvest (9/23/2024), three "bag" trees and three "control" trees were picked, keeping apples separated accordingly to the different bearing woods and classifying them as "spur", "brindilla", and "ramo misto". The apples were stored at 34 °F and graded approximately one month after harvest; data are reported as presented in Sheick et al., 2022 (Figure 5). When comparing the apples harvested from "bag" trees with those from the "control" trees, we confirmed the results from previous studies (Sheick et al., 2022) where apples, if bagged early enough in the season (beginning of June), did not develop any signs of "Severe" green spot (green spot stages 3, 4 or 5 that leads to unmarketable apples). On the other hand, the 29.7 % of the "control" apples developed severe symptoms (culled apples).

When looking at the incidence of "Mild" green spot (green spot 1, 2, or 6 affecting below 5% of apple surface) between "bag" and "control" apples, the former presented just a green halo (GH) around the lenticels (classified as green spot stage 1) in 30.1 % of the apples, while the latter developed higher GS incidence of GS equal to 62.5 % of graded apples (Figure 5). The presence of GH in the "bag" apples can be explained by the fact we observed the onset of GH around lenticels on 6/4/2024 before carrying out the bagging procedure for the whole experiment; therefore, we can state that GH did not evolve in any possible worsen stages once apples were bagged for over 15 weeks. We noticed that green spots symptoms appeared earlier in 2024, and this can explain why some GH has been observed in bagged fruit, too.

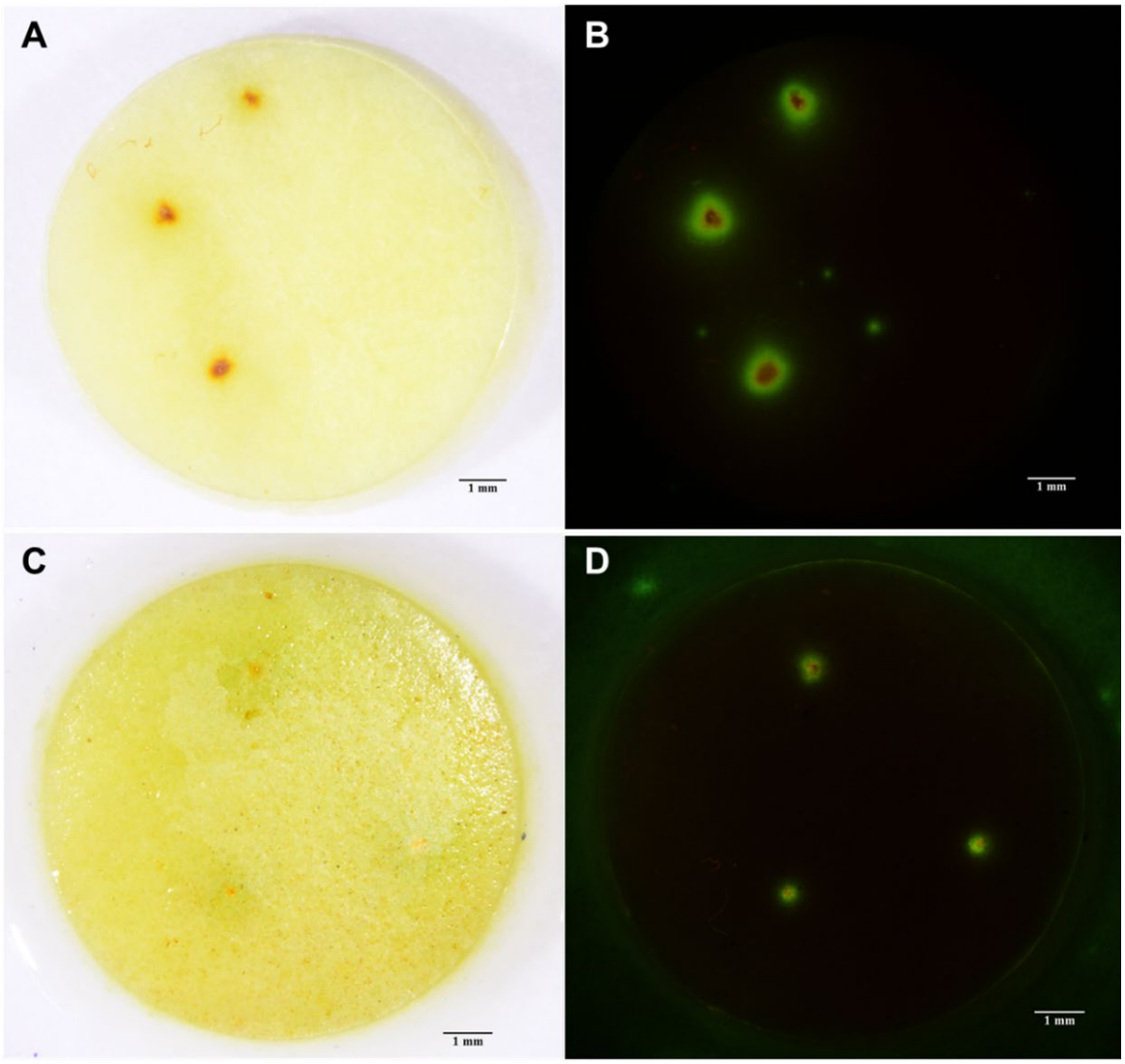


***Figure 4:*** *Visible and fluorescent disks (epidermis) after Acridine orange (AO) staining at timepoint 2 (07/25/2024) of "bag" (A and B) and "control" (C and D) 'WA 38' apples. Disks are 10 mm in diameter, the magnification of the microscope was 0.75X for all the images. Analog gain used was 1.0X for visible (no fluorescence) and 14.0X for fluorescence light (green) both with an exposure of 30ms. (A) 10 mm disk of "bag" fruit after AO staining in visible light, and (B) in emission fluorescence green light showing the respective AO infiltrated lenticels. (C)10 mm disk of "control" fruit after AO staining in visible light, and (D) in fluorescence (green) light showing the respective AO infiltrated lenticels. White and black scale bars represent 1 mm in each respective image.*

The 2024 graded production (left on trees after the abovementioned samplings) was distributed mainly on spurs (84.5% in the "control" trees and 88.7% in the "bag" trees), followed by brindilla (11.7% in the "control" trees and 6.3% in the "bag") and ramo misto (3.8% in the "control" trees and 5% in the "bag" trees). No significant differences were found in the incidence of GS within each type of bearing wood, nor were any specific trends observed (data not shown).

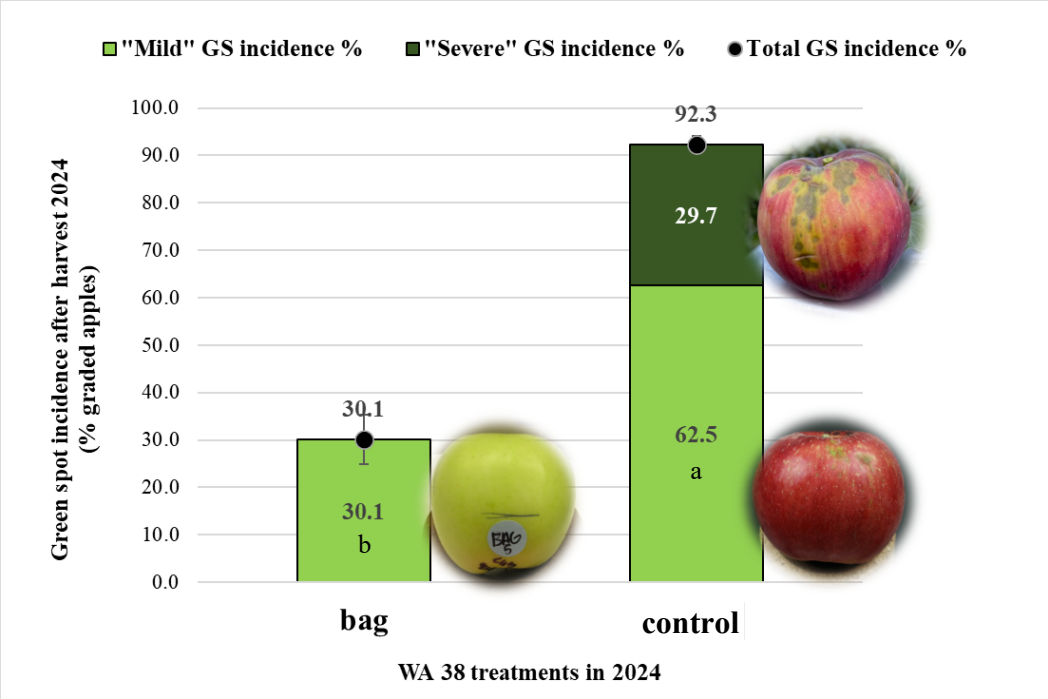


***Figure 5:*** *'WA 38' Green spot incidence after harvest by "bag" versus "control" treatments in 2024. Light green shaded bars represent the proportion of "mild" GS, and dark green shaded bars represent the proportion of "severe" GS over the total incidence. Total incidence (%) in each treatment combination is reported above each bar and indicated by a round black marker with an associated error bar representing the standard error. Separation of means are indicated by lowercase italics letters for significance of p< 0.05. On the right side of each column bar the corresponding example of GS grading is reported. For more details consult Sheick et al., 2022.*

## References:


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## EXECUTIVE SUMMARY

**Project Title**: Finding peel surface properties linked with green spot of 'WA 38'

***Keywords*:** green spot, bags, lenticel, acridine orange, wax, cutin.

**Abstract:**
Our research focused on two main objectives: understanding the role of cuticle deposition during the development of 'WA 38' apples compared with the two parents ('Enterprise' and 'Honeycrisp') and studying the development of lenticels. To study 'WA 38' green spot (GS), we utilized trees grafted on G.41 that, in previous trials carried out at Sunrise farm (SRO) showed a higher percentage of fruit affected by "Severe" GS than Nic29. This tendency has also been confirmed in 2024.
We better defined the onset of Green Spot (GS) and we were able to determine a new early symptom of Green Spot. We named it "green halo", this symptom was first observed on 6/4/2024 = 47 DAFB. Furthermore, we noticed three differences in apple growth among varieties across the 18 weeks we collected measures. Fruit growth in the green spot region (GS) is inhibited, as indicated by slight depressions of the fruit surface in those regions where green spots form. This confirms the results of the previous project. Preliminary results showed that cuticle mass per unit area is higher within green spot areas.
The results from previous studies about "bag" apples were confirmed, in particular, in 2024 the early bagged fruits did not develop signs of "Severe" green spot, while the "control" apples showed 29.7 % of "Severe" symptoms (culled apples). No significant differences were found in the incidence of GS within each bearing wood. Lenticels in "bag" fruit presented a higher Acridine Orange (AO) infiltrated area than the "control" ones at 98 and 155 DAFB.

## PROJECT OUTCOMES

*Manuscript:*

- A manuscript explaining the difference in the cuticle and a possible theory on the development of green spot will be written as soon as possible (beginning of 2025), and it will be considered part of this report.

# **Project Title:** Improving Apple Fruit Quality and Postharvest Performance

**Report Type:** Continuing Project Report

**Primary PI: Manoella Mendoza**
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**Cooperators**: Peter Balk (Wageningen University and Research), Luca Galatro and Raffaele Romano (Vertigo Technologies), Loren Honaas (USDA – ARS). Dave Rudell (USDA - ARS), and Stemilt.

**Project Duration:** 2-Year

**Total Project Request for Year 1 Funding:** $ 36.661
**Total Project Request for Year 2 Funding:** $ 39,802
**Total Project Request for Year 3 Funding:** $

**Other related/associated funding sources:** in-kind contributions = $30K.
**Notes:** Stemilt conducts the application of SmartFresh and donates and applies postharvest fungicides. The WA 38 apples are harvested from two Stemilt blocks. Vertigo-Tech provided the microwave sensor prototype. USDA is providing CA storage (chambers). The industry donates other miscellaneous supplies (trays, boxes, bags, etc.). The Dutch government, via Orchard of the Future collaboration: "Developing bilateral innovation projects (BIS) for technology development and application," provides funding for Vertigo Tech (traveling, machine shipping).

**Primary PI:** Manoella Mendoza
**Organization Name:** WA Tree Fruit Research Commission
**Contract Administrator:** Paige Beuhler
**Telephone:** (509) 665-8251
**Contract administrator email address:** paigeb@treefruitresearch.com
**Station Manager/Supervisor:**
**Station manager/supervisor email address:**

**Budget**

| Item | 2023 | 2024 |
|---|---|---|
| Salaries | | |
| Benefits | | |
| Wages | $24,802.00 | $27,011.00 |
| Benefits | $10,629.00 | $11,576.00 |
| RCA Room Rental | $1,180.00 | $1,215.00 |
| Shipping | | |
| Supplies | | |
| Travel | | |
| Plot Fees | | |
| Miscellaneous | | |
| | | |
| | | |
| | | |
| Total | $36,611.00 | $39,802.00 |

**Footnotes:** Wages/Benefits: calculated based on expected staff wage adjustments.

## Justification

One of the WTFRC internal program targets is to tackle high-priority industry needs that are not covered elsewhere. The Commission has used this project structure for several years, which enables us to respond swiftly to significant industry topics related to apple quality and postharvest issues. This includes the development of variety-specific starch scales (Honeycrisp, WA 38), testing various methodologies (e.g., bitter pit prediction methods for Honeycrisp), products (e.g., NSure sampling kit, Accuvin malic acid test), and equipment (e.g., Felix F750, and DA meter).

This project structure is also beneficial to decreasing required funding, as harvest and quality analysis for multiple objectives are executed concomitantly. In this funding cycle, the WA 38 was used for the starch degradation and the Fresco testing. Additionally, a new objective was added, and data was collected for the Granny software (WA 38, Honeycrisp, and Gala) at no additional cost, as it was integrated into the Fresco testing protocol. Granny Smith apples were also harvested this season and used for testing the Fresco and the Granny Software. The outcomes of this project lead to straightforward, directly actionable results benefitting both organic and conventional growers, regardless of the size or scale of their operations.

## Objectives

1. Evaluate new technologies to assess fruit quality parameters
    a. Testing Fresco (microwave sensor) to measure apple quality parameters non-destructively
    b. **(New)** Collaborate with Honaas lab to test the Granny software (image-based analysis) for starch degradation
2. Investigate the effect of 1-MCP on WA 38 starch degradation during RA and CA storage
3. Assess the influence of 1-MCP on WA 38 fruit flavor

**Significant Findings**

*Fresco Microwave Sensor*

- Wavelengths correlated with firmness, soluble solids, and titratable acidity were identified in the microwave spectra region
- Positive correlation of soluble solids, firmness, and titratable acidity are weak, moderate, and strong, respectively, when predicting quality parameters for a randomly selected set of apples, unseen by the model
- Soluble solids prediction was affected by the narrow distribution range, but the mean absolute percent error (MAPE) is low, indicating that the error between prediction and observed value is low.
- The best-fitting model was for titratable acidity, with a prediction power of about 70%, with more accurate predictions for the independent set of values than that achieved by the training set.

*WA 38 Research*

- WA 38 starch clearance rate and variability are influenced by tree age, with fruit from mature trees displaying a more homogeneous starch degradation compared to fruit from young trees
- Fruit from young trees (2 and 3 years) might need additional time in storage to achieve 90% of the fruit with a starch clearance of 5.0 required for packing and shipping
- 1-MCP treatment and CA storage did not impact the starch degradation rate
- 1-MCP had an adverse effect on the flavor of fruit harvested from mature trees in 2023, while fruit from mature trees that were not treated with 1-MCP was preferred among all tree age and treatment combinations

1. Evaluation of new technologies

**a. <u>Fresco microwave sensor</u>**

**Methods**

Fresco is a new sensor developed by Vertigo Tech, a startup, spin-off of Delft University of Technology in the Netherlands, and part of the Orchard of the Future Dutch-USA collaboration. The sensor is a hand-held device that uses low-energy microwaves to non-destructively measure fresh fruit quality parameters such as Brix, titratable acidity, firmness, juiciness, dry matter, and browning. One differential is that microwaves penetrate deeper into the fruit flesh in comparison with, for instance, NIR sensing. The sensor is in the pilot stage, with prototypes tested by Vertigo Technologies and the Wageningen University in the Netherlands (part of the Next Fruit 4.0 Cool data) and the WTFRC.

In 2023, one bin each of Gala, Honeycrisp, and WA 38 was harvested at commercial maturity and treated with postharvest fungicide before storage. The WA 38 and Gala were stored in a Stemilt RCA room (RA, 33°F), and Honeycrisp was conditioned and stored in the WTFRC cold room at 37°F. Fruit was not treated with 1-MCP. A total of 700 apples per variety were sampled during four sessions, from January to April. The apples were taken out of storage the day before quality assessment, numbered, placed in trays, and left at room temperature for one day. Each apple was processed individually.

In this first phase, we collected data for three apple quality parameters: firmness (lb.), soluble solids content (% Brix), and titratable acidity (% m.a.). The goal is to use the data to pinpoint the wavelength associated with each quality parameter and test the accuracy of the prediction by comparing the results of the destructive measurements with the values predicted by Fresco. The Fresco measurements were taken on the sun-exposed and shaded sides of each apple, at the same location where firmness was measured with the Fruit Texture Analyzer. The Brix and titratable acidity were measured from the apple juice made with the sun-exposed and shaded pieces. The Felix 750 produce quality meter was used to collect non-destructive Brix values, and the results will be compared with the Brix predictions provided by Fresco. Peter Balk at Wageningen University is evaluating the same quality parameters as the WTFRC for two apple varieties (Gala and Elstar), with the addition of dry matter assessment.

A partial least square (PLS) analysis was conducted to appraise the correlation between prediction (non-destructive) and destructive methods and to assess the accuracy of the non-destructive method. The regression model was validated using a K-fold and a Train/Test split. A 10-fold cross-validation performs the fitting procedure ten times, with each fit being performed on a training set consisting of 90% of the total training set selected at random, with the remaining 10% used as a "hold-out" set for validation. When using Train/Test split, part of the data is not used in training but only as a test set, unseen by the model.

## Results and discussion

Figure 1 shows the data distribution of soluble solids (A), firmness (B), and titratable acidity (C) assessed via destructive methods. The overall range of variability between time points is low, especially for soluble solids. This range restriction can affect the correlation coefficient. Titratable acidity had the highest variability among apple varieties, but the average mean values of each session are similar, except for WA 38 and Gala First's time points.

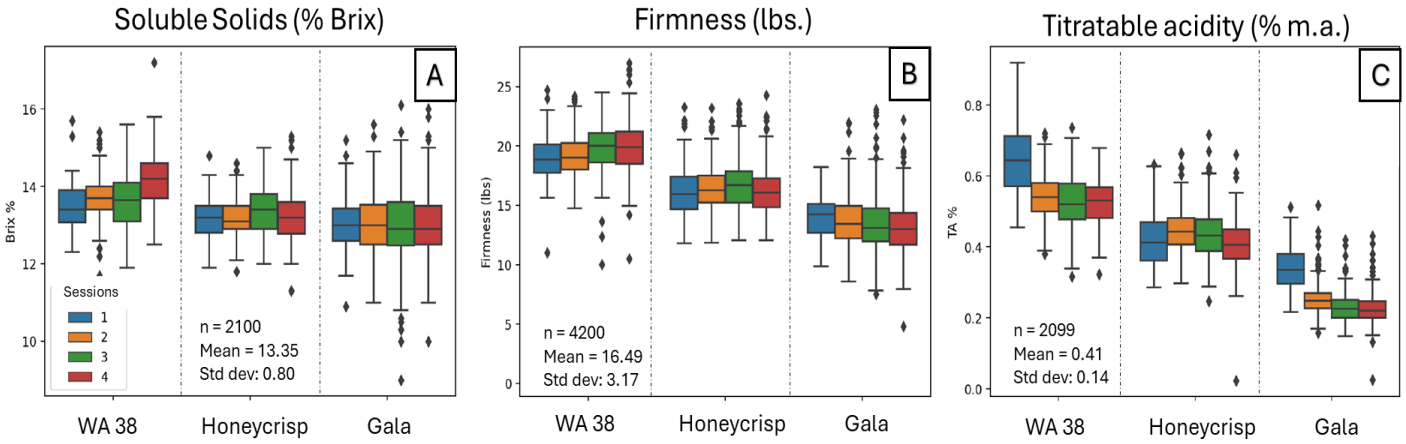


Figure 1. The data distribution of soluble solids (A), firmness (B), and titratable acidity (C) was measured using destructive methods for WA 38, Gala, and Honeycrisp apple varieties. Quality parameters were evaluated at four different times during the storage period. The graph displays the total data points (n) and the mean and standard deviation (Std dev) for each quality parameter.

The data from the three cultivars were combined to perform the model training and validation for each quality parameter. The training is conducted using 90% of the dataset and cross-validated using

the remaining 10% unseen by the model. The model training and validation scatterplots (Figure 2) and the correlation coefficient and error (Table 1) show variability in prediction accuracy between quality parameters. Positive correlation for soluble solids, firmness, and titratable acidity are weak, moderate, and strong, respectively, when predicting quality parameters for a randomly selected set of apples unseen by the model.

Among the three tested quality parameters, the best-fitting model was for titratable acidity, with a prediction power of about 70%. The $r^2$ is higher, and the error is lower for the validation set than the model training set, indicating that predictions were more accurate for the independent set of values. The mean absolute percentage error (MAPE) decreased by 3.8% for the validation set. Firmness predictions for the validation set were equivalent to those for the training set, with an $r^2$ of 0.5 and a MAPE of 10%.

Soluble solids have the lowest correlation and error. The low correlation coefficient ($r^2$ = 0.405 and 0.331) can be associated with the range restriction of Brix values, as the lower absolute error indicates that the predicted values are close to the measured values. Thus, increasing the range of values for soluble solids might generate a higher correlation coefficient.

The prediction of soluble solids was assessed using the Felix F-750 instrument based on a generic apple model (data not shown). The correlation coefficient for the Felix prediction was lower ($r^2$ = 0.194) than the Fresco prediction ($r^2$ = 0.331). It's important to emphasize that high correlation coefficients (above 0.7) can be achieved with the Felix instrument when developing a variety-specific model or conducting a Partial Least Squares (PLS) analysis using the data generated by Felix. However, this experiment evaluated the instrument's ability to deliver quality parameter results promptly without requiring further data manipulation by the user. Additional data analysis will be conducted throughout this project to better compare the predictive power of both instruments.

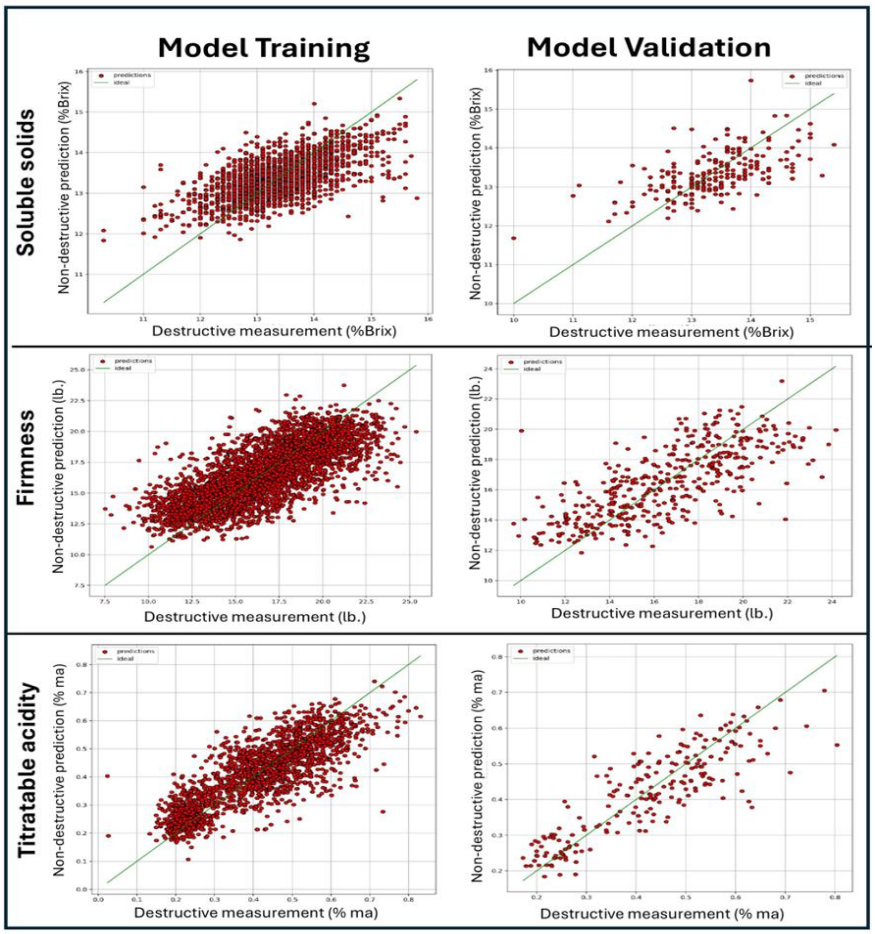


Figure 2. Soluble solids (%Brix), firmness (lbs.), and titratable acidity (% ma) correlation between non-destructive predicted values calculated by Fresco and destructive measurements for model training and validation using PLS regression.

Table 1. Correlation coefficient ($r^2$), root mean square error (RMSE), maximum absolute error (MAE), and maximum absolute percent error (MAPE) for the sample sets used by Fresco in the model training and validation to predict soluble solids, firmness, and titratable acidity values.

| Parameter | sample set | $r^2$ | RMSE | MAE | MAPE |
|---|---|---|---|---|---|
| soluble solids (%Brix) | model | 0.405 | 0.598 | 0.461 | 3.5% |
| | validation | 0.331 | 0.658 | 0.511 | 3.8% |
| Firmness (lb.) | model | 0.555 | 2.095 | 1.649 | 10.0% |
| | validation | 0.518 | 2.096 | 1.657 | 10.0% |
| Titratable acidity (% malic acid) | model | 0.677 | 0.080 | 0.062 | 17.7% |
| | validation | 0.723 | 0.074 | 0.056 | 13.9% |

## Conclusion

The wavelengths associated with firmness, soluble solids, and titratable acidity were identified in the microwave spectra region. A positive correlation was achieved for the three quality parameters with a varying prediction power range. The strongest correlation was found for titratable acidity, followed by firmness and soluble solids concentration. The narrow distribution range affected the prediction of

soluble solids, but the MAPE is low, indicating a low error between prediction and observed value. Overall, the results are promising, but more data is needed to evaluate the instrument's accuracy.

**Upcoming work**

In 2024, Granny Smith apples were included in the experiment to widen the data distribution range. Pre-harvest samples were collected for all apple varieties, and the potential for using Fresco as a pre-harvest assessment tool will be evaluated. In addition to the three quality parameters, dry matter will be evaluated during storage in 2025. After collecting data for two years, the generic apple model will be re-evaluated. Based on the results, the need for a variety-specific model will also be assessed.

**b. Granny Software**

The Granny Software is an image analysis software developed through a collaboration between the Honaas lab at the USDA Agricultural Research Service and the Flicking Research Program at Washington State University. This software employs an instance segmentation algorithm to identify individual fruits in photos. It then processes the images to extract individual fruit sub-images and remove the background for downstream analyses. The goal of Granny Software is to provide reliable and consistent results for assessing superficial disorders and determining the maturity of various apple and pear cultivars. This project is funded by the WTFRC.

The WTFRC internal program partnered with the Honaas lab to provide pictures of starch degradation to test and increase the robustness of the starch reading model. In 2023, Gala, WA 38 and Honeycrisp apples were stained with iodine following industry standards, placed in a clean apple tray located in the Granny imaging station, and photographed with an RGB camera in the lab. The apples were numbered, and the starch readings were conducted by visual assessment in accordance with the proper starch degradation scale: Honeycrisp – Honeycrisp starch scale (1-6), WA 38 – WA 38 starch scale (1-6), and Gala – Cornell Starch Scale (1-8). The pictures and visual grading were shared with the Honaas lab and will be part of a training dataset. Refer to the project report titled “Granny – Image-based Analysis of Fruit Quality” submitted to the Technology Committee for additional information.

2. WA 38 Research

Starch degradation is one of the most used procedures to determine apple harvest time. For WA 38 apples, the recommendation is that fruit should be harvested at a minimum starch of 2.0 (WA 38 starch scale: 1 to 6). In 2023, the requirement for packing and shipping **was** that 90% of the apples must reach or surpass starch 5.0. Previous studies have reported that fruit picked at 2.0 starch clearance will take an average of six weeks in refrigerated air (RA) storage to reach the required clearance (Musacchi et al., 2019). Preliminary data collected in 2021 by the WTFRC internal program indicates a slower starch degradation rate that may be influenced by the age of the trees (see Improving Apple Fruit Quality and Postharvest Performance, final project report, 2021). In addition, no data is available regarding the effect of 1-MCP treatment or CA storage on the starch-clearing rate.

**Methods**

WA 38 apples were harvested from one young and one mature orchard near Quincy in 2023. The young trees were planted in 2020 in a vertical trellis on G41 rootstock, and the mature trees were planted in 2012 in a spindle system on M9 337 rootstock. The apples were harvested in bins, treated with postharvest fungicide, and immediately stored in RA. The apples were sorted and placed in blue crates within 48 hours of harvest, and half of the batch was treated with SmatFresh (1-MCP, 1000ppb)

at Stemilt. Samples were stored in refrigerated air (RA, 33°F) or controlled atmosphere (CA, 34°F, 1% CO2, 2% O2). Because the fruit needed to be sampled weekly, CA and CA+1-MCP samples were stored in the USDA-ARS CA chambers.

Starch degradation (WA 38 starch scale, 1-6), color (red color coverage and intensity), background color, greasiness, firmness, soluble solids, flavor, and titratable acidity were assessed at harvest and weekly for eight consecutive weeks with the first storage analysis conducted two weeks after harvest. Flavor evaluations were conducted weekly in the laboratory, and a taste panel was held near the first ship date established by PVM. Quality parameters were assessed for each apple sampled during the taste panel in 2023 and 2024. This report will focus on the 2023 data, as data analysis for the apples in 2024 is ongoing.

## Results and discussion

a. Starch degradation

On average, fruit harvested from young trees had less advanced clearance at harvest than fruit from mature trees, 2.8 (±0.9) and 3.2 (±0.6), respectively. However, the initial starch clearing was more uniform, and degradation occurred more quickly in the fruit from mature trees. From harvest to the first starch assessment during storage, two weeks later, starch clearance levels in mature fruit increased by an average of 1.4 (±0.3). Starch clearance in fruit from young trees increased by 0.7 (±0.2) during the same period. A similar pattern was observed in 2022 when fruit harvested from young trees at an average starch content of 2.5 (±0.8) showed a starch clearance increase of 0.7 (±1.0) after two weeks of harvest (data not shown).

During storage, the starch clearance averages oscillated between sampling dates rather than a sequential stepwise increase. Fruit from mature trees exhibited a more uniform pattern of starch degradation (Figure 3B and Table 2) than fruit from young trees. Apples harvested from young trees also displayed higher variability within treatments, as evidenced by a higher standard deviation (Table 2). The starch deviation within treatment per sampling time decreased over time, showing that apple-to-apple variability declined as fruit matures in storage regardless of treatment (Table 2).

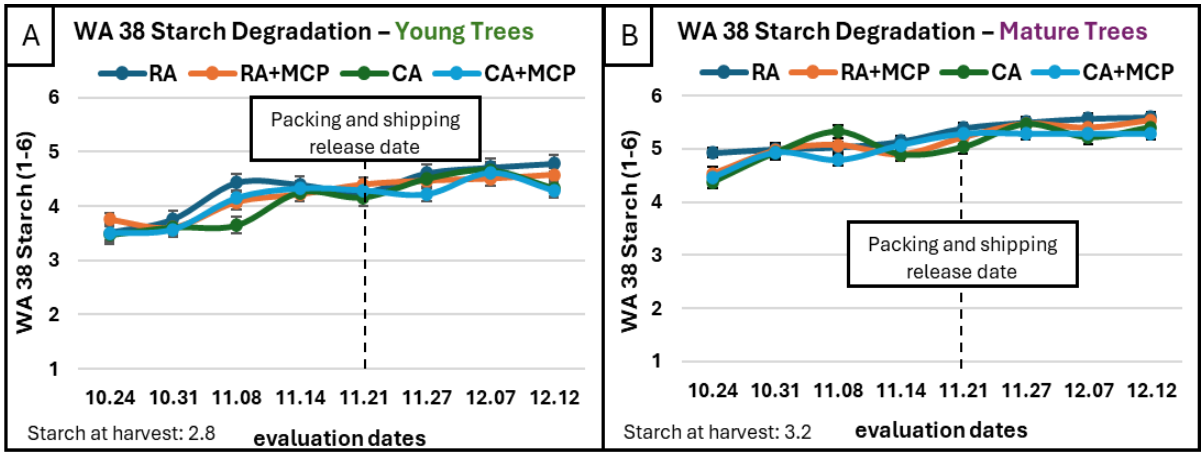


Figure 3. Starch degradation average for WA 38 harvested from young (A) and mature (B) trees and stored in RA or CA, with and without 1-MCP. Apples were sampled at harvest and during storage for eight consecutive weeks.

Table 2. Starch degradation variability (standard deviation), variance by timepoint (Timepoint var.), and treatment (Treatment var.) of WA 38 apples harvested from young or mature trees and stored in RA or CA for eight weeks. A darker to lighter yellow shade is used to identify higher to lower variance.

**Standard Deviation - Young trees**

| Treatments | 10.24 | 10.31 | 11.08 | 11.14 | **11.21** | 11.27 | 12.07 | 12.12 | Treatment. var. |
|---|---|---|---|---|---|---|---|---|---|
| RA | ± 1.0 | ± 0.5 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.4 |
| RA+MCP | ± 0.7 | ± 0.4 | ± 0.5 | ± 0.4 | ± 0.3 | ± 0.2 | ± 0.2 | ± 0.2 | ± 0.4 |
| CA | ± 0.5 | ± 0.9 | ± 0.2 | ± 0.5 | ± 0.2 | ± 0.1 | ± 0.3 | ± 0.3 | ± 0.4 |
| CA+MCP | ± 0.9 | ± 0.2 | ± 0.4 | 0.5 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.4 |
| Timepoint var. | ± 0.7 | ± 0.5 | ± 0.4 | ± 0.4 | ± 0.3 | ± 0.2 | ± 0.2 | ± 0.3 | |

**Standard Deviation - Mature trees**

| Treatments | 10.24 | 10.31 | 11.08 | 11.14 | **11.21** | 11.27 | 12.07 | 12.12 | Treatment. var. |
|---|---|---|---|---|---|---|---|---|---|
| RA | ± 0.3 | ± 0.1 | ± 0.4 | ± 0.4 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.2 | ± 0.3 |
| RA+MCP | ± 0.5 | ± 0.2 | ± 0.5 | ± 0.4 | ± 0.3 | ± 0.2 | ± 0.3 | ± 0.2 | ± 0.3 |
| CA | ± 0.5 | ± 0.3 | ± 0.4 | ± 0.6 | ± 0.2 | ± 0.2 | ± 0.3 | ± 0.2 | ± 0.3 |
| CA+MCP | ± 0.4 | ± 0.2 | ± 0.4 | ± 0.5 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.3 |
| Timepoint var. | ± 0.4 | ± 0.2 | ± 0.4 | ± 0.5 | ± 0.3 | ± 0.3 | ± 0.3 | ± 0.2 | |

Fruit harvested from mature trees achieved a 5.0 starch average in week 2 (10.31) and reached the 90% equal to or above 5.0 required for shipping at the release date in week 5 (11.21). At that time, apples were ready to be packed, regardless of treatment. A similar occurrence was observed in 2022 when fruit from mature trees achieved shipping clearance 3 days before the release date.

Analogous to 2022, the fruit from young trees did not reach the 5.0 starch average or the 90% starch clearance required for shipping during the 8 weeks of evaluation in the 2023 season. It is important to mention that the data collected does not indicate the inadequacy of the chosen packing and shipping date. Rather, it emphasizes the need for starch assessment for every fruit lot before packing.

a. Flavor evaluation

Only apples stored in RA were sampled for flavor evaluation, as fruit in CA is typically stored for at least 6 months and is not selected to be sold at the packing release date. Seven people sampled three apples per location/treatment combination after 7 days at room temperature. Starch degradation, quality parameters (firmness, soluble solids, and titratable acidity), and flavor classification based on overall liking (yuck, poor, ok, good, outstanding) were recorded for each apple. Quality parameters results will be summarized in the final report after the conclusion of the 2024 storage evaluations.

Fruit from mature trees that were not treated with 1-MCP was preferred among the tree age and treatment combinations, achieving 79% ratings as good or outstanding (Figure 4). On the other hand, fruit from mature trees treated with 1-MCP had the lowest overall liking, with the highest incidence of unpleasant flavor (yuck and poor categories). These results indicate that 1-MCP treatment had a negative impact on fruit flavor of apples from mature trees. This can also be observed in the flavor raking (Figure 4 B), where the fruit treated with 1-MCP was more often classified as the least favorite (4$^{th}$ place).

Fruit harvested from young trees achieved the second-highest overall liking, with most participants rating the apple flavor as pleasant (good). There were no noticeable differences in the flavor

of young fruit, whether treated with or without 1-MCP. This indicates that the 1-MCP treatment had no effect on flavor for this particular lot.

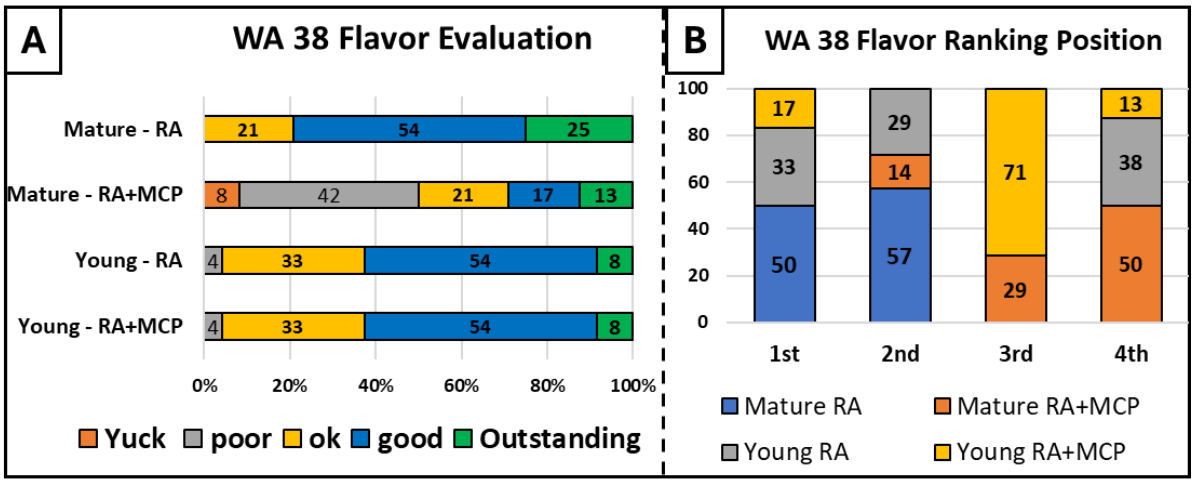


Figure 4. Flavor classification (A) in percentage by category (yuck, poor, ok, good, outstanding) of WA 38 apples harvested from young or mature trees and stored in RA, with and without 1-MCP treatment. The ranking indicates the overall preference for each tree age and treatment combination, with 1st position being the best and 4th position the worst.

## Conclusion

Although several conditions can affect starch degradation in apples, such as crop load, temperature, growing degree days, and ethylene production, these findings suggest that the rate of starch clearance in WA 38 apples is influenced by tree age, with fruit from mature trees displaying a faster and more homogeneous clearance rate. In this experiment, climate conditions' influence and year-to-year variability were accounted for by sampling orchard blocks in the same growing region over three years (2022 to 2024).

The use of 1-MCP did not affect the starch degradation rate of fruit stored in either RA or CA during the study period. However, a negative impact on fruit flavor was noted in fruit treated with 1-MCP that was harvested from mature trees. No benefits were observed for fruit harvested from young trees.

## Future work

Data collection for 2024 is ongoing. Starch degradation, flavor evaluation and a summary of the quality data collected from 2022 to 2024 will be included in the final report.

## Outcomes

The results of this experiment have been included in the *Recommended Harvest Criteria for Commercial WA-38 Storage in 2024*, with the recommendation that fruit from young trees be sold in January or February due to the variability of starch clearance rate. This study, along with the historical data collected in the Apple Breeding Program phase three and other WA 38 research projects contributed to the adjustment of the packing and shipping requirement to 90% of the fruit with starch clearance of 4.5 (instead of 5.0).

**Project Title:** Mitigating WA 38 greasiness and related quality defects

**CONTINUING PROJECT REPORT** **YEAR**: 3 of 3

**Primary PI**: Carolina Torres
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**City/State/Zip**: Wenatchee/WA/98801
**Cooperators**: Anne Plotto, USDA-ARS, USHRL

**Project Duration:** 3 Years
**Total Project Request for Year 1 Funding:** $ 84,050.00
**Total Project Request for Year 2 Funding:** $ 84,012.00
**Total Project Request for Year 3 Funding:** $ 86,092.00

**Other related/associated funding sources:** None

**WTFRC Collaborative Costs:** None

**Budget 1**
**Primary PI:** Carolina Torres
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**Station Manager/Supervisor:** Kimi Lucas
**Station manager/supervisor email address:** kimi.lucas@wsu.edu

| Item | Type year of project start date here | (Type year start date of year 2 here if relevant) | (Type year start date of year 3 here if relevant) |
|---|---|---|---|
| **Salaries** | 54,000 | 56,160 | 58,406 |
| **Benefits** | 20,050 | 20,852 | 21,686 |
| **Wages** | | | |
| **Benefits** | | | |
| **Equipment** | | | |
| **Supplies** | 4,000 | 1,000 | |
| **Travel** | | | |
| **Miscellaneous** | | | |
| **Plot Fees** | | | |

| | | | |
|---|---|---|---|
| **Total** | 78,050 | 78,012 | 80,092 |

**Footnotes:**

Salaries: Research personnel to carry out field and laboratory work, fruit evaluations and data analyses in years 1, 2, and 3.

Benefits: $20,050, $20,852, and $21,686 are requested for benefits tied to the research personnel.

Supplies: Supply costs of $4,000 in year 1 and $1,000 in year 2 are requested to pay for supplies for fruit quality evaluation.

**Budget 2**

**Co-PI:** David Rudell

**Organization Name:** USDA-ARS

**Contract Administrator:** Chuck Myers and Sharon Blanchard

**Telephone:** (510) 559-5769 (CM, 509-664-2280 (SB)

**Contract administrator email address:** Chuck.Myers@usda.gov, Sharon.Blanchard@usda.gov

| **Item** | **Type year of project start date here** | **(Type year start date of year 2 here if relevant)** | **(Type year start date of year 3 here if relevant)** |
|---|---|---|---|
| **Salaries** | | | |
| **Benefits** | | | |
| **Wages** | | | |
| **Benefits** | | | |
| **Equipment** | | | |
| **Supplies** | 3,000 | 3,000 | 3,000 |
| **Travel** | 3,000 | 3,000 | 3,000 |
| **Miscellaneous** | | | |
| **Plot Fees** | | | |
| **Total** | 6,000 | 6,000 | 6,000 |

**Footnotes:**

Supplies: Supply costs of $4,000 in year 1 and $1,000 in year 2 are requested to pay for supplies for fruit quality evaluation.

Travel: $3,000 is requested in years 1, 2, and 3, respectively, for associated travel for Dr. Anne Plotto.

**Objectives**:

1. Further, define harvest maturity guidelines limiting greasiness in the cold chain.
2. Establish ethylene mitigation protocols that reduce greasiness for both conventional and organic production.
3. Determine the limitations of wax/detergent for mitigating greasiness in the post-storage cold chain.
4. Identify and determine protocols for mitigating off-flavors associated with greasiness.

**Significant Findings:**

1. Maturity progression varied between growing sites and seasons. The starch degradation rate was slow during 2024 due to low temperatures at the end of the growing period.
2. Overall, fruit from 2022 season had lower greasiness incidence and severity than that of 2023. Fruit greasiness appears to be more related to fruit maturity and seasonal weather than just tree age.
3. There was a higher incidence (and severity) of greasiness in fruit from air storage compared to CA, but more consistently on that from H2 (1 week after commercial harvest), and in all cases, greasiness increased during the shelf-life period (7 days at 68$^{o}$F).
4. 1-MCP and AVG treatment altered maturity and ripening. Retain® reduced greasiness incidence and severity after 1 day at 68$^{o}$F, but not during 'shelf-life'. Only Harvista$^{TM}$ 1x plus SmartFresh was able to reduce greasiness severity postharvest.
5. Detergents were able to remove skin greasiness for 1-3 days, and coatings continued to control greasiness during the cold chain.

**Objective 1**: Further define harvest maturity guidelines limiting greasiness in the cold chain.

During 2024, as well as in the previous 2 years, fruit was harvested twice, at starch index ~2 and ~4 from four commercial orchards (4$^{th}$-6$^{th}$-leaf trees: Mattawa, Quincy, Royal city, Zillah). Maturity progression was evaluated weekly starting four weeks before commercial harvest. Following each harvest, fruit was stored 33°F in air or in controlled atmosphere (2.5% $O_2$, 1.5% $CO_2$) for six months. Fruit quality (ripeness, skin greasiness, physiological disorders) was evaluated monthly. Skin greasiness was rated using a 4-point subjective scale, rubbing the fruit against the hand, and rated as (0) no greasiness, (1) slight greasiness/tackiness, (2) moderate greasiness, and (3) severe greasiness. As in year 1 (2022), fruit maturity progression pre and postharvest in year 2 (2023/2024) varied between sites, particularly in ethylene production, starch degradation index (SI), and chlorophyll degradation rate (change in background color). Fruit harvested in 2024 is being evaluated postharvest. Table 1 shows the maturity indices at harvest from both locations in all three seasons, and Figure 1, the frequency of SI values in fruit harvested commercially and one week later in 2022 and 2023, and up to three weeks later in 2024. The latest was a consequence of low temperatures at the end of the growing season. Skin greasiness at harvest was absent in all sites in 2022, and present (in some fruit, severity (1)) in all sites in 2023 and 2024.

During the 2023-2024 storage season, CA reduced fruit softening (~1.5 lb), ethylene production, and background color change ($I_{AD}$ values) compared to air storage (Table 2). The starch index dispersion after 2 months of storage was the highest in CA-stored fruit from H1 (commercial harvest). Skin greasiness incidence and severity were lower in fruit stored in CA compared to that in air (Table 2). Greasiness increased during the 'shelf-life' period (7 days at 68$^{o}$F).

Table 1. Apple maturity and ripeness at commercial harvest and 1 or 3 weeks afterward (WAH) until the median for starch index (SI) was ~4, for fruit from Mattawa and Quincy blocks during seasons 2022, 2023, and 2024.

| | Location | Harvest | Weight (g) | Red skin coverage (%) | Backg. Color (1-4) | Soluble Solids (°Brix) | SI (1-6) | $I_{AD}$ (0-2.2) | Ethylene (ppm) | Firmness (lb) |
|---|---|---|---|---|---|---|---|---|---|---|
| 2022 | Mattawa | Harvest | 283.8±38.6[Y] | 91.9±5.1 | 3.6±0.5 | 13.3±1.2 | 3.0±0.9 | 0.7±0.3 | 0.5±0.3 | 15.4±0.9 |
| | | 1 WAH | 268.9±40.1 | 90.5±5.7 | 3.8±0.4 | 13.3±1.2 | 3.3±0.8 | 0.6±0.2 | 0.8±0.4 | 17.5±1.4 |
| | Quincy | Harvest | 313.1±62.3 | 87.7±6.4 | 3.5±0.5 | 13.8±0.8 | 2.1±0.7 | 1.2±0.3 | 0.0±0 | 19.5±1.1 |
| | | 1 WAH | 317.5±41.3 | 90.3±4.7 | 3.6±0.5 | 13.9±0.9 | 1.8±0.6 | 0.7±0.3 | 0.3±0.5 | 15.8±0.8 |
| 2023 | Mattawa | Harvest | 268.8±43.9 | 85.7±5.7 | 3.7±0.5 | 12.8±0.9 | 2.1±0.5 | 0.7±0.3 | 0.4±0.3 | 21.9±0.4 |
| | | 1 WAH | 296.4±34.1 | 94.7±1.2 | 3.9±0.2 | 16.2±1.2 | 2.9±0.5 | 0.4±0.2 | 5.9±1.5 | 23.7±0.4 |
| | Quincy | Harvest | 312.9±27.3 | 82.7±6.4 | 3.7±0.5 | 14.6±1.6 | 2.0±1.0 | 1.0±0.3 | 0.2±0.3 | 22.9±0.3 |
| | | 1 WAH | 249.4±27.3 | 93.6±2.9 | 3.9±0.2 | 14.5±0.5 | 3.1±0.7 | 0.4±0.1 | 11.8±1.9 | 20.4±0.2 |
| 2024 | Mattawa | Harvest | 262.9±26.1 | 92.6±3.9 | 3.9±0.2 | 13.6±1.2 | 2.6±0.6 | 0.8±0.4 | 3.1±4.8 | 20.4±1.4 |
| | | 3 WAH | 300.8±36.9 | 86.6±9.7 | 3.8±0.3 | 14.1±0.9 | 3.8±0.9 | 0.2±0.1 | 3.5±8.8 | 16.8±1.2 |
| | Quincy | Harvest | 226.6±32.03 | 88.8±6.3 | 2.7±0.7 | 13.2±1.3 | 2.4±0.4 | 1.1±0.3 | 0.5±0.5 | 20.4±1.5 |
| | | 3 WAH | 257.8±44.6 | 81.6±7.8 | 2.8±0.3 | 12.8±0.9 | 3.3±0.5 | 0.9±0.3 | 1.2±1.9 | 18.1±1.2 |

[Y]Average ± Standard Error

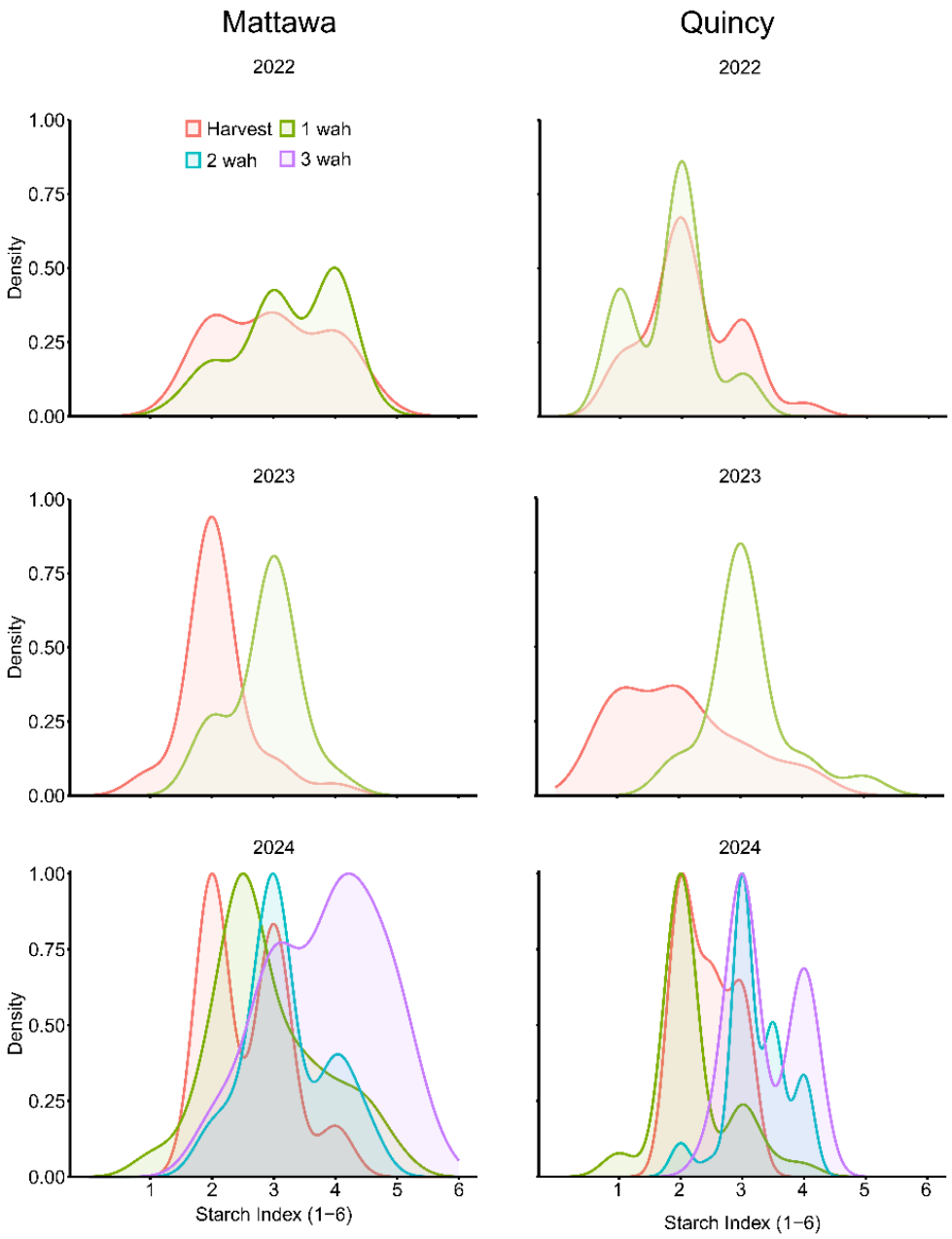


Figure 1. Density plots from the starch index of fruit from Mattawa and Quincy in 2022 (Top), 2023 (middle), and 2024 (bottom) at commercial harvest and one week later (1 wah) (2022-2023) or up to three weeks later (3 wah) (2024). Overlap shapes show no differences in the starch degradation population in fruit sampled at each time point.

Table 2. Relative ripeness and greasiness (average values) during air and CA storage at 33ºF during the 2023-2024 season.

| | Harvest | Storage Eval. | Firmness (lb) | | Backg. Color (1-4) | | $I_{AD}$ (0-2.2) | | SI (1-6) | | Ethylene (ppm) | | Grease score (0-3) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | RA | CA | RA | CA | RA | CA | RA | CA | RA | CA | RA | CA |
| Mattawa | H1 | 1mo+1d | 21.4 | 20.4* | 3.9 | 3.9 | 0.6 | 0.5 | 3.6 | 4.0 | 4.3 | 6.1 | 0.2* | 0.4 |
| | | 2mo+1d | 25.9 | 20.3* | 4.0 | 3.9 | 0.1* | 0.5 | 5.0 | 4.8 | 29.2 | 5.5* | 0.3 | 0.3 |
| | | 3mo+1d | 20.3* | 25.3 | 4.0 | 3.9 | 0.3* | 0.5 | 5.9 | 5.9 | 45.7 | 21.5* | 0.2 | 0.3 |
| | | 4mo+1d | 17.8* | 19.1 | 4.0 | 3.8* | 0.2* | 0.4 | 5.9 | 5.9 | 45.0 | 12.1* | 0.5 | 0.3 |
| | | 5mo+1d | 19.4 | 18.6* | 4.0 | 3.9 | 0.1* | 0.5 | 5.8* | 6.0 | 68.6 | 11.5* | 0.8 | 0.2* |
| | | 6mo+1d | 18.9* | 20.8 | 4.0 | 3.9* | 0.2* | 0.4 | 6.0 | 6.0 | 51.1 | 2.2* | 0.6 | 0.0* |
| | H2 | 1mo+1d | 21.0 | 20.9 | 4.0 | 4.0 | 0.4 | 0.4 | 3.5 | 3.5 | 1.1 | 1.3 | 0.1* | 0.4 |
| | | 2mo+1d | 23.5 | 23.5 | 3.9 | 4.0 | 0.3* | 0.4 | 4.7 | 4.6 | 30.8 | 12.3 | 0.3 | 0.2 |
| | | 3mo+1d | 19.8* | 24.8 | 4.0 | 4.0 | 0.3* | 0.4 | 5.9 | 5.8 | 55.6 | 10.1* | 0.5 | 0.2* |
| | | 4mo+1d | 16.8* | 17.6 | 3.3* | 4.0 | 0.1* | 0.2 | 6.0 | 6.0 | 51.4 | 13.1* | 1.0 | 0.4* |
| | | 5mo+1d | 18.5 | 17.6* | 4.0 | 4.0 | 0.2* | 0.4 | 6.0 | 6.0 | 70.2 | 10.0* | 1.4 | 0.1* |
| | | 6mo+1d | 17.3* | 19.6 | 4.0 | 4.0 | 0.2* | 0.3 | 6.0 | 6.0 | 79.8 | 6.7* | 0.3 | 0.1* |
| Quincy | H1 | 1mo+1d | 22.3 | 22.5 | 3.9 | 4.0 | 0.6 | 0.6 | 3.5 | 3.6 | 40.3 | 16.1* | 0.5* | 1.1 |
| | | 2mo+1d | 23.7 | 22.1* | 3.9 | 3.8 | 0.5* | 0.7 | 4.6 | 4.2 | 34.7 | 16.3* | 0.5 | 0.2* |
| | | 3mo+1d | 21.2* | 28.2 | 4.0 | 3.9 | 0.4* | 0.6 | 5.7 | 5.7 | 116.6 | 16.8* | 0.3* | 1.0 |
| | | 4mo+1d | 17.4* | 19.3 | 3.9 | 3.6* | 0.4 | 0.5 | 5.7 | 5.7 | 90.4 | 17.2* | 1.1 | 0.7* |
| | | 5mo+1d | 20.2 | 20.1 | 4.0 | 3.8* | 0.2* | 0.6 | 5.7* | 6.0 | 75.1 | 13.4* | 0.5* | 0.9 |
| | | 6mo+1d | 17.7* | 22.0 | 3.9 | 3.7 | 0.1* | 0.6 | 6.0 | 6.0 | 12.4 | 3.3* | 0.7 | 0.4 |
| | H2 | 1mo+1d | 21.5 | 22.1 | 4.0 | 3.9 | 0.5 | 0.5 | 3.8 | 4.0 | 6.9 | 11.6 | 1.4 | 0.8* |
| | | 2mo+1d | 25.8 | 21.6* | 3.9 | 3.6* | 0.5* | 0.8 | 4.7 | 4.7 | 43.0 | 20.1* | 1.5 | 0.3* |
| | | 3mo+1d | 25.2 | 24.7 | 4.0 | 3.9 | 0.3* | 0.5 | 5.8* | 6.0 | 45.4 | 20.4* | 1.1 | 0.9 |
| | | 4mo+1d | 18.8 | 19.1 | 3.5* | 3.9 | 0.4* | 0.5 | 5.8 | 5.7 | 90.8 | 25.1* | 1.4 | 1.0 |
| | | 5mo+1d | 19.5 | 18.7 | 4.0 | 4.0 | 0.3 | 0.3 | 6.0 | 6.0 | 68.3 | 13.1* | 1.7 | 0.8* |
| | | 6mo+1d | 19.5* | 21.4 | 4.0 | 4.0 | 0.3* | 0.4 | 6.0 | 6.0 | 58.9 | 11.0* | 0.5 | 0.5 |

* Indicates significant differences between storage regimes (RA, CA) (ANOVA, P≤0.05)

**Objective 2**: Establish ethylene mitigation protocols that reduce greasiness for both conventional and organic production.

Table 3 shows treatments applied in 2023 and 2024. Maturity and ripening progression postharvest are ongoing for the 2024 harvest.

Preharvest application of Retain®, either at half or full rate, suppressed ethylene production and delayed the starch degradation at harvest (Table 4). Fruit greasiness was present at the time of the first application (2 weeks before harvest) in 2023. In 2024, it appeared in the UTC one week after commercial harvest. There were no differences between treatments in ethylene production or starch degradation postharvest (Table 4). All Retain® reduced greasiness incidence (~15%) and severity after 1 day at 68ºF. The effect disappears after 7 days at 68ºF (Table 5).

As in 2022, in 2023 Harvista™, either applied at a single (1x) or double (2x) rate, was not able to reduce ethylene production nor affect the starch degradation pattern on the fruit compared to the UTC. Nevertheless, in 2023 Harvista™ 2x was able to better retain firmness after 4 months of air storage (Table 6). Harvista™ 1x plus SF was able to retain flesh firmness for up to 12 months of storage (+3.0 lb than UTC and Harvista™ 1x). No differences in skin greasiness between treatments were observed at harvest. Ethylene production was suppressed using Harvista™ 1x plus SF (T5) and

UTC plus SF (T4) for up to 8 months plus 7 days at 68ºF. Skin greasiness severity was reduced by Harvista™ 1x plus SF treatment (Table 7).

Table 3. PGR treatments (rate and time of application) during 2023 and 2024 seasons.

| Year | Treat. | Material/a.i. | Rate | Timing | Others |
|---|---|---|---|---|---|
| 2023 | 1 | Untreated control (UTC) | NA | NA | • Orchard location: Zillah, WA<br>• Sylcoat (0.1% OSi) added to Retain® applications.<br>• Two harvests: At the time of UTC (commercial), and 1 week later. |
| | 2 | ReTain® | Full rate (24 oz/acre) | 21 DBH | |
| | 3 | ReTain® | Full rate (24 oz/acre) | 14 DBH | |
| | 4 | ReTain® | Full rate (24 oz/acre) | 7 DBH | |
| 2024 | 1 | Untreated control (UTC) | NA | NA | |
| | 2 | ReTain® | Full rate (24 oz/acre) | 14 DBH | |
| | 3 | ReTain® | Half rate (12 oz/acre) | 14 DBH | |
| | 4 | ReTain® | Full rate (24 oz/acre) | 7 DBH | |
| 2023 | 1 | UTC | | | • Orchard location: Royal City, WA<br>• Two harvests: At the time of UTC (commercial), and 1 week later.<br>• Only harvest 1 for SF treats. |
| | 2 | 1-MCP (Harvista™ 1.3 SC) (1x) | Full dose | 14 DBH | |
| | 3 | Harvista™ (2x) | Full dose | 14 & 7 DBH | |
| | 4 | UTC plus 1-MCP (SmartFresh™; SF) | 100 ppm | At harvest | |
| | 5 | Harvista™ 1x plus SF | T2 plus SF (100 ppm) | At harvest | |
| 2024 | 1 | UTC | | | |
| | 2 | UTC plus 1-MCP (SmartFresh™; SF) | 100 ppm | At harvest | |

AVG: Aminoethoxyvinylglicine; 1-MCP: 1-methylcyclopropene; DBH: Days before harvest

Table 4. Fruit maturity/ripeness during storage at 33ºF of WA 38 apples (3rd-leaf trees) treated with AVG (Retain®), Zillah, WA. 2023.

| | Eval. | Treat. | SI (1-6) | Firmness (lb) | | Ethylene (ppm) | | Titratable Acidity (% malic acid) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | +1d | +7d | +1d | +7d | +1d | +7d |
| H1 | Harvest | UTC[Y] | 2.7 a[Z] | 22.6 ab | - | 5.5 b | - | 0.7 ab | - |
| | | 21 DBH | 2.0 b | 23.5 a | - | 0.4 a | - | 0.8 a | - |
| | | 14 DBH | 2.3 ab | 22.5 b | - | 1.4 a | - | 0.6 b | - |
| | | 7 DBH | 2.3 ab | 22.4 b | - | 0.1 a | - | 0.7 ab | - |
| | 4 m | UTC | 6.0 | 20.1 a | 18.0 | 100.9 | 237.9 | 0.5 | 0.5 |
| | | 21 DBH | 5.9 | 18.9 b | 17.7 | 63.5 | 266.2 | 0.6 | 0.5 |
| | | 14 DBH | 6.0 | 18.8 b | 17.7 | 48.5 | 246.2 | 0.5 | 0.5 |
| | | 7 DBH | 5.9 | 19.3 ab | 17.9 | 86.5 | 220.7 | 0.5 | 0.5 |
| | 8 m | UTC | 6.0 | 17.1 ab | 16.4 | 51.0 b | 405.7 ab | 0.4 | 0.4 a |
| | | 21 DBH | 6.0 | 16.5 b | 15.8 | 68.2 a | 363.7 ab | 0.4 | 0.4 a |
| | | 14 DBH | 6.0 | 17.7 a | 16.5 | 36.6 b | 453.2 a | 0.4 | 0.4 a |
| | | 7 DBH | 6.0 | 16.7 b | 15.7 | 39.6 b | 286.7 b | 0.4 | 0.3 b |
| H2 | Harvest | UTC | 3.7 a | 22.9 a | - | 2.6 | - | 0.8 | - |
| | | 21 DBH | 2.7 b | 21.9 b | - | 1.7 | - | 0.7 | - |
| | | 14 DBH | 3.1 b | 22.3 ab | - | 1.2 | - | 0.7 | - |
| | | 7 DBH | 3.8 a | 20.2 c | - | 9.8 | - | 0.7 | - |
| | 4 m | UTC | 6.0 | 19.9 a | 17.9 | 95.0 ab | 329.7 | 0.6 | 0.5 |
| | | 21 DBH | 6.0 | 18.7 b | 17.1 | 91.1 ab | 408.1 | 0.5 | 0.5 |
| | | 14 DBH | 6.0 | 18.9 ab | 17.5 | 58.2 b | 341.5 | 0.5 | 0.5 |
| | | 7 DBH | 6.0 | 18.6 b | 17.6 | 111.3 a | 327.5 | 0.5 | 0.5 |
| | 8 m | UTC | 6.0 | 16.6 | 16.1 | 6.0 | 190.5 | 0.4 | 0.2 b |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 21 DBH | 6.0 | 16.6 | 16.1 | 3.8 | 211.6 | 0.4 | 0.3 a |
| | 14 DBH | 6.0 | 16.5 | 16.3 | 8.1 | 251.1 | 0.4 | 0.3 a |
| | 7 DBH | 6.0 | 16.9 | 16.3 | 0.5 | 229.8 | 0.4 | 0.3 a |

[Z] ANOVA (P≤0.05). Different letters indicate significant differences between treatments (Tukey, P≤0.05).
[Y] UTC= Untreated Control

Table 5. Skin greasiness incidence and severity of WA 38 apples (3rd-leaf trees) treated with AVG (Retain®), Zillah, WA. 2023.

| | Evaluation | Treatment | No grease (% fruit) | | Grease score 1 (%) | | Grease score 2+3 (%) | |
|---|---|---|---|---|---|---|---|---|
| | | | +1d | +7d | +1d | +7d | +1d | +7d |
| H1 | Harvest | UTC | 87 a[Z] | - | 9 a | - | 4 | - |
| | | 21 DBH | 100 b | - | 0 b | - | 0 | - |
| | | 14 DBH | 100 b | - | 0 b | - | 0 | - |
| | | 7 DBH | 100 b | - | 0 b | - | 0 | - |
| | 4 m | UTC | 54 a | 9 | 41 | 42 | 5 | 50 |
| | | 21 DBH | 67 ab | 49 | 32 | 31 | 1 | 19 |
| | | 14 DBH | 71 b | 8 | 28 | 48 | 1 | 44 |
| | | 7 DBH | 73 | 20 | 26 | 54 | 1 | 26 |
| | 8 m | UTC | 77 | 75 | 20 | 22 | 3 | 3 |
| | | 21 DBH | 72 | 69 | 25 | 28 | 3 | 3 |
| | | 14 DBH | 83 | 70 | 16 | 27 | 1 | 3 |
| | | 7 DBH | 78 | 67 | 18 | 25 | 4 | 8 |
| H2 | Harvest | UTC | 60 a | - | 40 | - | 0 | - |
| | | 21 DBH | 97 b | - | 3 | - | 0 | - |
| | | 14 DBH | 93 b | - | 7 | - | 0 | - |
| | | 7 DBH | 80 ab | - | 20 | - | 0 | - |
| | 4 m | UTC | 45 ab | 28 ab | 40 | 41 | 15.4 | 9 b |
| | | 21 DBH | 33 a | 7 a | 57 | 37 | 10.05 | 56 ab |
| | | 14 DBH | 59 b | 62 c | 35 | 14 | 6.15 | 24 ab |
| | | 7 DBH | 61 b | 51 bc | 32 | 13 | 7.02 | 36 a |
| | 8 m | UTC | 74 ab | 64 | 26 | 36 a | 0 | 0 b |
| | | 21 DBH | 86 b | 32 | 14 | 38. b | 0 | 29 a |
| | | 14 DBH | 59 a | 43 | 41 | 50 ab | 0 | 7 ab |
| | | 7 DBH | 82 b | 39 | 18 | 41 ab | 0 | 20 ab |

[Z] ANOVA (P≤0.05). Different letters indicate significant differences between treatments (Tukey, P≤0.05)

Table 6. Fruit maturity/ripeness during storage at 33ºF of WA 38 apples (6th-leaf trees) treated with 1-MCP (Harvista™ and SmartFresh (SF)), Royal City, WA. 2023.

| Eval. | Treat. | SI (1-6) | Firmness (lb) | | Ethylene (ppm) | | TA (% malic acid) | |
|---|---|---|---|---|---|---|---|---|
| | | | +1d | +7d | +1d | +7d | +1d | +7d |
| Harvest | UTC | 2.9 | 19.8 b[Z] | - | 0.3 a | - | 0.6 | - |
| | Harvista™ 1x | 3.5 | 19.4 a | - | 0.6 b | - | 0.6 | - |
| | Harvista™ 2x | 2.9 | 20.9 b | - | 0.5 b | - | 0.8 | - |
| 4 m | UTC | 6.0 | 17.7 ab | 17.8 ab | 20.4 ab | 86.0 b | 0.3 a | 0.4 |
| | Harvista™ 1x | 6.0 | 16.9 a | 17.0 a | 36.5 b | 85.7 b | 0.3 a | 0.3 |
| | Harvista™ 2x | 6.0 | 18.6 bc | 18.5 bc | 29.9 b | 100.5 b | 0.4 ab | 0.4 |
| | UTC+SF | 6.0 | 19.3 c | 19.1c | 5.2 a | 2.6 a | 0.5 b | 0.4 |
| | Harvista™ 1x+SF | 6.0 | 18.1 b | 18.1 bc | 4.0 a | 1.1 a | 0.4 ab | 0.4 |
| 8 m | UTC | 6.0 | 19.3 b | 18.1 b | 28.9 b | 226.1 b | 0.3 | 0.3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Harvista™ 1x | 6.0 | 17.5 a | 16.5 a | 11.5 a | 221.2 b | 0.2 | 0.4 |
| | Harvista™ 2x | 6.0 | 18.6 ab | 18.4 b | 19.7 ab | 163.3 b | 0.3 | 0.3 |
| | UTC+SF | 6.0 | 20.7 c | 21.0 c | 10.1 a | 44.7 a | 0.3 | 0.4 |
| | Harvista ™ 1x+SF | 6.0 | 20.5 c | 20.8 c | 12.4 a | 27.5 a | 0.4 | 0.3 |
| 12 m | UTC | 6.0 | 17.1 b | 15.9 a | 12.9 b | 284.0 b | 0.2 a | 0.2 |
| | Harvista™ 1x | 6.0 | 15.8 a | 15.0 a | 1.1 a | 224.1 ab | 0.2 a | 0.2 |
| | UTC+SF | 6.0 | 20.3 c | 20.1 b | 16.6 b | 208.8 ab | 0.3 b | 0.3 |
| | Harvista ™ 1x+SF | 6.0 | 20.9 c | 19.8 b | 11.0 b | 193.5 a | 0.3 b | 0.2 |

[Z] ANOVA (P≤0.05). Different letters indicate significant differences between treatments (Tukey, P≤0.05)

Table 7. Skin greasiness incidence and severity of WA 38 apples (6th-leaf trees) treated with 1-MCP (Harvista™ and SmartFresh (SF)), Royal City, WA. 2023.

| Evaluation | Treatment | No grease (% fruit) | | Grease 1 (% fruit) | | Grease 2+3 (% fruit) | |
|---|---|---|---|---|---|---|---|
| | | +1d | +7d | +1d | +7d | +1d | +7d |
| Harvest | UTC | 61 | - | 39 | - | 0 | - |
| | Harvista™ 1x | 67 | - | 33 | - | 0 | - |
| | Harvista™ 2x | 78 | - | 22 | - | 0 | - |
| 4 m | UTC | 94 | 41 a[Z] | 6 | 57 b | 0 | 2 |
| | Harvista™ 1x | 94 | 41 a | 6 | 43 b | 0 | 16 |
| | Harvista™ 2x | 93 | 75 b | 7 | 25 b | 0 | 0 |
| | UTC+SF | 100 | 92 b | 0 | 8 a | 0 | 0 |
| | Harvista ™ 1x+SF | 100 | 100 b | 0 | 0 a | 0 | 0 |
| 8 m | UTC | 36 | 46 | 59 | 29 | 5 | 24 |
| | Harvista™ 1x | 64 | 80 | 36 | 10 | 0 | 10 |
| | Harvista™ 2x | 49 | 69 | 46 | 15 | 5 | 16 |
| | UTC+SF | 54 | 59 | 41 | 27 | 5 | 14 |
| | Harvista ™ 1x+SF | 67 | 75 | 31 | 18 | 2 | 6 |
| 12 m | UTC | 90 | 67 b | 10 | 28 a | 0 a | 6 a |
| | Harvista™ 1x | 77 | 18 a | 23 | 55 ab | 0 a | 27 ab |
| | UTC+SF | 59 | 18 a | 28 | 32 a | 12 b | 50 b |
| | Harvista ™ 1x+SF | 67 | 2 a | 26 | 63 b | 8 b | 35 ab |

[Z] ANOVA (P≤0.05). Different letters indicate significant differences between treatments (Tukey, P≤0.05)

**Objective 3**: Determine the limitations of wax/detergent for mitigating greasiness in the post-storage cold chain.

During 2023, two experiments (Table 8) were conducted with fruit stored for 6 (April 2023) and 10 months (June 2023). Evaluations were done for up to 7 days (detergents) and 21 days (coatings) at room temperature (68ºF) after treatment, and after 30 days in cold storage (33ºF) after treatment in the case of coatings. For the coating experiment, the fruit was cleaned with Epi-Clean (60 s), dried in air, and then coated with the different formulations (Table 8).

For the detergent trial, in general, all treatments were able to remove the natural greasiness of the fruit for up to 2-3 days at 68ºF in fruit stored for 6 months and 1 day for that stored for 10 months (Fig. 2). All coatings tested were able to effectively reduce greasiness on the fruit during the shelf-life period with and without 30 days in cold storage post-application (Fig. 3).

Table 8. Detergent and coating treatments 2023 season.

| Product | Treat. | Material | Rate | Application Time |
|---|---|---|---|---|
| Detergents | 1 | UTC (Water) | NA | 30 s |
| | 2 | Acidex Duo | 25 ml / L | 30 & 60 s |
| | 3 | Epi-Clean | 25 ml / L | 30 & 60 s |
| Coatings | 1 | UTC | NA | - |
| | 2 | PrimaFresh 360 HS | 0.4 g /fruit | 30 s |
| | 3 | Shield-Brite AP-450 | 0.4 g /fruit | 30 s |
| | 4 | Xedasol | 0.4 g /fruit | 30 s |

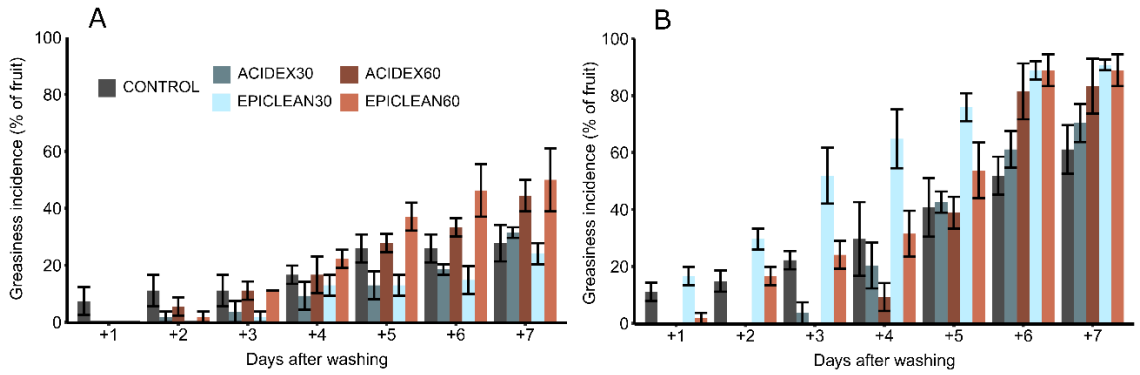


Fig. 2. Greasiness incidence (% fruit) in WA 38 apples stored for 6 months (A) or 10 months (B) for up to 7 days at 68 ºF after washing with different detergent treatments (Average ± Standard Error).

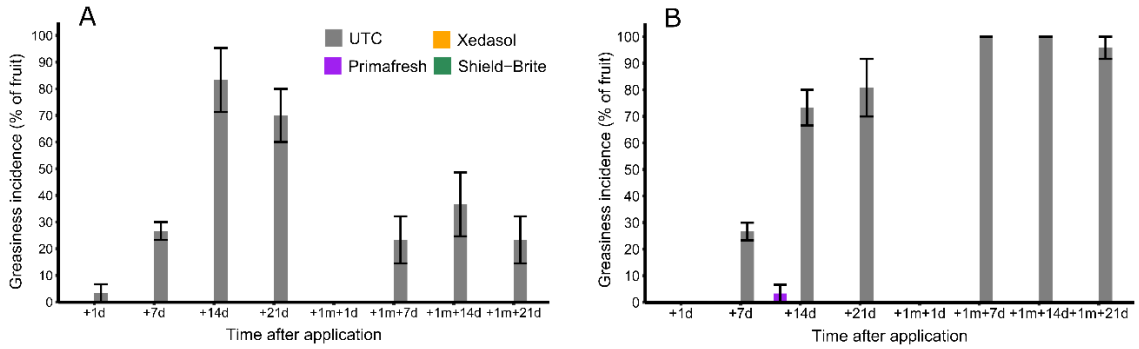


Fig 3. Greasiness incidence (% fruit) in WA 38 apples stored for 6 months (A) or 10 months (B) for up to 21 days at 68ºF with or without 1 month of air storage (33 ºF) after the coating treatment.

**<u>Objective 4</u>**: Identify and determine protocols for mitigating off-flavors associated with greasiness.

No bitterness or off flavor was detected in fruit from any of the experiments performed during 2023.